·Basic Research·

The protective role of tacrine and donepezil in the retina of acetylcholinesterase knockout mice

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Abstract

• **AIM: To determine the effect of different concentrations of the acetylcholinesterase (AChE) inhibitors tacrine and donepezil on retinal protection in AChE$^{+/-}$ mice (AChE knockout mice) of various ages.**

• **METHODS: Cultured ARPE-19 cells were treated with hydrogen peroxide (H_2O_2) at concentrations of 0, 250, 500, 1000 and 2000 μmol/L and protein levels were measured using Western blot. Intraperitoneal injections of tacrine and donepezil (0.1 mg/mL, 0.2 mg/mL and 0.4 mg/mL) were respectively given to AChE$^{+/-}$ mice aged 2mo and 4mo and wild-type S129 mice for 7d; phosphate buffered saline (PBS) was administered to the control group. The mice were sacrificed after 30d by *in vitro* cardiac perfusion and retinal samples were taken. AChE-deficient mice were identified by polymerase chain reaction (PCR) analysis using specific genotyping protocols obtained from the Jackson Laboratory website. H&E staining, immunofluorescence and Western blot were performed to observe AChE protein expression changes in the retinal pigment epithelial (RPE) cell layer.**

• **RESULTS: Different concentrations of H_2O_2 induced AChE expression during RPE cell apoptosis. AChE$^{+/-}$ mice retina were thinner than those in wild-type mice ($P<0.05$); the retinal structure was still intact at 2mo but became thinner with increasing age($P<0.05$); furthermore, AChE$^{+/-}$ mice developed more slowly than wild-type mice ($P<0.05$). Increased concentrations of tacrine and donepezil did not significantly improve the protection of the retina function and morphology ($P>0.05$).**

• **CONCLUSION: *In vivo*, tacrine and donepezil can inhibit the expression of AChE; the decrease of AChE expression in the retina is beneficial for the development of the retina.**

• **KEYWORDS:** retinal pigment epithelium; acetylcholinesterase; apoptosis; AChE$^{+/-}$ animal models; acetylcholinesterase inhibitors
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INTRODUCTION

Age-related macular degeneration (AMD) is the most common cause of severe loss of vision in developed countries [1]. It is a progressive, delayed, degenerative disease that affects the central vision. Apoptosis is considered to be one of the causes of retinal pigment epithelium (RPE) death in AMD [2], but the specific molecular mechanisms and signalling pathways have yet to be described[3].

Acetylcholinesterase (AChE, EC 3.1.1.7) is a type of β carboxylesterase, which exists in cholinergic synapses and neuromuscular junctions; it is capable of rapid hydrolysis and inactivation of the neurotransmitter acetylcholine (ACh) thus terminating the cholinergic transmission signal [4,5]. Previous studies have shown that during the process of induced apoptosis, AChE will induce the expression in various types of cells [6], suggesting that it may be involved in AMD in the pathogenesis of RPE cell apoptosis [7]. We found that hydrogen peroxide (H_2O_2) induced apoptosis of adult RPE cells (ARPE-19), and expression of AChE was also increased as well as the apoptosis marker poly (ADP ribose) polymerase (PARP).

To further confirm that AChE is indeed involved in the process of apoptosis in ARPE-19 cells, we inhibited the expression and activity of AChE in DNA and protein expression levels [8] and found that H_2O_2-induced apoptosis was reduced. To further study the function of AChE in RPE cell apoptosis *in vivo*, we used a heterozygous AChE knockout mouse model (AChE$^{+/-}$ mice)[9] (purchased from Jackson Laboratory). AChE$^{+/-}$ mice exhibit many age related symptoms of organophosphate poisoning including body

tremors, abnormal gait, restlessness, muscle weakness. Homozygous AChE$^{-/-}$ mice also showed photoreceptor degeneration, resulting in the defects of the retina (http://jaxmice.jax.org/query). The retina of AChE$^{+/-}$ mice is thinner than that of wild-type (WT) mice, it develops more slowly, and pred is posed to age-related retinal diseases[10].

The study found that, in contrast with WT mice, AChE$^{+/-}$ mice have reduced AChE expression and also express fewer apoptosis related proteins in RPE cells. It is thought that reduced AChE expression in the mouse retina has a protective effect. In the normal phosphate buffered saline (PBS) control group, there was no significant difference in the RPE cell structure. We can infer that AChE is indeed involved in the process of apoptosis through H_2O_2-induced apoptosis of RPE cells in the *in vitro* model. Particularly noteworthy is that lack of AChE expression or inhibition of the activity can protect cells from apoptosis in AChE$^{+/-}$ mice models to some extent[11].

