• Basic Research •

Activation of the TRAAK two-pore domain potassium channels in rd1 mice protects photoreceptor cells from apoptosis

Lei Wang, Kang-Pei Shi, Han Li, Hao Huang, Wen-Bin Wu, Chu-Sheng Cai, Xiao-Tong Zhang, Xiao-Bo Zhu

State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, Guangdong Province, China

Correspondence to: Xiao-Bo Zhu. State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University 54S Xianlie Road, Guangzhou 510060, Guangdong Province, China. zhuxbo@mail.sysu.edu.cn

Received: 2019-05-23 Accepted: 2019-07-24

Abstract

• AIM: To investigate the expression of TWIK-related arachidonic acid-stimulated K^+ channel (TRAAK) in retinal degeneration mice (rd1) and further evaluate how TRAAK affect photoreceptor cell apoptosis.

• METHODS: The rd1 mice were distributed into blank (no treatment), control (1.4% DMSO, intraperitoneal injection) and riluzole groups (4 mg/kg·d, intraperitoneal injection) from postnatal 7d to 10, 14 and 18d; C57 group (no treatment), as age-matched wild-type control. The thickness of the outer nuclear layer (ONL) of retina was detected by paraffin section hematoxylin and eosin staining. The expression of TRAAK and the apoptosis of the ONL cells were detected by immunostaining, Western blotting, and real-time polymerase chain reaction.

• RESULTS: The channel agonist riluzole activated TRAAK and delayed the apoptosis of photoreceptor cells in ONL layer of rd1 mice. Both at mRNA and protein levels, after riluzole treatment, TRAAK expression was significantly upregulated, when compared with the control and blank group. Then we detected a series of apoptosis related mRNA and protein. The anti-apoptotic factor Bcl-2 downregulated and the pro-apoptotic factors Bax and cleaved-caspase-3 upregulated significantly.

• CONCLUSION: Riluzole elevates the expression of TRAAK and inhibits the development of apoptosis. Activation of TRAAK may have some potential effects to put off photoreceptor apoptosis.

• KEYWORDS: TRAAK; riluzole; photoreceptor cell; apoptosis

DOI:10.18240/ijo.2019.08.03

Citation: Wang L, Shi KP, Li H, Huang H, Wu WB, Cai CS, Zhang XT, Zhu XB. Activation of the TRAAK two-pore domain potassium channels in rd1 mice protects photoreceptor cells from apoptosis. *Int J Ophthalmol* 2019;12(8):1243-1249

INTRODUCTION

Retinitis pigmentosa (RP) is a large class of retinal disease caused by a group of inherited gene mutations that cause photoreceptor cell death[1]. The main manifestations are night blindness, progressive visual field damage, fundus pigmentation, abnormal or wave-free retinal electroretinogram, and central vision loss[2]. Retinal metabolism is active, and photoreceptor cells are at a high oxygen level for a long time, which may result in their own oxidative stress[3]. Bcl-2 and caspase-3 are two important members in regulating cell apoptosis[4]. Activation of Bcl-2-related family members and changes in mitochondrial permeability and membrane potential may occur when cells are stimulated by extracellular apoptosis signals or related factors, such as increases in reactive oxygen species (ROS) and widely changes of oxidative stress injury. All of the alteration lead to cascade activation of the caspase-3 family, which inevitably leads to cell apoptosis[5]. During the occurrence and development of RP, oxidative stress injury always occurs, which induced attentional apoptotic effect in photoreceptor cells.

The TREK-TRAAK channel belongs to two-pore potassium ion channels family. This member contains TREK-1, TREK-2, and TRAAK[6]. Activation of TRAAK generates background potassium leakage currents that are participated in regulating excitability of cells and the resting membrane potential[7-10]. TRAAK is widely expresses in the central nervous system. Activation of TRAAK by polyunsaturated fatty acids in brain tissue has a protective effect on neuronal death induced by cerebral ischemia[11]. In addition, TRAAK also widely expresses in the mouse retina[12]. Our previous studies have shown that apoptosis of A-RPE19 and human-RPE (hRPE) cells induced by t-BH can be inhibited by TRAAK agonists by activating of TRAAK *in vitro*[13-14]. In this study, the channel agonist riluzole was used to activate this channel in the retina

of a RP mouse model (rd1 mouse) to observe the changes in apoptosis in the retina-related cell layer and the expression of apoptosis related factors.

MATERIALS AND METHODS

Ethical Approval All of the animal experiments conformed to the relevant regulations of the ARVO Center on Animal Feeding, Ophthalmology and Visual Research and ethics committee of the Zhongshan Eye Center (ethics code: 2015-131).

