• Basic Research •

# Acetylcholinesterase inhibition ameliorates retinal neovascularization and glial activation in oxygen-induced retinopathy

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## Abstract

● **AIM:** To investigate whether inhibition of acetylcholinesterase (AChE) by donepezil ameliorate aberrant retinal neovascularization (RNV) and abnormal glial activation in oxygen-induced retinopathy (OIR).

● **METHODS:** A mouse model of RNV was induced in postnatal day 7 (P7) mice by exposure to 75% oxygen. Donepezil was administrated to P12 mice by intraperitoneal injection. Expression and localization of AChE in mouse retinas were determined by immunofluorescence. RNV was evaluated by paraffin sectioning and hematoxylin and eosin (HE) staining. Activation of retinal Müller glial cells were examined by immunoblot of glial fibrillary acidic protein (GFAP). rMC-1, a retinal Müller cell line, was used for *in vitro* study. Expression of hypoxia-induced factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) were determined by Western-blot analysis, enzyme-linked immunosorbent assay (ELISA) or immunostaining.

● **RESULTS:** Aberrant RNV and glial activation was observed after OIR. Of note, retinal AChE was mainly expressed by retinal Müller glial cells and markedly increased in OIR mice. Systemic administration of donepezil significantly reduced RNV and abnormal glial activation in mice with OIR. Moreover, ischemia-induced HIF-1α accumulation and VEGF upregulation in OIR mouse retinas and cultured rMC-1 were significantly inhibited by donepezil intervention.

● **CONCLUSION:** AchE is implicated in RNV with OIR. Inhibition of AChE by donepeizl is likely to be a potential therapeutic approach for retinal neovascular diseases.

● **KEYWORDS:** acetylcholinesterase; oxygen-induced retinopathy; neovascularization; glosis; mice

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## INTRODUCTION

Pathological retinal neovascularization (RNV) is wildly implicated in a variety of eye disorders, such as retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and retinal vasculitis with subsequent non-perfusion area[1]. Neovessel outgrowth is commonly potentiated by tissue ischemia and upregulation of vascular endothelial growth factor (VEGF).

ROP is the major cause of visual impairment in premature infants[2]. In ROP, immature retinal vasculature was arrested by supplemental oxygen therapy for respiratory support; thus, the peripheral avascular area become ischemic and triggered pathological neovascularization, which may lead to retinal hemorrhage, fibrovascular proliferation, tractional retinal detachment and even permanent vision loss. Currently, laser coagulation for peripheral retina ablation and intravitreous injection of anti-VEGF agents were recommended for high-risk prethreshold ROP and aggressive posterior ROP, respectively[3]. However, side effect after laser ablation such as depressed foveal development and high myopia susceptibility and disruption of normal vessel homeostasis after anti-VEGF agents may compromised therapeutic expectation[4-5]. Thus, to identify the key factor contributing to RNV is quite critical for seeking the novel treatment medication.

Müller cells, the most abundant glial cells in the retina, span across the entire thickness of the retinas. Müller cell is activated by pathological changes of the retina, which is called Müller cell gliosis[6]. Müller cell gliosis is characterized by increased expression of glial fibrillary acidic protein (GFAP), one of the intermediate filament proteins and decreased expression of glutamine synthetase (GS), a Müller cell-specific enzyme involved in recycling of neurotransmitter[7]. Retinal capillaries were ensheathed by Müller cells. Previous

studies suggested that VEGF released by activated Müller cells may induce retinal vascular leakage and neovascularization, suggesting a close association of reactive Müller cell gliosis with retinal vascular dysfunction[8].

Apart from its enzymatic function in hydrolyzing acetylcholine, acetylcholinesterase (AChE) was demonstrated to be closely associated with cell proliferation, migration, neurite outgrowth, apoptosis and angiogenesis[9-12]. However, there is limited evidence as to whether AChE is involved in RNV. Donepezil is a specific and reversible inhibitor of AChE and used for treatment of Alzheimer's disease (AD)[13]. A previous study demonstrated donepezil inhibited beta-amyloid-induced microglial activation[14]. However, whether donepezil impacts retinal gliosis remains to be elucidated.

