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Effect of light deprivation on expression of extended vascular endothelial growth factor and neovascularization in retina of neonatal rats

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Abstract. – OBJECTIVE: The aim of this study is to analyze the effect of light deprivation on the expression of vascular endothelial growth factor (VEGF) and neovascularization in the retina of neonatal rats.

MATERIALS AND METHODS: Thirty-six neonatal SD rats (male and female) were used in this study. These rats were numbered randomly and assigned into 3 groups (12 rats in each group), ie. 10-day group (routine feeding after birth, eyeball enucleation on 10th day), 14-day group (routine feeding after birth, eyeball enucleation on 14th day) and light deprivation group (routine feeding within 1st week after birth, feeding with light deprivation within 2nd week after birth, eyeball enucleation on 14th day). The expression level of VEGF mRNA was measured by RT-PCR, and the percentage of the retinal vascular area was calculated by PAS staining, and the number of vascular endothelial cells was counted with a microscope in a double-blind manner.

RESULTS: It was found that the expression levels of VEGF mRNA and the number of vascular endothelial cells in 10-day group and light deprivation group were significantly higher than 14-day group ($p < 0.05$), while the difference between the 10-day group and light deprivation group was not significant. The percentage of retinal vascular area in the 10-day group and light deprivation group was significantly lower than 14-day group ($p < 0.05$), while the difference between the 10-day group and the light deprivation group was not significant.

CONCLUSIONS: The light deprivation delayed the growth of neovessels in the retina.

Key Words:
Light deprivation, Retina, VEGF, Neovascularization, Extended vascular endothelial cells.

Introduction

Retinal neovascularization is an important cause of many oculopathies that lead to vision loss, such as diabetic retinopathy, retinal vein occlusion and retinopathy of premature children[1]. Both lighting and oxygen level are important controlling factors in retinal neovascularization model[2]. Currently, most models used high-oxygen environment, early lighting and strong light irradiation, to stimulate retinal neovascularization, however, retinal neovascularization usually occurred under natural lighting and at natural oxygen level. Therefore, these models are defective. The maturation of the retinal vessels in normal mice typically occurred 2 weeks after birth. This study aims to clarify the effect of light deprivation on retinal neovascularization.

Materials and Methods

Animals

36 neonatal SD rats (male and female, mean weight 12 ± 3 g) were randomly assigned into 3 groups (12 rats in each group), i.e. 10-day group (routine feeding after birth, eyeball enucleation on 10th day), 14-day group (routine feeding after birth, eyeball enucleation on 14th day) and light deprivation group (routine feeding within 1st week after birth, feeding with light deprivation within 2nd week after birth, eyeball enucleation on 14th day). The animals were given 12 h of lightening and 12 h of dark, and the room temperature was 23 ± 2°C. Light deprivation was indicated a brightness < 0.0004 cd/m^2.

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The expression level of VEGF mRNA was measured by RT-PCR by using GAPDH as the internal control. The percentage of the retinal vascular area was calculated by PAS staining, and the number of vascular endothelial cells was counted with a microscope in a double-blind manner.