Animals The rd1 mice (FVB/rd1) and C57BL/6 mice were purchased respectively from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and Southern Medical University (Guangzhou, China). All animals were kept in the SPF laboratory of Zhongshan Ophthalmic Animal Center and were fed with clean food and water (15℃-25℃ and under a 12h light/12h dark schedule. There were 4 groups in the experiment: a C57BL/6 mouse group (C57, *n*=8) and 24 rd1 mice were stochastically, equally distributed into 3 groups, including blank group (blank), control group (control), and riluzole group (riluzole, Sigma-Aldrich, USA). From postnatal 7d, the riluzole group was injected intraperitoneally riluzole [with 4 mg/kg, dissolved in 1.4% dimethylsulfoxide (DMSO)] every day, and the control group was intraperitoneally injected with 1.4% DMSO (MP Biomedicals, USA) until 14d[15-16]. Mice in the blank and C57 groups received no treatment. Mice were received cervical dislocation to dead at 10, 14, and 18d, respectively, and fresh eyeballs were collected for later use.

Frozen Sections Fresh eyeballs were rapidly enucleated and placed in liquid nitrogen to snap frozen. Then the tissue was preserved at -80℃ condition until sectioned with optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan). The tissue sections were sliced vertically with a cryostat at 6 μm, every sections were acquired through or a serrata and optic nerve head.

Immunostaining The immunofluorescence staining assay of sections was described previously[17]. First, the tissue sections were deparaffinized and rehydrated, then, at room temperature, the sections were incubated with 1% BSA (MP Biomedicals, USA) and 0.5% Triton X-100 (MP Biomedicals, USA) for 30 min. After that, primary antibodies against TRAAK (1:100; Alomone labs, Israel) in 1% BSA were incubated at 4℃ overnight. Anti-rabbit IgG secondary antibodies (Alexa Fluor® 555 Conjugate; 1:1000; CST, USA were added for 1h. We used fluorescence microscope (Olympus, Japan) to acquire immunostaining images.

H&E Staining for Paraffin Sections The eyeballs of rd1 and C57BL/6 mice were paraffin embedded. The 5 μm thickness of tissue sections were sliced for stained with hematoxylin and eosin (H&E). Then sections were H&E, and images were acquired using a digital imaging system (Olympus, Japan). The outer nuclear layer (ONL) thickness were analyzed and calculated at 200 μm far away from optic nerve head.

TUNEL Assays The above sections were double-stained with a TUNEL kit (Roche, Switzerland) and 4',6-diamidino-2-phenylindole (DAPI; 1:2000; Beyotime Biotechnology, China). TUNEL images were photographed by fluorescence microscope (Olympus, Japan).

RT-PCR Total retina tissue RNA was extracted with Trizol reagent (MP Biomedicals, USA). Reverse transcription procedures according to HiScript® II Q RT SuperMix kit (Vazyme, China) manufacture's protocol. And then quantitative RT-PCR was conducted *via* a ChamQ SYBR Color qPCR Master Mix kit (Vazyme, China) following the manufacture's protocol. The PCR cycle conditions for the reaction were 95℃×30s, followed by 40 cycles of 95℃×10s, 60℃×30s and a melting curve at 95℃×15s, 60℃×60s, and 95℃×15s. Amplification specificity was determined by analyzing the melting curves. GAPDH was used to normalize the relative mRNA levels. Then the expression of mRNA was calculated using the $2^{-\triangle\triangle CT}$ method. The RT-PCR specific primers were following: GAPDH, forward: 5'-TGGCCTTCCGTGTTCCTAC-3' and reverse: 5'-GAGTTGCTGTTGAAGTCGCA-3'; TRAAK, forward: 5'-AACCACGTGGAACAAAAGAGG-3' and reverse: 5'-CATCCAAAAAGCCTTCCAG-3'; BCL-2, forward: 5'-GTCGCTACCGTCGTGACTTC-3' and reverse: 5'-CAGACATGCACCTACCCAGC-3'; BAX, forward: 5'-CCGGCGAATTGGAGATGAACT-3' and reverse 5'-CCAGCCCATGATGGTTCTGAT-3'.

Protein Extraction and Western Blotting Mouse retinal tissues were homogenized 100:1 in cold radio immunoprecipitation assay (RIPA) buffer and phenylmethanesulfonyl fluoride (PMSF) buffer (Beyotime Institute of Biotechnology, China) using an ultrasonic disruptor. Then, 20 μg proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels. Next, proteins samples were transferred into polyvinylidene fluoride (PVDF) membranes (PVDF) membranes (Roche, USA). Phosphate buffer saline (PBS) containing 5% nonfat-dried milk were used to block the membranes, then incubated with primary antibodies TRAAK (1:200; Alomone Labs, Israel), GAPDH (1:5000; Abcam, USA), Bcl-2 (1:1000; Abcam, USA), Bax (1:1000; Abcam, USA), and cleaved caspase-3 (1:1000; CST, USA). Anti-rabbit IgG HRP-linked antibodies (1:20000; CST, USA) was used to incubated membranes. After wash with 1×Tris-buffered saline containing Tween-20 (TBST) buffer for 6 times×10min, chemiluminescence signals were visualized with a ChemiDoc™ MP Imaging System (BioRad, USA).