In the present study, we investigated expression and localization of AChE in retinas of oxygen-induced retinopathy (OIR) mice, with a specific focus on the preventive and potential mechanism of AChE inhibition on aberrant neovascularization and abnormal glial activation in pathogenesis of OIR.

## MATERIALS AND METHODS

**Ethical Approval** All animal experiments were performed according to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of Animals in Ophthalmic and Vision Research and protocols approved by the Institutional Animal Care and Use Committee at Nanchang University.

**Animals** OIR was setup by exposing mouse pups with their nursing mom to 75% oxygen from postnatal day (P) 7-12. At P12, the neonatal mice were returned to room air and given daily intraperitoneal injection of donepezil [15 mg/mL in phosphate buffer saline (PBS), Sigma-Aldrich, St. Louis, USA] at dose of 60 mg/kg. Mice were administrated with PBS as vehicle control. At P17, mice were humanly euthanized and subjected to biomedical assays.

**Paraffin Sectioning and Hematoxylin and Eosin Staining** Nucleated eyeballs were immersed in Perfix (4% paraformaldehyde, 20% isopropanol, 2% trichloroacetic acid, 2% zinc chloride) for 24h. The eyeballs were embedded in paraffin and sagittal cut through the cornea parallel to the optic nerve with microtome (Leica Biosystems, Wetzlar, Germany). The sections were stained hematoxylin and eosin (HE) and photographed under light microscopy (Olympus, Germany). Preretinal neovascular nuclei eye were counted.

**Immunofluorescence** Mouse eyes were briefly fixed in 4% paraformaldehyde (PFA; Electron Microscopy Science, Hatfield, PA, USA). After cornea, iris and lens were dissected, the eyecups were further fixed with 4% PFA and cryoprotected with sequential immersion in 10%, 20%, and 30% sucrose. The eyecups were then embedded in Tissue-Tek Optimal Cutting Temperature (OCT, Sakura Finetek, Torrance, CA, USA) and frozen by immersion in dry ice-cold alcohol. Sections in thickness of 7 microns were harvested with Cryostat (Leica) and mounted on slides. The sections were blocked and permeabilized with 10% normal donkey serum and 1% Triton X-100 in PBS for 1h. The species were incubated with mouse anti-AChE (Thermofisher Scientific, Waltham, MA, USA) and rabbit anti-GFAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-GS (Abcam, Cambridge, MA, USA), rabbit anti-Iba1 (Wako, Richmond, VA, USA) and immune-detected with Alexa Fluor 488 donkey anti-mouse (Thermofisher Scientific) and Alexa Fluor 594 donkey anti-rabbit secondary antibodies (Thermofisher Scientific). The slides were cover slipped with VECTASHIELD anti-fade mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were acquired with fluorescence microscopy (Olympus, Germany).

**Enzyme-linked Immunosorbent Assay** Briefly, retinas were dissected and grinded by mortar and pestle in liquid nitrogen. Protein was extracted in PBS. Protein concentration was quantified by BCA (Thermofisher Scientific) method. Mouse retinal VEGF content was measured by VEGF Quantikine Enzyme-linked Immunosorbent Assay (ELISA) Kit (R&D systems, Minneapolis, MN, USA) following manufacturer's instruction.

**Cell Culture** rMC-1, a rat retinal Müller cell line, were kindly gifted by Dr Vijay Sarthy (Northwestern University, Evanston, IL) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS; Thermofisher Scientific) and 1% antibiotic/antimycotic (Thermofisher Scientific). After quiescent in DMEM with 1% FBS, rMC-1 were preincubated with different doses of donepezil (Sigma-Aldrich) for 2h and then treated with cobalt chloride (CoCl2, Sigma-Aldrich) for 24h to mimic hypoxic condition. Cells were harvested for immunocytochemistry or Western-blot analysis.