Methods

RT-PCR

Reagents used were DEPC stock solution (Sigma-Aldrich, St. Louis, MO, USA), Trizol (Invitrogen, Carlsbad, CA, USA), reverse transcription kit (Fermentas, Glen Brunie, MD, USA), PCR reagents (TIANGEN, Shanghai, China). Instruments used were tissue homogenizer (Kimble, Finland,), Nucleic Acid and Protein detector (Bio-Rad, Hercules, CA, USA), qPCR amplifier (Bio-Rad, Hercules, CA, USA). The procedure includes (1) Collection of retinal tissues. Neonatal rats were anesthetized by intraperitoneal injection of 2% chloral hydrate (0.02 ml/g) and, then, were sacrificed for eyeball enucleation. (2) Extraction of RNA. RNA samples were treated with liquid nitrogen, and were homogenized in 200 µl Trizol for 3 min, 300 µl Trizol for 2 min, and 500 µl Trizol for 2 min, sequentially. Then 0.2 ml chloroform was added to 1 ml of homogenate and samples were incubated at room temperature without agitation for 5 min, 12 000 rpm, 4°C, 15 min. After that, the colorless aqueous phase (500 µl) in the middle and upper layer was harvested and transferred into a new tube. Subsequently, an equal volume of isopropanol was added, mixed and incubated at 30°C for 10 min, 12 000 rpm, 4°C, 10 min. The supernatant was discarded. 1 ml of 75% ethanol was added and centrifuged at 7500 rpm at 4°C for 5 min, repeated once. The ethanol was removed and RNA was precipitated for 5-10 min at room temperature. DEPC-treated H_2O (10 µl) was added and incubated at 55-60°C for 10 min. The purity and concentration of extracted RNA were determined by measuring the OD values at 260 nm, 280 nm, 230 nm and 320 nm with a Nucleic Acid and Protein detector. (3) Reverse transcription of RNA. The list of primers is given in Table I. PCR reaction mixture consists of IQ SYBR Green Supermix (10 µl), forward primer (10 µM) 1 µl, reverse primer (10 µM) 1 µl, cDNA (8 µl). PCR Cycle conditions includes 50.0°C for 3 min; 95.0°C, 15 min; 95.0°C, 10 s; 62.5°C, 25 s; 72°C, 25 s; n = 40, Tm 70-95°C, increment 0.5°C, 0.05 s.

Immunohistochemistry (IHC)

Tissues were fixed in formaldehyde solution, acetic acid, absolute ethyl alcohol and distilled water (1:1:1:1). The rats were anesthetized by intraperitoneal injection of 2% chloral hydrate (0.1 ml/10 g). A 27-G syringe needle was used to mark the retina at 12 o'clock of the eyeball, which was enucleated. The optic nerve (3 mm) was left as the location marker for retina. Periocular tissues were removed with a corneoscleral scissors. The eyeball was fixed in fixation solution for 72 h and then dehydrated. Cornea, iris, and lens were removed under a surgical microscope. The optic cup was left and then dehydrated in absolute ethyl alcohol for 30 min twice. After incubation in cedar oil for 72 h and in paraffin (60°C) for 2 h, the eyeball was embedded in sagittal position at 12 o'clock. After cooling, sections (4 µm thick) were made along the plane across 12 o'clock, 6 o'clock and optic nerve. One section was selected from 8 sections for each eyeball, a total of 5 sections was subject to hematoxylin-eosin (HE) staining. The number of vascular endothelial cell nuclei that extended through and connected with the internal limiting membrane was counted by blind analysis of two experts.

PAS Staining of Stretched Preparation

Reagents include PAS staining kit (Sunbiote, Shanghai, China), trypsin (Sigma-Aldrich, St. Louis, MO, USA). For digestion of retina, 72h after fixation in formalin solution, the eyeball was cut along the corneosclera under a surgical microscope, then cornea and lens were removed. The posterior segment of the eyeball was placed in a plate filling with distilled water; retina and

Table I. List of primers used in study.

Gene name	Forward primer	Reverse pimer	PCR product length (bp)
Rat-VEGF	GTGACCACATTCACTGTGAGCCT	GGCTCACCGCCTTGGCTTGT	167
GAPDH	CAGTGCCAGCCTCGTCTCAT	AGGGGCCATCCACAGTCTTC	595

Table II. Relative expression level of VEGF mRNA.

Group	C_t value of internal control	C_t value of VEGF	$2^{-\Delta\Delta}C_t$
10-day group	15.7425 ± 0.4843	15.3133 ± 0.5194	1.0032 ± 0.0835
14-day group	16.0408 ± 0.7015	16.0317 ± 0.7606	0.7544 ± 0.1039
Light deprivation group	17.0233 ± 1.0699	16.4625 ± 1.0052	1.0996 ± 0.0993
F			41.342
p			< 0.001

choroid were bluntly dissected with a scalpel. The optic nerve was cut off with a Venus Thinning scissors. The intact retina was taken out of the optic cup and was placed in a plate filling with distilled water, then was cut radially on eyeball side. The retina was flushed by running water for 24 h, and then the intact retina was incubated in 3% trypsin (10 ml) in 37°C water bath for 2-3 h, with shaking 3-4 times.