Statistical Analysis Data were presented with the means± standard deviation (SD). One-way ANOVA was used to assess differences between experimental and control group. Each experiment was repeated 3 times, as indicated "*n*=3" in figure

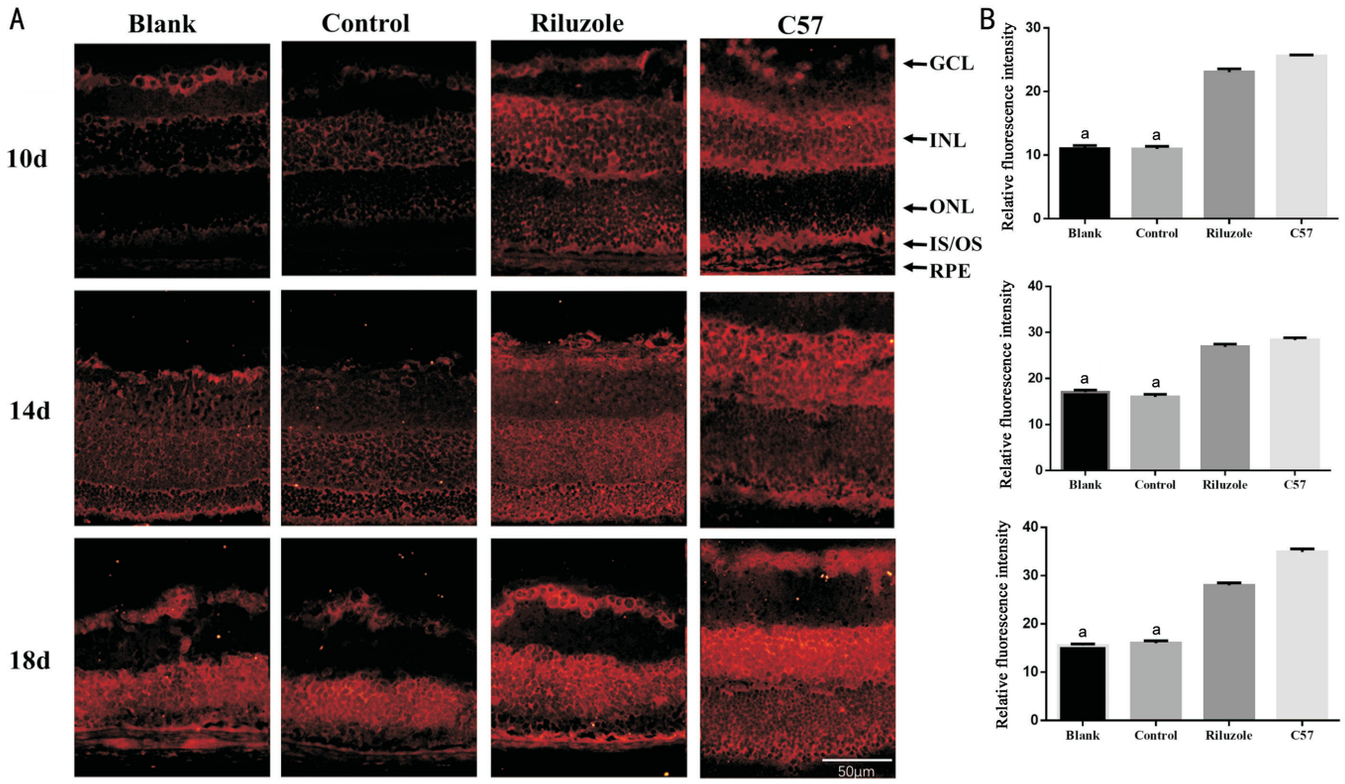

Figure 1 Immunofluorescence analysis demonstrated the expression of TRAAK on the retina A: The expression and distribution of TRAAK on the retina of mice at 10, 14, and 18d, TRAAK presented red fluorescence; B: Relative fluorescence intensity of TRAAK K2P on the retina of mice at 10, 14, and 18d. GCL: Ganglion cells layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; IS/OS: Inner and outer segments; RPE: Retinal pigment epithelial. The bar graphs show the means±SD (n=3). ^{a}P<0.01 *vs* riluzole group. Magnification 200×.

captions. Data were analyzed by SPSS statistical software (version 13.0; SPSS Inc., Chicago, IL, USA). P value <0.05 had statistically difference.