Therefore, in this study we focused on the evaluation of AChE expression in the retinal pigmented layer along with the change in expression of AChE during the process of apoptosis *in vitro* and the effect of AChE inhibitors (AChEI) in the animal model.

MATERIALS AND METHODS

Materials AChE knockout mice (stock number 005987) were purchased from the Jackson Laboratory (Bar Harbor, Maine, Sacramento, California, USA). WT and AChE$^{+/-}$ mice of different ages were used for the experiments. Animals were housed under standard conditions of a light and dark cycle with free access to food and water. ARPE-19 was obtained from the American Type Culture Collection (ATCC; Rockville, MD). H_2O_2 was purchased from the Shanghai CASB (Shanghai Academy Biotechnology Research Center, China). Rabbit polyclonal anti-cleaved PARP antibodies (human specific #9541; rat specific #9545) were purchased from Cell Signaling Technology (Beverly, MA, USA). The mouse monoclonal anti-β-actin antibody (A2228) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The AChE monoclonal antibody used to detect endogenous levels of the 68 kDa fragment of human AChE was from BD Biosciences (San Jose, CA, USA). Rhodamine coupled anti-mouse IgG and fluorescein isothiocyanate (FITC) coupled anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell pellets were suspended in a TUNEL reaction mixture (Roche Diagnostics Corporation Indianapolis, IN, USA) for 1h and stained with Hoechst 33258. Stained cells were studied using a Nikon fluorescence microscope (Nikon Inc.).

Methods

Western blot analysis RPE cells were homogenized using 200 μL of lysis-buffer (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin and 10 μg/mL aprotinin) on ice. The protein concentration of the sample was determined using the Bradford assay. Equal amounts (50 μg) of protein from each sample were loaded for SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA) at 100 mA for 2h. After blocking of nonspecific binding sites with 5% skim milk for 1h, the membrane was incubated with primary antibodies with the following dilutions: mouse monoclonal antibody to AChE at 1:200 (Santa Cruz, CA, USA) and rabbit polyclonal antibodies to PARP-1 at 1:200 overnight at 4℃. Antibody dilutions were made in a solution of 5% skim milk/0.1% tris-buffered saline (TBS) with Tween-20. Subsequently, membranes were washed with TBS and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Bioss Biotechnology, Beijing, China) in a blocking buffer for 1h at room temperature. After four washes, the proteins were detected using an ECL kit (Thermo Fisher, USA). β-actin staining served as the internal standard for all membranes.

Hematoxylin & eosin staining Slides were deparaffinized and went through a series of xylene and alcohol wash. Then Harris hematoxylin solution was used to stain the nucleus, while eosin-phloxine solution was used for counterstaining. Slides were mounted with xylene based mounting medium.

Double staining of acetylcholinesterase and TUNEL assays To ensure that the AChE protein did indeed exist in apoptotic cells, we performed immunohistochemical double staining for AChE and TUNEL reaction. Briefly, after 20min fixation at 4℃ with paraformaldehyde solution (3% in PBS, pH 7.4), the harvested cells were washed three times with TBST [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.1% Tween]. The cells were incubated with 1 mL of blocking buffer (5.5% normal goat serum in TBST) for 45min and then incubated with 100 μL of primary antibody [1:100 dilution in TBST containing 2% bovine serum albumin (BSA)] for 24h at 4℃. Following incubation, the cells were washed and incubated with 100 μL of secondary antibody (1:100 dilution, rhodamine conjugated anti-mouse IgG-R, Santa Cruz, CA, USA) for 60min at 37℃ in the dark. The cells were then washed and re-suspended in a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2min on ice. After washing, the pellet was re-suspended in 50 μL TUNEL reaction mixture (Roche) and incubated for 60min at 37℃ in the dark. The labelled cells were then washed, transferred onto glass slides and observed under a fluorescence microscope (OLYMPUS).