**Immunocytochemistry** rMC-1 grown on culture coverslips with desired treatment were fixed with 10% formaldehyde at room temperature for 30min. After permeabilization with 0.5% Triton X-100 in PBS on ice, the cells were blocked with 10% normal donkey serum for 1h at room temperature and incubated with rabbit anti-HIF-1α (Novus Biologicals, Littleton, CO, USA) for overnight at 4℃. The cells were rinsed with PBS and immunostained with Alexa Fluor 488 donkey anti-rabbit (Thermofisher Scientific) for 1h at room temperature. Nuclei were counterstained with DAPI. Immunosignals were imaged under fluorescence microscopy (Olympus, Germany).

**Western-blot Analysis** Dissected retinas or cultured cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Roche, Applied Science,

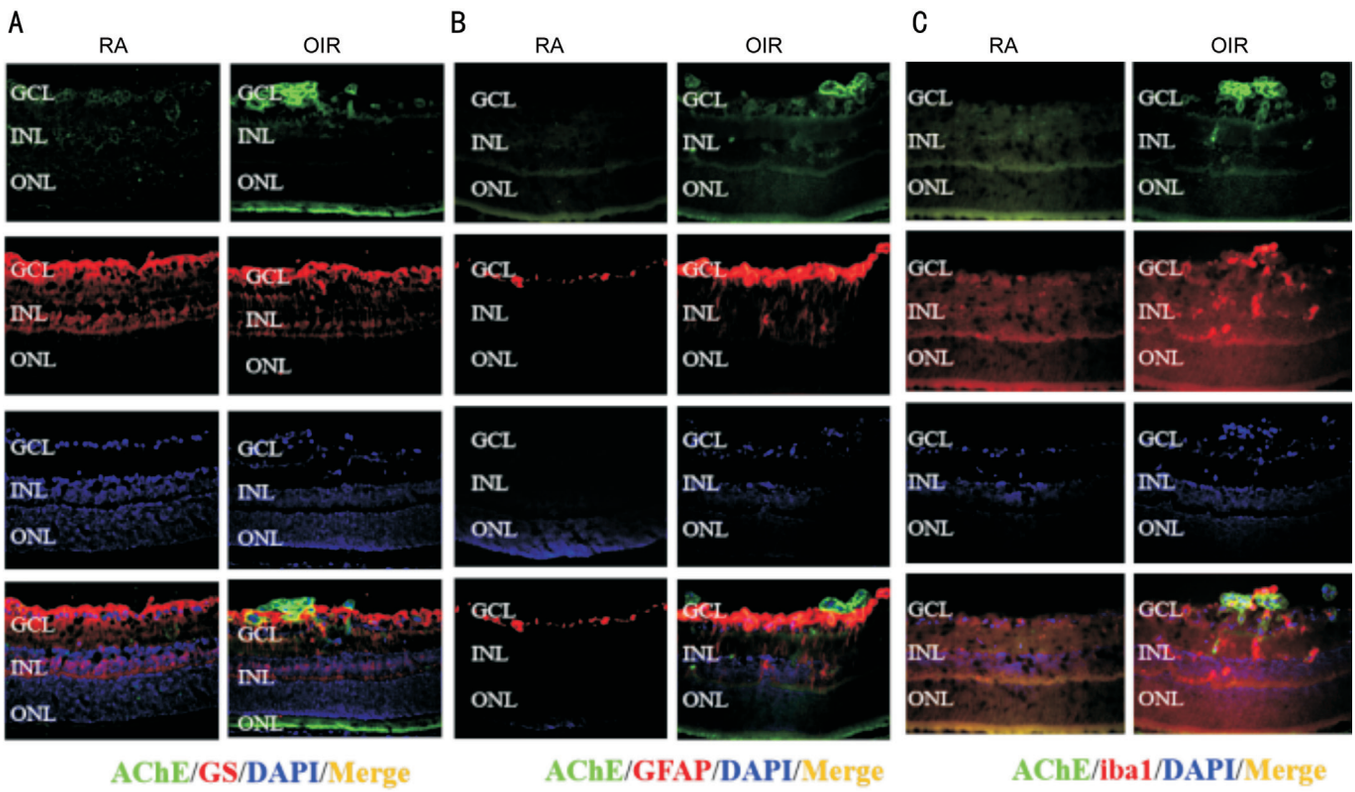


**Figure 1 Upregulation of AChE in retinal Müller cells in OIR mice** OIR was induced by exposing neonatal mice to 75% oxygen from P7 to P12. Expression of AChE in the retinas were examined by immunostaining at P17. Müller cell and microglia activation were determined with anti-GFAP and anti-Iba1 staining, respectively. Increased AChE immunoreactivity was found in OIR retinas. A, B: Immunofluorescence showed that AChE (green) were partially colocalized with Müller cell marker GS (A, red) and GFAP (B, red). Decreased GS immunoreactivity (A) and increased GFAP immunoreactivity (B) were observed in OIR retinas compared with room air retinas. C: Immunofluorescence showed that AChE (green) were not coimmunostained with microglia marker Iba1 (red). The representative images were taken from 6 different animals.

Mannheim, Germany). Twenty-five micrograms of total protein were separated on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blotted with following primary antibodies: rabbit anti-GFAP (Santa Cruz), rabbit anti-HIF-1α (Novus Biological) and anti-VEGF (Abcam) at 4°C for overnight. After washing with tris buffered saline tween (TBST) for three times, the blots were incubated with