RESULTS

Localization and Expression of TRAAK on Mouse Retinas TRAAK is represented by red fluorescence and is widely expressed on the retina in rd1 mice and C57BL/6 mice (Figure 1A). The relative fluorescence intensity of the riluzole group was increased versus the control and blank group at the three time points. There existed no significant difference between control and blank group (Figure 1B).

Riluzole Reduced the Apoptosis of Photoreceptor Cells The riluzole group was more elevated compared with control and blank group in the thickness of ONL at the three time points; there existed no significant difference between control and blank group (Figure 2). In the ONL of the riluzole group at the 3 time points, fewer TUNEL-positive (TUNEL+) cells was detected. Like previous result, no significant differences in the number of TUNEL+ cells between control and blank groups, and the C57 group had almost no TUNEL+ cells (Figure 3A-3D).

Riluzole Activated the Expression of TRAAK and Inhibited the Development of Apoptosis In riluzole group, the mRNA and protein expression levels of TRAAK were significantly upregulated than those in the blank and control group at three time points. The mRNA and protein expression levels of TRAAK between the control and blank group had no significant differences. Versus with the control and blank groups, the mRNA and protein expression levels of Bcl-2 in the riluzole group at the three time points were significantly increased; while the mRNA and protein expression levels of Bax in this group at the three time points were significantly decreased than control and blank groups. Protein expression levels of cleaved caspase-3 in the riluzole group were decreased than control and blank groups at the three time points (Figure 4A-4D).

DISCUSSION

The rd1 mouse model is a successful and representative animal model for RP. There are morphological changes mainly in the retinal ONL layer, with gradual thinning over time that leads to the complete disappearance of the ONL. At approximately 8d, the outer segment starts to die, followed by the death of the inner segment and photoreceptor cells, which peaks around 14d and is nearly complete by 21d[18]. In light of this sequence, this study evaluated the occurrence and development of photoreceptor apoptosis from the perspective of morphology and molecular change mechanisms at the early (10d), peak (14d) and later (18d) stages of mouse apoptosis.

Cell shrinkage usually happens at early phase of apoptosis and always accompanies with decreasing in intracellular potassium concentration regulating by TRAAK[19]. Hence, we

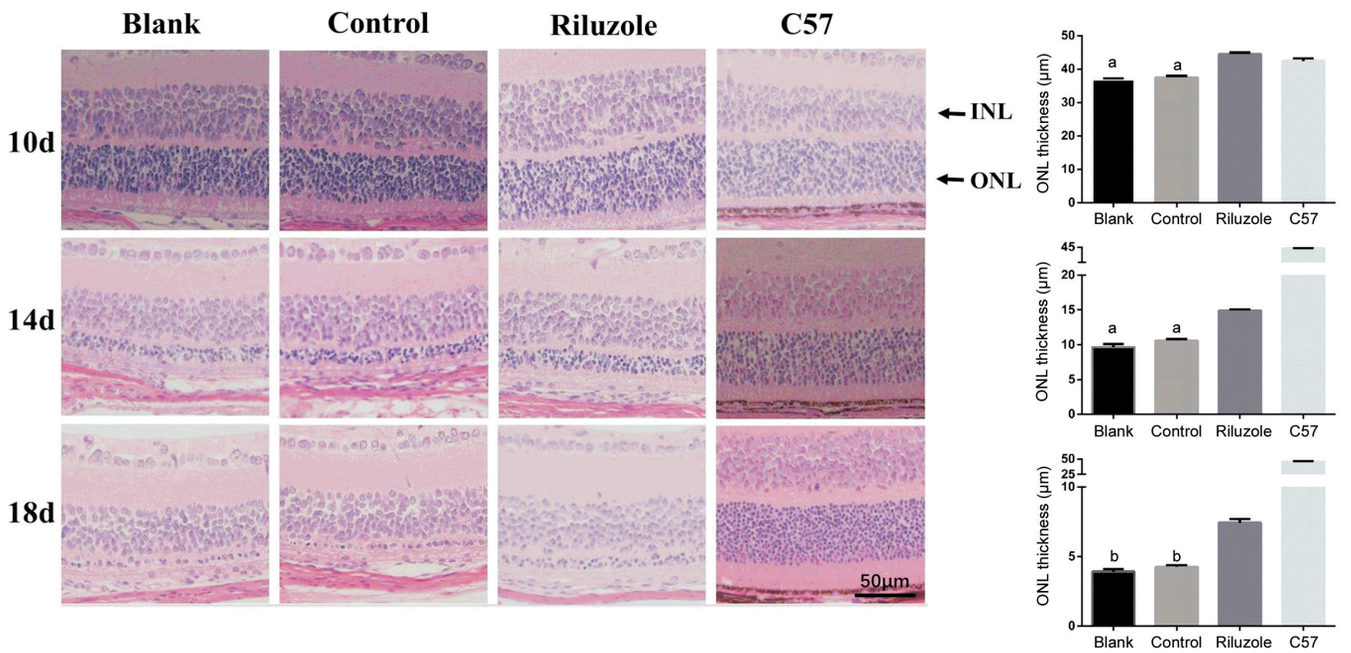

Figure 2 H&E staining in paraffin section detected the thickness of ONL in all groups At 10, 14, and 18d, the thickness of the ONL in all groups were measured. [a]*P*<0.05 *vs* riluzole group; the bar graphs show the means±SD (*n*=4). ONL: Outer nuclear layer. [b]*P*<0.01 *vs* riluzole group. Magnification 200×.