Identification of acetylcholinesterase -deficient mice AChE-deficient mice were identified by PCR analysis [11] using specific genotyping protocols obtained from the Jackson Laboratory website. PCR primers were as follows: oIMR4266, 5'-AAC ATT GGC CGC CTC CAG CTC-3';

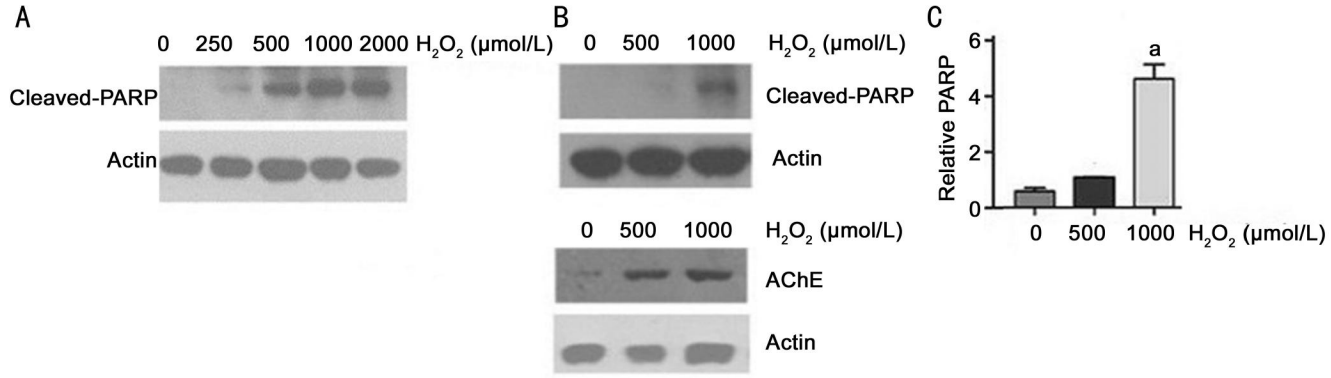

Figure 1 After different concentrations of H_2O_2 were incubated for 2h, the AChE protein was detected by Western blot At higher concentrations of H_2O_2, expression of cleaved-PARP (A, B) and AChE increased (C) Western blot grey values were quantitatively analyzed (ImageJ software); [a] P<0.05 was considered as statistically significant.

oIMR4267, 5'-TGG AAG GTG CCA CTC CCA CTG-3'; oIMR4268, 5'-AAT GAC ACC GAG CTG ATA GCC-3'; oIMR4269, 5'-CCA GTA TTG ATG AGA GCC TCC G-3' (http://jaxmice.jax.org/protocolsdb/f?p=116:2:0::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:13279,005987).

Tacrine and donepezil effect on the retina of acetylcholinesterase –deficient mice PBS containing the preconfigured concentrations of tacrine and donepezil was injected according to body weight into mice once a day for seven days, using a sterile intraperitoneal method; the injection time was 30s. Throughout the process, no experimental mice died, thus 54 mice were used in the final statistical analysis. All animal procedures were approved by the Nanchang University Eye Hospital Animal Ethics Committee. Specific pathogen free (SPF) mice of different ages were randomly divided into the following groups, each consisting of 18 animals:

PBS control group: 4mo, 2mo, WT.

Tacrine group (tacrine concentrations were 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, each concentration included 4-mo- and 2-mo-old mice).

Donepezil group (donepezil concentrations were 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, each concentration included 4-mo- and 2-mo-old mice).

Statistical Analysis All experiments were repeated at least three times. Data were expressed as means±SEM for the four replicate samples. The results were analysed using the Student's unpaired t-test, using the software SPSS16.0 analysis the significant differences between means, or by two-way ANOVA followed by a least-significance procedure, to determine the significance of the response. P< 0.05 was considered significant.

RESULTS

Induction of Acetylcholinesterase Expression During Apoptosis Triggered by Hydrogen Peroxide Western blotting indicated that the AChE protein was slightly expressed in normal human RPE cells. After 2h, the expression of AChE was slightly enhanced in the 500 μmol/L H_2O_2 group and significantly enhanced in the 1000 μmol/L H_2O_2 group (t=7.358, P=0.032). PARP cleavage was found starting at 2h after treatment (Figure 1A, 1B). Relative intensity was calculated as the ratio to actin control. A comparison of PARP expression in the 500 μmol/L H_2O_2 group, the 1000 μmol/L group and the control group, showed that PARP relative expression increased by statistically significant levels (t=10.714, P=0.014; Figure 1C).