horseradish peroxide (HRP)-conjugated secondary antibodies (Vector Laboratories) at room temperature for 1h. Signals were detected with SuperSignal West Dura Substrate (Thermofisher Scientific) using Bio-imaging System (Syngene, Frederick, MD, USA). The same membranes were stripped and re-blotted with anti-β-actin (Sigma-Aldrich) as internal control. The bands were semi-quantified with Genetool (Syngene) by densitometry.

**Statistical Analysis** Data were expressed as mean±standard derivations. Difference among multiple groups were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's post hoc test and using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). Comparison between two groups were performed by paired student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

**Acetylcholinesterase mainly Expressed by Retinal Müller Glials and Upregulated in OIR Mouse** OIR was a commonly used animal model to study RNV. To investigate whether AChE is involved in regulating aberrant RNV, we first examined the expression and localization of AChE in retinas of mice with OIR. Immunofluorescence staining demonstrated that AChE is faintly expressed in retinas of room air (RA) mice (Figure 1A-1C), which was markedly increased in that of OIR mice (Figure 1A-1C). Intriguingly, the immunosignal of AChE was distributed across the entire retinal layers in the retinas of RA mice. Müller cells, the principal glia in retinas, span through the whole retinal tissue and facilitate angiogenesis. Thus, we hypothesized that AChE may be prominently expressed by Müller cells and involved in regulating retinal angiogenesis. To test this hypothesis, we performed double-staining experiment with anti-AChE antibody and anti-GS antibody. In retinas, GS is exclusively expressed by Müller cells and catalyze glutamine synthesis for retinal neuron excitation. As shown in Figure 1A, immunosignals of AChE was partially colocalized with GS in RA retinas, suggesting that AChE was expressed by Müller cells. Moreover, GS was