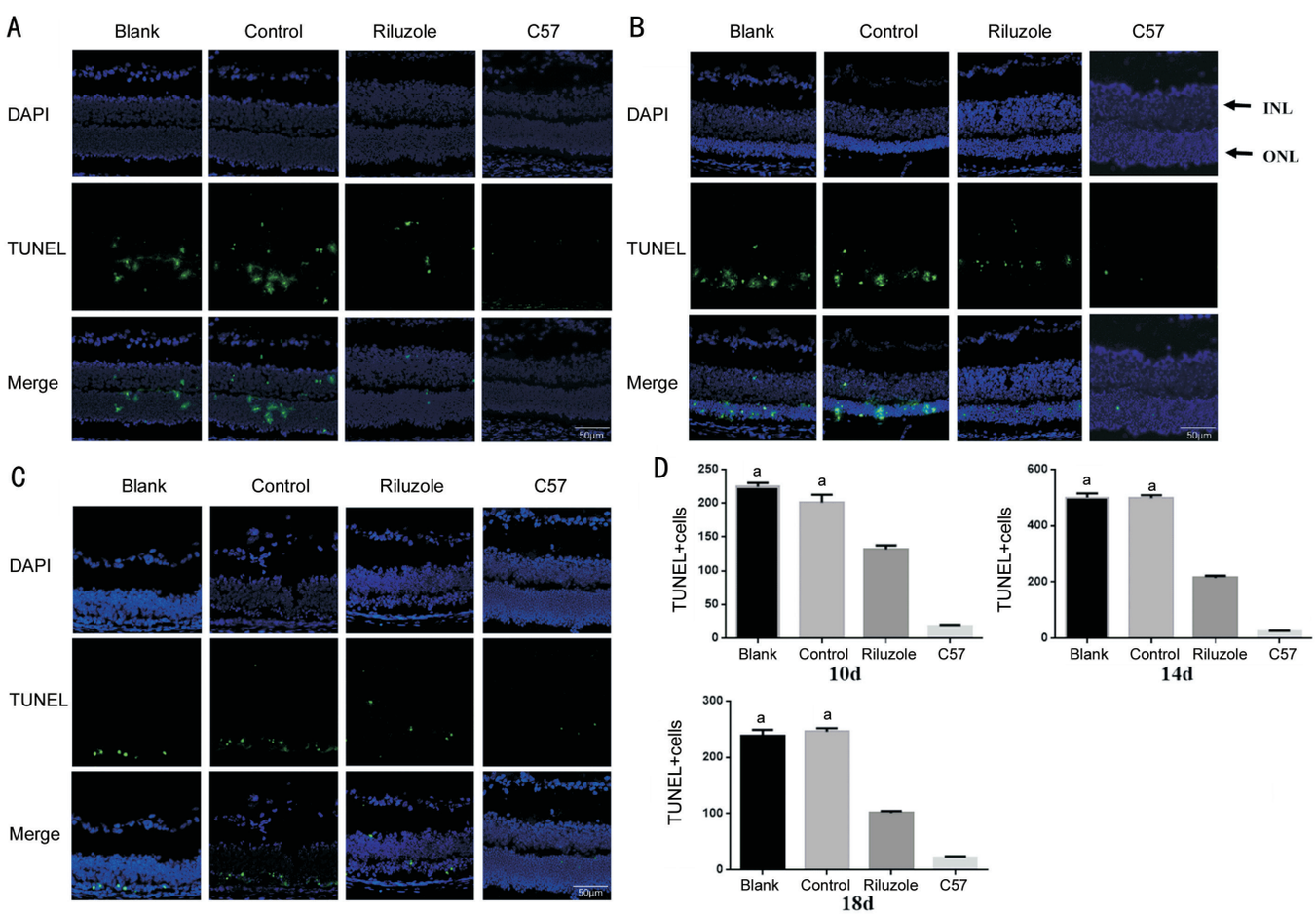

Figure 3 TUNEL staining revealed the distribution of apoptotic cells in the retina A-C: Apoptosis of cells in all group at 10, 14, and 18d. The cell nucleus stained with DAPI showed blue fluorescence, and the TUNEL+ cells were distributed in the ONL and INL with green granular fluorescence. D: Quantity of TUNEL+ cells in the ONL of all groups at 10, 14, and 18d. DAPI: 4',6-diamidino-2-phenylindole. The bar graphs show the means±SD (*n*=4). INL: Inner nuclear layer; ONL: Outer nuclear layer. [a]*P*<0.01 *vs* riluzole group. Magnification 200×.