The internal limiting membrane with the vitreous body was peeled off and the retina was flipped. The retinal nervous tissues on the capillary network were flipped with whisker and washed. For PAS staining, 5-8 drops of fixation solution were dropped onto the section and were then washed for 5 min followed by drying. PAS solution I was added, then the section was washed for 10 min and dried. PAS solution II was added, the section was incubated in the dark at room temperature for 30 min, then was washed with running water for several minutes. After HE staining for 1-2 min, the section was washed and dried, and was mounted by neutral gum. The retinal vessels were observed under a microscope, the whole retinal area was measured by Image Pro-Plus software, and the percentage of retinal vascular area in the whole retinal area was calculated.

Statistical Analysis

SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Measurement data was represented by mean ± SD. The differences between 3 groups were analyzed by one-way ANOVA, $\alpha < 0.05$ was considered statistically significant. The inter-group comparison was performed by Fishers Least Significant Difference test (LSD test) and Bonferroni's test, 1/3 $\alpha < 0.017$ was considered statistically significant.

Results

Relative Expression Level of VEGF mRNA

As shown in Table II, the relative expression levels of VEGF mRNA in 10-day group and light deprivation group were significantly higher than 14-day group ($p < 0.05$), while the difference between the 10-day group and the light deprivation group was not significant (LSD test: $p = 0.019$; Bonferroni's test: $p = 0.058$).

Number of Extended Vascular Endothelial Cells

As shown in Figure 1, the numbers of extended vascular endothelial cells in the 10-day group and light deprivation group were significantly higher

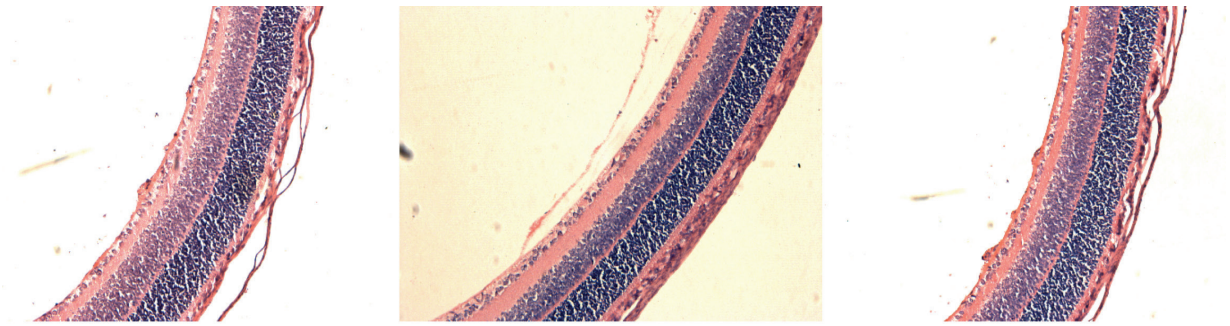


Figure 1. Numbers of extended vascular endothelial cells in 10-day group, 14-day group and light deprivation group (200×).

than the 14-day group [10-day group: (6.2167 ± 1.3630), the 14-day group: (0.9167 ± 0.3129), light deprivation group: (6.1667 ± 1.4792), F = 80.589, $p < 0.001$], while the difference between the 10-day group and light deprivation group was not significant (LSD test: $p = 0.918$; Bonferroni's test: $p = 1.000$).