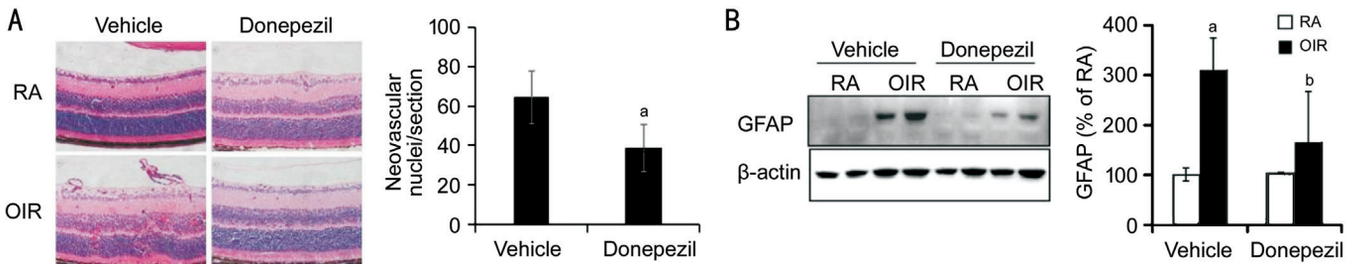


**Figure 2 Inhibitory effect of donepezil on RNV and Müller gliosis in OIR** Donepezil were peritoneally injected to mouse pups from P12 to P17. A: RNV were quantified by counting pre-retinal vascular nuclei on HE-stained paraffin section; B: The expression of GFAP in retinas were determined by Western-blot analysis. $n$=6, $^aP$<0.01 *vs* RA+vehicle; $^bP$<0.01 *vs* OIR+vehicle.

downregulated in OIR retinas, which may lead to glutamate loss of retinal neurons and indicates the detrimental gliosis. To further verify whether AChE is involved in retinal Müller gliosis, we double stained the retina with anti-AChE and anti-GFAP antibodies. As, Müller cell displayed colocalization of positive AChE and GFAP immunosignals. GFAP fluorescence was confined in the ganglion cell layer (GCL) in RA retinas and dramatically increased in OIR retinas. Moreover, GFAP immunoreactivity extending from their inner processes throughout the soma toward to outer process, suggesting activation of Müller glia in OIR. In addition, double staining with anti-Iba1 antibody indicated barely colocalization of AChE with microglial marker. All these suggested that AChE is expressed by Müller glial cells and upregulation of AChE during OIR may be associated with Müller gliosis and RNV.

**Inhibition of AChE by Donepezil Attenuated Retinal Neovascularzation and Müller Gliosis in OIR** To test whether upregulation of AChE promotes RNV and Müller gliosis, we treated the OIR mice with donepezil, a specific AChE inhibitor, from P12 to P17. Neovascular formation between vitreous and retina interface were examined by HE staining. Donepezil significantly suppressed preretinal neovascularization in OIR mice as shown in Figure 2A. Meanwhile, intraretinal hemorrhage within the inner nuclear layer (INL) and outer nuclear layer (ONL) were detected in vehicle treated OIR mice, indicating severe retinal vascular damage. Treatment with donepezil dramatically ameliorated OIR-induced intraretinal hemorrhage. In addition, a robust increase of GFAP were observed in vehicle-treated OIR retinas, suggesting Müller glial activation. Invention with donepezil significantly inhibited GFAP upregulation in OIR mice. Collectively, inhibition of AChE by donepezil could benefit OIR by reducing RNV and Müller gliosis.

**Suppression of AChE by Donepezil Inhibited HIF-1α Activation and VEGF Expression** Previous studies demonstrated that the activated Müller cell is a major source of VEGF production in ischemic retinopathy including ROP and diabetic retinopathy (DR). HIF-1α, the pivotal transcription factor, regulates tissue response towards hypoxia and VEGF expression. We evaluated whether inhibition of AChE by donepezil affected accumulation of HIF1-α. As shown in Figure 3, HIF-1α was significantly increased in retinas of OIR mice treated with vehicle. Injection of donepezil abolished hypoxia-induced HIF-1α expression in OIR retinas. Moreover, we found the immunoreactivity of VEGF were increased in retinas of vehicle treated OIR mice. This increase was attenuated by donepezil as shown in Figure 3B. Finally, suppression of hypoxia-induced VEGF in OIR retinas by donepezil treatment was also confirmed by ELISA assay (Figure 3C).