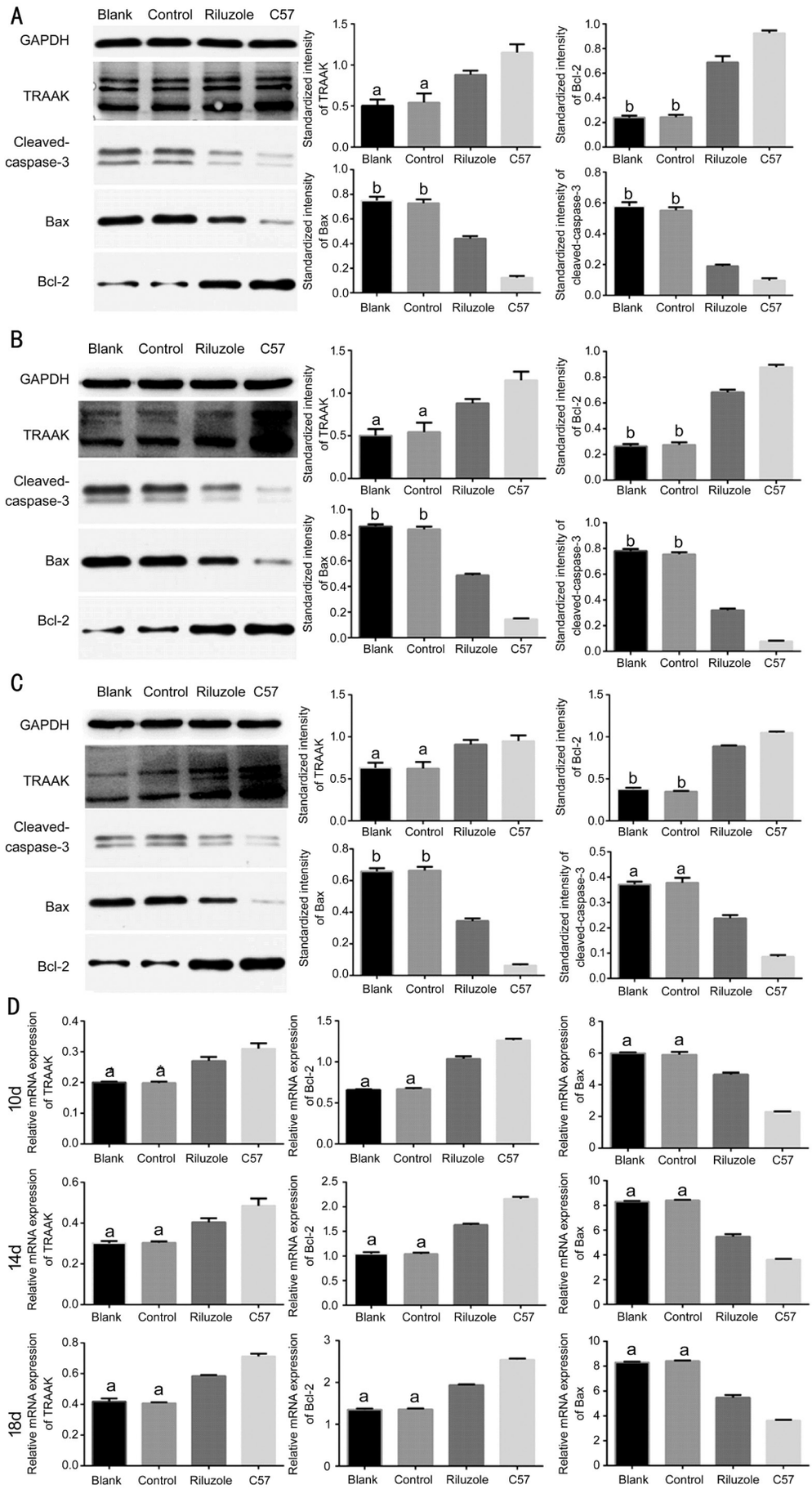

Figure 4 Western blotting and RT-PCR demonstrated the variations of TRAAK, and apoptosis-related factors expression levels A-C: Western blotting detected protein expression of TRAAK, Bcl-2, Bax and cleaved caspase-3 at 10, 14, and 18d; D: RT-PCR detected the mRNA expression levels of TRAAK, Bcl-2 and Bax at 10, 14, and 18d. The bar graphs show the means±SD (n=3). [a]P<0.05 *vs* riluzole group; [b]P<0.01 *vs* riluzole group.

