

## Original Article

# Protective effect of TBN, a free radical scavenger, against excessive light-induced retinal damage in vivo and vitro

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**Abstract:** Light-induced retinal damage is a common cause of retina degeneration. Here, we use *in vivo* and *in vitro* model to examine projective effects of 2-[[[(1,1-dimethylethyl)oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN), a radical scavenger with neuro protective properties, on light-induced retinal damage. White cool light (3500±200 Lux) was used to induce retinal damage of SD rats for 48 hour, and then the animals were randomly assigned to different groups either injected with vitamin C or TBN. The electro retina gram (ERG) had been evaluated of different groups and the number and thickness of outer nuclear layer (ONL) had been measured. The tert-butyl hydro peroxide (t-BHP) was used to induce apoptosis of ARPE-19 cells, and then the pre-protection was given at different time points and concentrations of vitamin C and TBN. The activity of different group of cells was detected by CCK8. Intracellular mitochondrial membrane potential ( $\Delta\Psi_m$ ), ROS and  $Ca^{2+}$  level was detected with flow cytometry. The oxidative stress related proteins expression was detected by western analysis. After intense exposure of SD rats to light, a- and b-wave of ERG almost disappears, number of ONL significantly reduced. After TBN treatment, the a- and b-wave amplitude of ERG was obviously increased. Number of ONL significantly increased. ARPE-19 cells treated with t-BHP exhibited slow cell activity and  $\Delta\Psi_m$  loss, increased ROS and  $Ca^{2+}$  level, and upregulation of caspase-3, Bax, CHOP, Bip and CaMKII protein as well as downregulation of Bcl-2 protein. Vitamin C and TBN treatment can significantly decrease ROS and  $Ca^{2+}$  level, increase cell activity and  $\Delta\Psi_m$  in ARPE-19 cells treated with t-BHP and ameliorate the expression of Bcl-2 protein caspase-3, Bax, CHOP, Bip and CaMKII. These findings suggest that the TBN has protective effects on excess light-induced retinal damage and t-BHP induced apoptosis of ARPE-19 cells, via inhibition of mitochondria and endoplasmic reticulum (ER) oxidative stress.

**Keywords:** Tert-Butyl hydroperoxide (t-BHP), light-induced retinal damage, oxidative stress, 2-[[[(1,1-dimethylethyl)oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN), retinal pigment epithelium (RPE)

## Introduction

Light-induced retinal damage has long served as a model of retinal dysfunction and visual cell loss arising from inherited disease or caused by oxidative stress [1, 2]. Its utility resides in the fact that nearly the entire complement of retinal photoreceptors is simultaneously involved in a now well-defined progression of cellular degeneration and active cell death [3, 4]. Numerous extrinsic factors are known to influence the extent of visual cell loss, including previous light-rearing history, light intensity and duration, and diet [5]. Many lines of evidence suggest that mitochondria have a central role

in ageing-related neurodegenerative diseases [6-8]. Mutations in mitochondrial DNA and oxidative stress both contribute to ageing, which is the greatest risk factor for neurodegenerative diseases [9]. A variety of key events in apoptosis focus on mitochondria, including the release of caspase activators (such as ROS and  $Ca^{2+}$ ), changes in electron transport, loss of mitochondrial trans-membrane potential, altered cellular oxidation-reduction, and participation of pro- and anti-apoptotic Bcl-2 family proteins [10-12]. The different signals that converge on mitochondrial trigger or inhibit these events and their downstream effects delineate several major pathways in physiological cell death.

Thus, therapies targeting basic mitochondrial processes, such as energy metabolism or free-radical generation, or specific interactions of disease-related proteins with mitochondria, hold great promise.

The mechanism of apoptosis is not yet fully understood, it is generally believed that there are three main pathways involved in the regulation of apoptosis: death receptor pathway, mitochondrial pathway and endoplasmic reticulum pathway. The endoplasmic reticulum pathway is different from the other two pathways, is the recent studies of apoptosis pathway. The central content of this pathway is endoplasmic reticulum stress (ER stress) [13]. Research confirmed, ER stress is the earliest response of cells in stress, is the common initial pathway of cellular stress response, such as oxidative stress and so on [14, 15]; ER stress may be independent of other pathways to induce apoptosis [16]. About neuronal apoptosis induced by ER stress, has been studied in the pathogenesis of many neurodegenerative diseases, such as Parkinson disease, Alzheimer disease, Huntington disease and so on.