**Donepezil Ameliorated Hypoxia-induced HIF-1α Stabilization and Nuclear Accumulation as well as Downstream VEGF expression** Finally, the inhibitory effect of donepezil on hypoxia-induced HIF-1α activation were assessed by cultured rMC-1. We found that treatment with $CoCl_2$ to induce hypoxia resulted in a robust cellular accumulation of HIF-1α, which was significantly suppressed by donepezil (Figure 4A). In addition, immunocytochemistry study showed that HIF-1α is weakly expressed by rMC-1 under normoxia condition. $CoCl_2$ treatment induced a significantly accumulation and translocation of HIF-1α in the nuclei. Treatment with donepezil greatly blocked hypoxia-induced HIF-1α nuclei translocation. Consistently, donepezil significantly blocked hypoxia-induced VEGF expression in rMC-1. Taken together, inhibition of AChE by donepezil attenuated VEGF expression through attenuation of HIF-1α activation.

## DISCUSSION

The function of AChE is not restricted to catalyze acetylcholine hydrolysis, but also involved in several biological processes that are associated with neurite growth, angiogenesis, and tumor development. However, its role in ischemic retinopathy remains to be elucidated. In the present study, we, for the first time, demonstrated AChE was colocalized with Müller glial markers and upregulated by OIR. Inhibition of AChE by donepezil significantly attenuated hypoxia-induced RNV and Müller gliosis, suggesting noncatalytic properties of AChE on ischemic retinas.

AChE has three different isoforms generated by alternative pre-mRNA splicing[15]. The AChE isoforms differ in multimeric

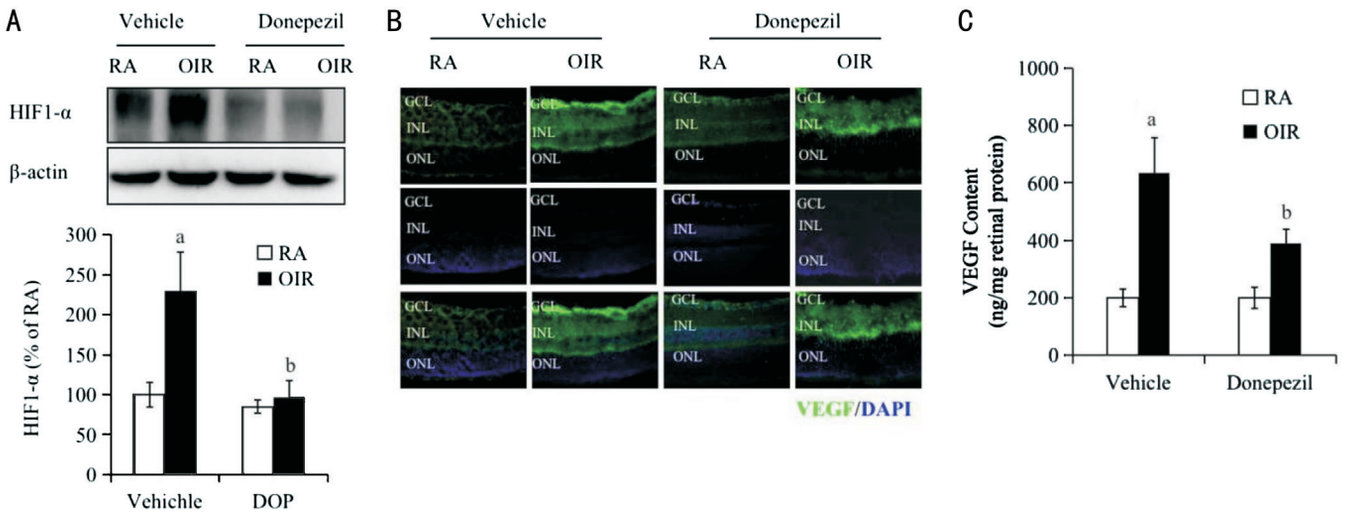


**Figure 3 Reduction of HIF-1α and VEGF expression retinas of OIR mice treated with donepezil** Mouse pups received daily peritoneal injection of donepezil from P12 to P17. A: Expression of HIF-1α in mouse retinas were examined by Western-blot analysis. B, C: Retina distribution and content of VEGF were detected by immunofluorescence (B) and ELISA (C), respectively. The representative images were taken from 6 different animals. $n$=6, $^aP$<0.01 *vs* RA+vehicle; $^bP$<0.01 *vs* OIR+vehicle.