speculate reversing potassium outflow at the early stage of apoptosis may alter the progression of apoptosis. Riluzole is a first-line drug for the treatment of lateral sclerosis of spinal cord and has a certain effect on cerebral ischemia, anxiety and other diseases[20-21]. The effect of riluzole on TRAAK is rapid, reversible, dose-dependent and, unlike TREK-1 or TREK-2, it is a sustained response[22]. A study reported that riluzole protected nerve cell from apoptosis and improved the recovery of retinal function in rats with ischemic injury [23]. Intraperitoneal injection and topical treatments of riluzole can delay the damage of retinal ganglion cells in the glaucoma model of rats with high intraocular pressure[24]. These protective effects may be partially own to activation of TRAAK. In this study, we used riluzole to activate TRAAK in rd1 mice to explore whether there is the same protective effect against apoptosis and to determine the possible mechanism.

The distribution and expression of TRAAK in mice retinas are shown in Figure 1. It can be seen that TRAAK is expressed in almost all retinal layers of the two kinds of mice, mainly in the ganglion cells, ONL, inner nuclear layer and retinal pigment epithelial, which is basically consistent with previous research[25]. TRAAK is widely involved in the physiological activities of the retina, but its specific mechanism of action on the retina remains unclear. In this study, the average fluorescence intensity of TRAAK in ONL in the riluzole group were higher than that in the control and blank groups at all time points. The thickness of the ONL in the riluzole group was thicker than that in the control and blank groups. In riluzole group, we found the quantity of TUNEL-positive cells in the ONL of the were decreased. These results demonstrated that riluzole downregulated the ONL photoreceptor cells' apoptosis by upregulating the expression of the TRAAK in the retina of rd1 mice. To further explore the possible relationship between the expression of TRAAK and apoptosis, Western blot and RT-PCR assays were performed. In the riluzole group, the protein expression of TRAAK was increased, and the mRNA expression showed the same trend. The increase of this expression was consistent with the change in the previous relative fluorescence intensity of TRAAK. The expression of Bax and cleaved-caspase-3 were decreased in the riluzole groups at the level of transcription and translation, and expression of Bcl-2 was elevated. These changes reversed apoptosis signaling in normal rd1 mice, indicating that increased expression of the TRAAK had a protective effect against apoptosis, demonstrating that this channel was involved in early and late regulation of apoptosis.

This study had certain limitations. We have manifested riluzole, as a TRAAK channel agonist can protect and even reverse the photoreceptor apoptosis *in vitro* and *vivo*. However, the specific mechanism about TRAAK channel regulating apoptosis needs further research. Besides, due to the experimental conditions and other factors, our experiment did not detect the changes of mouse visual function, such as electroretinogram or electrooculogram. Moreover, relative electrophysiological technique to future studies should be included to evaluate TRAAK effect sufficiently. In conclusion, our results show that the use of riluzole increases the expression of the TRAAK in the retina of rd1 mice, delays the apoptosis of photoreceptor cells in the ONL layer of rd1 mice, and inhibits the development of apoptosis. Therefore, TRAAK has potential research value in the treatment of RP.

ACKNOWLEDGEMENTS

Foundation: Supported by National Natural Science Foundation of China (No.81271012)

Conflicts of Interest: Wang L, None; **Shi KP,** None; **Li H,** None; **Huang H,** None; **Wu WB,** None; **Cai CS,** None; **Zhang XT,** None; **Zhu XB,** None.

REFERENCES

1 Campochiaro PA, Mir TA. The mechanism of cone cell death in retinitis pigmentosa. *Prog Retin Eye Res* 2018;62:24-37.

2 Narayan DS, Wood JP, Chidlow G, Casson RJ. A review of the mechanisms of cone degeneration in retinitis pigmentosa. *Acta Ophthalmol* 2016;94(8): 748-754.

3 Nita M, Grzybowski A. The role of the reactive oxygen species and oxidative stress in the pathomechanism of the age-related ocular diseases and other pathologies of the anterior and posterior eye segments in adults. *Oxid Med Cell Longev* 2016;2016:3164734.

4 Alhosin M, León-González AJ, Dandache I, Lelay A, Rashid SK, Kevers C, Pincemail J, Fornecker LM, Mauvieux L, Herbrecht R, Schini-Kerth VB. Bilberry extract (Antho 50) selectively induces redox-sensitive caspase 3-related apoptosis in chronic lymphocytic leukemia cells by targeting the Bcl-2/Bad pathway. *Sci Rep* 2015;5:8996.

5 Singh R, Letai A, Sarosiek K. Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins. *Nat Rev Mol Cell Biol* 2019;20(3):175-193.