We treated RPE cells with H_2O_2 (1000 μmol/L) for 2h. Leica confocal microscope photographs showed the normal growth of RPE cells with HoChest 33258 staining positive and AChE immunofluorescence and TUNEL staining negative. However, TUNEL and AChE immunofluorescence staining in the H_2O_2 group was positive (Figure 2). These data suggested that the expression of AChE was induced in apoptotic cells; this indicates that H_2O_2 (1000 μmol/L) treatment for 2h can induce RPE cell apoptosis with AChE expression, suggesting that AChE may play a key role in the apoptosis of human RPE cells (t=14.683, P=0.008).

Identification of Acetylcholinesterase Knockout Mice $AChE^{+/-}$ animals were initially identified using PCR analysis (Figure 3A). Western blot analysis confirmed that the level of AChE protein expression in the retinal tissues of $AChE^{+/-}$ mice was approximately half the level of AChE protein detected in wild type mice (t=8.176, P=0.025); in addition, AChE was not expressed in $AChE^{-/-}$ mice (Figure 3B).

Acetylcholinesterase Deficiency or Inhibition Attenuates Histomorphologic Changes of the Retina Cells of retina were confirmed present by the detection of shrunken and condensed nuclei using H&E staining. The WT group had no detectable histomorphologic abnormalities. In the retina of adult $AChE^{+/-}$ mouse, the retina became normal (Figure 4A).

By contrast, the retina of different age $AChE^{+/-}$ mice treated with PBS showed widespread disorder of the retinal structure (Figure 4B) including no tight connections between cells, dilation, thinning, cellular vacuolization, pyknotic nuclei and loose cell structure. $AChE^{+/-}$ treatment using different doses of tacrine and donepezil (Figure 4C, 4D) showed a