Percentage of Retinal Vascular Area

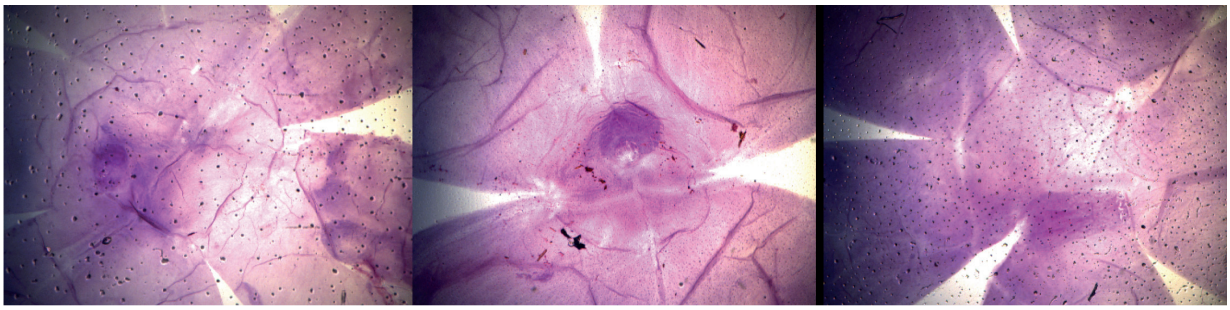
As shown in Figure 2, the percentage of retinal vascular area in 10-day group and light deprivation group was significantly lower than the 14-day group [10-day group: (0.0544 ± 0.0104 %), the 14-day group: (0.1110 ± 0.0133 %), light deprivation group: (0.0523 ± 0.0124 %), F = 91.208, $p < 0.001$], while the difference between 10-day group and light deprivation group was not significant (LSD test: $p = 0.668$; Bonferroni's test: $p = 1.000$).

Discussion

Neovascularization is a complex and multi-step process, involving proliferation and migration of endothelial cells, denaturation and dissolution of the extracellular matrix, the formation of endothelial tubular tissue structure and the growth of pre-existing vessels in a manner of budding or intussusception[3,4]. It is currently believed that ischemia, hypoxia and the change of shear stress on vascular wall by blood flow could trigger accumulation of inflammatory cells around vascular wall, induce secretion of cytokines, such as VEGF, matrix metalloproteinase-9, NF-κB and intercellular adhesion molecule-1, leading to proliferation, differentiation and migration of endothelial cells and vascular smooth muscle cells. As a result, vessels would bud and the new vascular network formed[5,6]. It was reported that retinal neovascularization was attributed to the combination of angiogenic stimulators, inhibitors, and antagonists[7].

Many animal models of retinal neovascularization had been established so far. Laser photocoagulation required large animal and a long time to induce retinal neovascularization, and may cause retinal vein occlusion[8]. Transgenic mouse model of retinal neovascularization is difficult to establish[9]. Animal models of vascular proliferative retinal neovascularization induced by oxygen induction[10] or carbon-dioxide acidosis had also been reported[11]. It had been demonstrated that rat hyaloid artery was still robust 7 days after birth, retinal vessels extended with development. The maximally developed vitreal vessels regressed, the development of the largest retinal vessel was not completely balanced, at this time, the development of retinal vessels was mostly close to that of premature[12]. Shi et al[13] established a model of retinal neovascularization by changing the duration of natural lighting; this method was characteristic of mild stimulation, the survivor rate was mostly close to that during the pathophysiological process in human retinal neovascularization.

This work demonstrated that the relative expression levels of VEGF mRNA in the 10-day group and light deprivation group were significantly higher than the 14-day group, the percentage of retinal vascular area in the 10-day group and light deprivation group was significantly lower than the 14-day group. VEGF was an extensively studied cytokine that was widely believed to be able to stimulate proliferation of vascular endothelial cells and neovascularization specifically. High-affinity receptors of VEGF were more abundant in retinal vascular endothelial cells than other endothelial cells[14]. VEGF was modulated by local oxygen level; hypoxia could induce high expression of VEGF, which could be pro-



10-day group (x100) 14-day group (x100) Light deprivation group (x100)

Figure 2. Percentage of retinal vascular area for different groups (100×).

duced and secreted in retinal vascular endothelial cells, pigment epithelial cells, perithelial cells and Müller cells[15]. VEGF could not only induce proliferation and migration of retinal endothelial cells, but also induce retinal neovascularization. The level of VEGF was high in patients with retinal neovascularization. Blocking the production and bioactivity of VEGF would treat retinal neovascularization[16,17]. Normally, retinal internal limiting membrane was homogenous, without any special structures or cells. Normal retinal vessels could not extend through the internal limiting membrane, the vessels extended through the internal limiting membrane and into vitreous chamber were considered neovessels. The development of retinal vessels in the 14-day group may be mature; thus, the number of extended vascular endothelial cells was significantly decreased.

Conclusions

Light deprivation delayed the growth of retinal neovessels, thus could be used to establish a stable model of retinal neovascularization.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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