6 Judge SI, Smith PJ. Patents related to therapeutic activation of K(ATP) and K(2P) potassium channels for neuroprotection: ischemic/hypoxic/anoxic injury and general anesthetics. *Expert Opin Ther Pat* 2009;19(4):433-460.

7 Brohawn SG. How ion channels sense mechanical force: insights from mechanosensitive K2P channels TRAAK, TREK1, and TREK2. *Ann N Y Acad Sci* 2015;1352:20-32.

8 Fink M, Lesage F, Duprat F, Heurteaux C, Reyes R, Fosset M, Lazdunski M. A neuronal two P domain K+ channel stimulated by arachidonic acid and polyunsaturated fatty acids. *EMBO J* 1998;17(12):3297-3308.

9 Noël J, Sandoz G, Lesage F. Molecular regulations governing TREK and TRAAK channel functions. *Channels (Austin)* 2011;5(5):402-409.

10 Feliciangeli S, Chatelain FC, Bichet D, Lesage F. The family of K2P channels: salient structural and functional properties. *J Physiol (Lond)* 2015;593(12):2587-2603.

11 Lauritzen I, Blondeau N, Heurteaux C, Widmann C, Romey G, Lazdunski M. Polyunsaturated fatty acids are potent neuroprotectors. *EMBO J* 2000;19(8):1784-1793.

12 Reyes R, Lauritzen I, Lesage F, Ettaiche M, Fosset M, Lazdunski M. Immunolocalization of the arachidonic acid and mechanosensitive baseline traak potassium channel in the nervous system. *Neuroscience* 2000;95(3):893-901.

13 Shen CL, Ma W, Zheng WB, Huang H, Xia RC, Li C, Zhu XB. The antioxidant effects of riluzole on the APRE-19 celll model injury-induced by t-BHP. *BMC Ophthalmol* 2017;17(1):210.

14 Huang H, Li H, Shi KP, Wang L, Zhang XT, Zhu XB. TREK-TRAAK two-pore domain potassium channels protect human retinal pigment epithelium cells from oxidative stress. *Int J Mol Med* 2018;42(5):2584-2594.

15 Heurteaux C, Laigle C, Blondeau N, Jarretou G, Lazdunski M. Alpha-linolenic acid and riluzole treatment confer cerebral protection and improve survival after focal brain ischemia. *Neuroscience* 2006;137(1): 241-251.

16 Melki SJ, Heddon CM, Frankel JK, Levitt AH, Momin SR, Alagramam KN, Megerian CA. Pharmacological protection of hearing loss in the mouse model of endolymphatic hydrops. *Laryngoscope* 2010;120(8):1637-1645.

17 Gao LX, Chen X, Tang YP, Zhao JH, Li QY, Fan XT, Xu HW, Yin ZQ. Neuroprotective effect of memantine on the retinal ganglion cells of APPswe/PS1ΔE9 mice and its immunomodulatory mechanisms. *Exp Eye Res* 2015;135:47-58.

18 Strettoi E, Porciatti V, Falsini B, Pignatelli V, Rossi C. Morphological and functional abnormalities in the inner retina of the rd/rd mouse. *J Neurosci* 2002;22(13):5492-5504.

19 Trimarchi JR, Liu L, Smith PJ, Keefe DL. Apoptosis recruits two-pore domain potassium channels used for homeostatic volume regulation. *Am J Physiol Cell Physiol* 2002;282(3):C588-C594.

20 Sámano C, Nistri A. Mechanism of neuroprotection against experimental spinal cord injury by riluzole or methylprednisolone. *Neurochem Res* 2019;44(1):200-213.

21 Zhou LY, Tian ZR, Yao M, Chen XQ, Song YJ, Ye J, Yi NX, Cui XJ, Wang YJ. Riluzole promotes neurological function recovery and inhibits damage extension in rats following spinal cord injury: a meta-analysis and systematic review. *J Neurochem* 2019;150(1):6-27.

22 Lamas JA, Fernández-Fernández D. Tandem pore TWIK-related potassium channels and neuroprotection. *Neural Regen Res* 2019;14(8):1293-1308.

23 Ettaiche M, Fillacier K, Widmann C, Heurteaux C, Lazdunski M. Riluzole improves functional recovery after ischemia in the rat retina. *Invest Ophthalmol Vis Sci* 1999;40(3):729-736.

24 Pirhan D, Yüksel N, Emre E, Cengiz A, Kürşat Yıldız D. Riluzole- and resveratrol-induced delay of retinal ganglion cell death in an experimental model of glaucoma. *Curr Eye Res* 2016;41(1):59-69.

25 Hughes S, Foster RG, Peirson SN, Hankins MW. Expression and localisation of two-pore domain (K2P) background leak potassium ion channels in the mouse retina. *Sci Rep* 2017;7:46085.