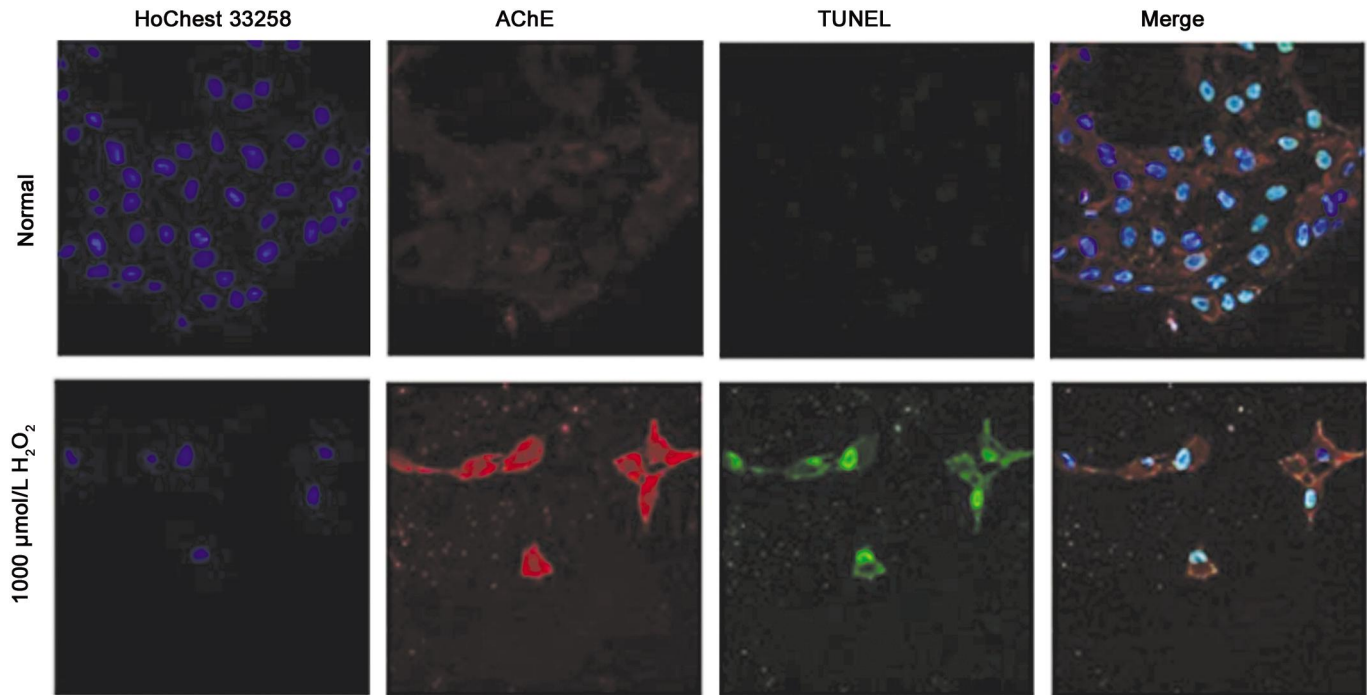

Figure 2 Apoptosis induced RPE cells treated with H_2O_2 as observed by morphology and TUNEL analysis In the control group, HoChest 33258 staining was positive; AChE immunofluorescence and TUNEL staining were negative. In the H_2O_2 (1000 μmol/L) group, AChE immunofluorescence and TUNEL were all positive (400×).

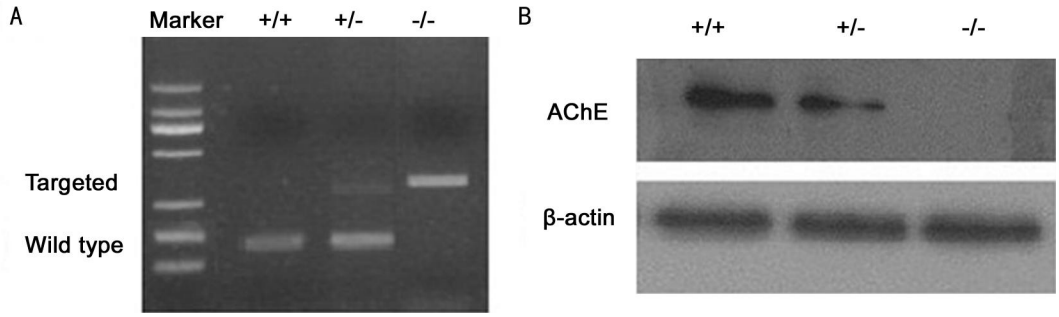

Figure 3 Identification of AChE knockout mice A: PCR-based genotyping of WT and AChE deficient mice; B: Western blot analysis of AChE protein expression in normal retinal tissues of WT and AChE deficient mice.

remarkable reduction of the histologic features, and the number of stained cells was reduced compared to the wild-type retina (t =16.552, P =0.008). The regular arrangement of membrane stacks in outer segments of the wild-type retina became completely disorganized with increased concentration; deterioration of the outer segments preceded that of the outer nuclear layer (ONL) and outer plexiform layer (OPL), and was therefore the first sign of retinal degeneration in the $AChE^{+/-}$ retina.

DISCUSSION

Using AChE knockout mice in the present study, we directly demonstrated that AChE plays a pro-apoptotic role *in vivo*[12]. However, we would like to know if AChE works in the retina. Starburst amacrine cells constitute the major cholinergic cell population in the vertebrate retina [13]. With their long horizontal processes organized within two sublaminae of the IPL, they are presynaptic to bipolar, ganglion and other amacrine cells. Moreover, a large variety of cells including horizontal cells are cholinoceptive, as shown by the distribution of muscarinic and nicotinic ACh receptor subtypes[14].

Tacrine may exert a neuroprotective effect by inhibiting or delaying the expression of Bax and promoting Bcl-2 expression. More studies are needed to elucidate the neuroprotection function of tacrine as to whether it inhibits AChE activity or protecting cholinergic neurons in the treatment of Alzheimer disease (AD)[15,16]. In our study, we used different concentrations of AChEI (0.1 mg/mL, 0.2 mg/mL and 0.4 mg/mL) each being tested on 4-mo-old and 2-mo-old mice. Tacrine and donepezil were injected intraperitoneally once a day for seven days. Following H&E staining we observe the morphology of the retina at the different concentrations of tacrine and donepezil and compared these to the PBS group.