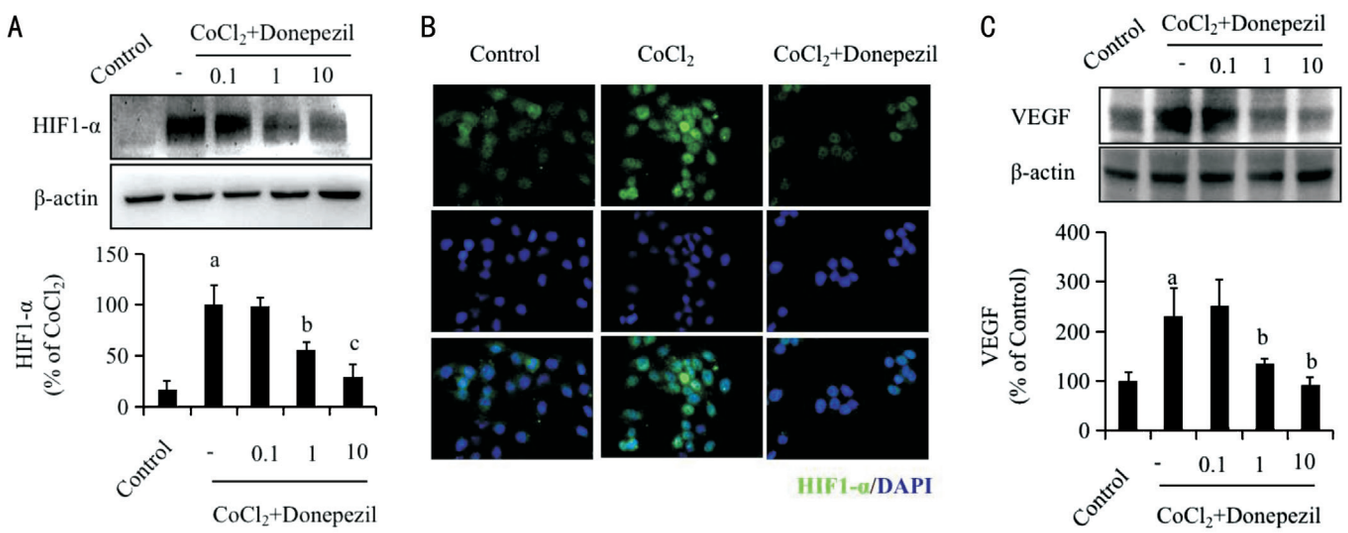


**Figure 4 Attenuation of HIF-1α activation and VEGF expression by donepezil in culture rMC-1** rMC-1 were treated with $CoCl_2$ in presence or absence of different doses of donepezil. A: Expression of HIF-1α were determined by Western-blot analysis. B: Cellular distribution of HIF-1α was examined by immunocytochemistry. C: Expression of VEGF were detected by Western-blot analysis. The representative images were taken from 3 independent experiments. $n$=3, $^aP$<0.01 *vs* control; $^bP$<0.05, $^cP$<0.01 *vs* $CoCl_2$.

assembly, membrane-associated pattern, tissue distribution and cellular localization. Overexpression of AChE stimulated neurite outgrowth on cultured R28, a retinal precursor cell line[16]. Notably, AChE is a membrane bound protein without transmembrane and intracellular domain; thus, it requires an anchor protein for membrane association. Co-overexpression of AChE and the anchor protein, PRiMA facilitated patchy-like localization of AChE to the cell membrane and strikingly promoted dendrites sprouting from multiple membrane sites[17]. Moreover, AChE interacted with the scaffold protein RACK1 to recruit PKC[18]. Activation of PKC is reported to be involved in astrogliosis[19]. All these findings provided a possible explanation how AChE induces active gliosis under pathological conditions.