Cellular structure was similar in tacrine and donepezil group, even with different concentrations of tacrine, and the protective effect on the retina did not change significantly from 0.1 mg/mL to 0.4 mg/mL. We suggest this may be due

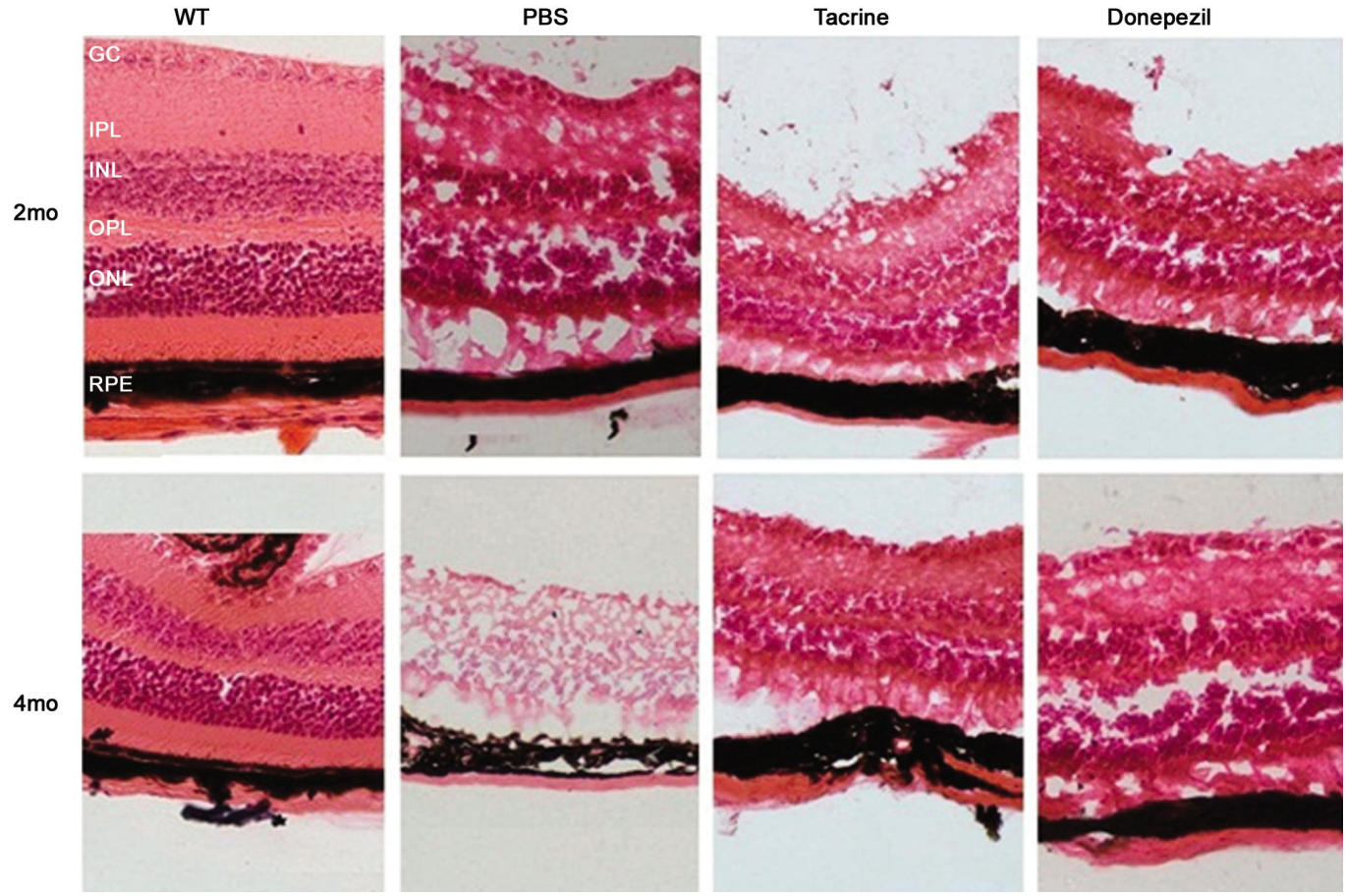

Figure 4 Retina sections from the tacrine and donepezil groups of mice or the PBS control, stained with H&E Retina cells of the WT group were in normal. The PBS group showed widespread disorder of the retinal structural. The tacrine and donepezil groups showed improvement on the PBS group but the number of stained cells was still reduced (40×).

to the intraperitoneal drug delivery resulting in a low drug concentration arriving at the eye. In addition, the blood-retinal barrier may restrict the drug effects, therefore tacrine and donepezil can only play a limited role. Because our results are the mean test of quantitative data of two levels single factors design, so we using the Student's unpaired *t*-test analysed the results. Further studies should involve the administration of tacrine and donepezil using eye drops or by injection into the retina-vitreus, with subsequent observation of retinal changes.

AChEI can inhibit a variety of neuronal apoptotic events, however AChEI on non-neuronal apoptosis requires further study [17]. With regard to AChEI on the role of non-neuronal cells *in vitro*, Wang *et al* [18] confirmed that inhibitors such as BW284e51 (a specific inhibitor of AChE), physostigmine and tacrine can inhibit the apoptotic process of neurons. Thus, the AChEI inhibition of apoptosis depends on the drugs used, the apoptosis inducer and the variety of cell lines; a similar phenomenon can be observed in nerve cells [19-21], and that is the reason why it may be used for induction of apoptosis of other cell lines. Donepezil is an AChEI, a novel drug that has been recently applied for the treatment of AD[22]. At the cellular level the ability to reduce the Aβ-induced cell apoptosis was inconclusive. Donepezil can reduce Aβ25-35 induced PC12 cell apoptosis and damage and can protect neurons; this suggests that donepezil may not only be an AChEI, but could also promote anti-apoptotic gene expression by reducing oxygen free radicals during neuronal damage or other methods of protecting neurons[23].

Although overexpression of AChE can increase the sensitivity of cells to apoptosis, it cannot initiate this process. In fact, there are a variety of normally growing cells that express AChE at basal levels including noncholinergic neurons and some hematopoietic cells, such as red blood cells and megakaryocytes. We know that certain differences exist in AChE between normal and apoptotic conditions, however the exact apoptotic pathways involved in AChE-mediated apoptosis remain to be further elucidated. Our previous studies have revealed that intracellular calcium dyshomeostasis and cytosolic Ca^{2+} play a key role in AChE regulation during apoptosis induced by two endoplasmic reticulum stress inducers, the calcium ionophore A23187 and the inhibitor of Ca^{2+}-ATPase thapsigargin [24]. Although the neuroprotective effects of several AChE inhibitors against apoptosis have been reported *in vitro*, these studies were limited to cell lines or primary cultured cells from the central

or peripheral nervous system. Explanations of the neuroprotective effect observed in these studies included activation of the central cholinergic system.

Eyes of AChE$^{-/-}$ mice are deformed; this study represents the first mammalian *in vivo* trial demonstrating a significant developmental role for AChE. Surprisingly, AChE $^{-/-}$ mice survived to adulthood in the absence of any AChE activity or AChE protein, although growth and behavioural problems were apparent. It is assumed that the related enzyme AChE, or possibly less specific esterases, compensate for the absence of AChE. Clearly, these mice are developmentally delayed, their eyes open on P13-14, one or two days later than in wild-type mice. AChE$^{-/-}$mice have pinpoint pupils throughout life; on visual inspection the eyes of young animals look normal, but in many individuals >P100, the eyes bulge out [25].

Using selective pharmacologic inhibitors of muscarinic acetyl cholinesterasereceptors (mAChR) or nerves acetyl cholinesterase receptors (nAChR), we previously demonstrated that galantamine-induced RGC survival occurred via activation of mAChR, whereas nAChRs were not involved[26]. Retinal and brain endothelial cells abundantly express M1 and M3 mAChR subtypes, which have been implicated in cholinergic-mediated vasomodulation in the brain, and ACh-dependent vascular relaxation is lost in M3 mAChR knockout mice. Intriguingly, adult retina ganglion cells (RGCs) do not express mAChR and muscarine does not elicit membrane currents measured in whole-RGC patch-clamp preparations[27-29].

In the AChE$^{-/-}$ retina all photoreceptors degenerated after three months [26,28], a somewhat extended period when compared with the animal models described above. At present it is unknown whether photoreceptors become physiologically active before their degeneration, however, behavioural observations indicate that the animals never have vision. This issue can currently be further analysed through electroretinograms in young and older animals. As photoreceptor degeneration is a late event, we speculated that deterioration of the outer retina could be a consequence of earlier wiring defects primarily afflicting the inner retina. Interestingly, AChE deficiency or inhibition not only decreases apoptotic cell death but also necrotic cell death. It appears that there is severe necrosis in WT or vehicle-treated animals but necrosis is attenuated in the retina of AChE$^{+/-}$ or AChEIs-treated groups. This finding may be due to the reduction of secondary necrosis.

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Conflicts of Interest: Yi YM, None; **Cai L,** None; **Shao Y,** None; **Xu M,** None; **Yi JL,** None.

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