Our *in vitro* evidence suggested that suppression of AChE in cultured Müller cell decreased hypoxia-induced VEGF expression. Müller cells as the most abundant glia in the retina regulate neovascularization, vascular leakage, inflammation during ischemic retinopathy through secretion of various factors such as VEGF[20]. VEGF is a potent angiogenic factor. Retinal Müller cell is a major source of VEGF[8]. First, *in situ* hybridization and immunohistochemistry demonstrated that VEGF was localized to the cell bodies of the INL in OIR retinas. The VEGF expression cell displayed Müller cell morphology. Conditional knockout Müller-derived VEGF in mouse led to 50% reduction of total retinal VEGF level, but

no detectable retinal abnormality, indicating Müller cell is a major VEGF producer in retina[8]. Importantly, OIR mice with conditional knockout of VEGF in Müller cell showed 40% reduction of RNV compared to wild type littermate controls[8], which further suggested that Müller cell-derived VEGF is a major contributor to RNV. VEGF secretion is regulated by multiple transcription factors. HIF-1α plays a central role in transcription of VEGF especially under hypoxia conditions[21]. In this study, we demonstrate that donepezil inhibited HIF-1α expression in OIR retinas and diminished its nuclear accumulation in Müller cell treated with hypoxia. All these suggested that inhibition of AChE by donepezil suppressed RNV and VEGF production at least partially through attenuation of HIF-1α activation.

Retina has a tightly orchestrated structure and the process of Müller cells cover the blood vessels. Thus, Müller cells plays a pivotal role in maintaining homeostasis of glia-vascular unit. Previous studies demonstrated that Müller cell could modulated angiogenesis-related activities of retinal endothelial cell including proliferation, migration and angiogenesis[6]. Müller cell, a potent source of angiogenic factors, regulates angiogenesis *via* paracrine mechanisms, as we just mentioned above. Of note, Müller cell may also affect the biological function of its immediate vessels *via* the extracellular environment. Previously, AChE has been reported to bind with extracellular matrix (ECM) laminin-1 and collagen IV[22-23]. Meanwhile, laminin-1 and collagen IV are two major components of mural basement membrane. Laminin were shown to mediate crosstalk between glial cells during retinal angiogenesis[24]. Collagen IV is the most abundant protein in basement membrane and associated with angiogenesis. Moreover, both laminin 1 and collagen IV could bind to integrin expressed by endothelial cells[25]. Collectively, we speculated that upregulated AChE in Müller glia may also promotes aberrant RNV through both VEGF-dependent and/or ECM dependent mechanism. However, AChE mediated cross talk between Müller glial and endothelial cells in ischemic retinas required future experiments to verify.

Donepezil exerted its therapeutic effect on AD by improving patients' cognitive function[13]. Recent study reported that donepezil significantly attenuated blood flow recovery and capillary density in mice after hindlimb ligation, unraveling donepezil's inhibitory effect on angiogenesis[12]. Here, we have proved that treatment with donepezil suppressed ischemic induced RNV and detrimental gliosis, suggesting the therapeutic potential of donepezil for ischemic retinopathy.

Taken together, our study demonstrated that AChE is dominantly expressed by retinal Müller glial cells and increased AChE expression in OIR retinas promotes Müller glial activation and aberrant angiogenesis, suggesting a proangiogenic effect of AChE in ischemic retinopathy such as ROP.

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**Conflicts of Interest: Liu QP,** None; **Zhang X,** None; **Qin YZ,** None; **Yi JL,** None; **Li JM,** None.

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