We previously reported on dual functional 2-[[[(1,1-dimethylethyl)oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN), a derivative of TMP armed with a powerful free radical-scavenging nitrogen moiety. TBN retains the thrombolytic activity of its parent TMP, exhibits stronger anti-oxidative activity [17]. After a lot of basic research, TBN not only can eliminate the excessive free radicals caused by various kinds of external effects, but it can protect the apoptosis of nerve cells by reducing the concentration of  $Ca^{2+}$  in the cells [18]. But TBN has not been used in the research of light-induced retina damage, so we hypothesis that TBN can be used as an antioxidant, protect the apoptosis of RPE cells by the inhibition of oxidative stress. To improve the occurrence and development of light-induced retina damage, and provide a theoretical basis and experimental basis for the new drug to become a candidate for the treatment of light-induced retina damage.

### Materials and methods

#### *Animals and treatment*

Male adult SD rats (180-220 g) were acclimated for one week under sterile conditions. All animal procedures were performed in strict

accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by competent ethics committees at Jinan University. All efforts were made to minimize the suffering of animals. 30 rats were randomly divided into six experimental groups: a control group (5 sham rats), Light-damage model group (5 rats), 250 mg/kg vitamin C group (5 rats), 10 mg/kg TBN group (5 rats), 50 mg/kg TBN group (5 rats) and 100 mg/kg TBN group (5 rats). Twenty-five SD rats were selected randomly and placed individually in clear plastic cages with wire tops scotopic for 12 h and then exposed to  $3500 \pm 200$  lx light (white cool light) for 48 h [19, 20]. The remaining five SD rats were normally fed in normal conditions, and then returned to the normal light/dark cycle for 1 week. After light-induced damage, SD rats had been selected as drug treatment groups. The drug was injected by tail vein, daily administration of twice for 1 week. 30SD rats were measured by ERG and then euthanized and the eyes processed for morphology detection.

#### *Electroretinography*

Flash ERGs were recorded with an ERG recording system (Brandenburg, Germany). Rats were maintained in total darkness overnight and prepared for ERG recording under dim red light. Rats should usually be adapted to the dark prior to ERG recordings. Following stabilization, ERGs were obtained. In brief, the control rats were anesthetized with 10% chloral hydrate (0.4 ml/100 g) and the pupils dilated with tropic amide. Recording electrodes were placed on the corneas. Two reference electrodes were inserted into subcutaneous tissue behind the ears and one ground electrode was inserted into subcutaneous tissue of the tail [21, 22].

Scotopic ERGs (a-wave, b-wave; 3.0 cds/m<sup>2</sup>, white flash) were first recorded after overnight dark adaptation. For the evaluation of cone function (photopic ERG), a strobe flash stimulus was presented to 5 min light-adapted, dilated eyes in a Ganzfeld with a 2000 cds/m<sup>2</sup> flash intensity. The measurement of different waves is illustrated in **Figure 1**. The incubation period of the a- and b-wave is the time of occurrence of a- and b-wave. The amplitude of the a-wave was measured from the baseline to the peak of the a-wave, and the amplitude of the b-wave

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was measured from the trough of the a-wave to the peak of the b-wave. Two waves were measured using pCLAMP9.2.

### *Histochemistry*

Sections for hematoxylin and eosin (HE) staining were first dewaxed in xylene, rehydrated in a series of descending alcohols, rinsed in ddH<sub>2</sub>O, and stained with HE. Stained sections were dehydrated in a series of ascending alcohols, cleared in xylene and mounted with cover slips [23]. The sections were observed with a OLYMPUS microscope (BX51T-PHD-J11, OLYMPUS, Japan).

### *Measurement of the ONL cell counts and thickness*

Image-Pro Plus6.0 software was used to select the ONL layer with an irregular selection. The ONL layer area and the number of cells were counted by the analysis of each photo, the number by dividing the area obtained cell density (number/mm<sup>2</sup>). Select 5 parts to measure the thickness of ENL layer, and the thickness of the 5 parts calculated the average value.

### *Cell culture*

A human ARPE-19 cell line was obtained from ATCC and was cultured in Dulbecco's modified eagle medium: nutrient mixture F-12 medium (BI, 01-053-1A, Israel) supplemented with 10% fetal bovine serum (PAN, P30-3302, Germany). Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Medium was changed every 2 days.

### *CCK8 assay*

ARPE-19 cells were seeded in 96-well plates at a density of 4×10<sup>4</sup> per well for 24 h. Cells were treated with different concentrations of t-BHP or vitamin C and TBN for the indicated time periods. The number of viable cells was then determined by the addition of CCK8 (Cell Counting Kit-8), it obtained from the Peptide Institute. Optical densities were read at 450 nm using a microplate spectrophotometer (Multiskan Ascent; Tecan Safire2, Switzerland).

### *Flow cytometry assay*

Different treated cells were collected, and FCM assay was employed to analyze apoptosis,

ΔΨ<sub>m</sub>, ROS and Ca<sup>2+</sup> level as previously reported [8]. ARPE-19 cells were treated with PL for 24 h and co-stained with annexin V-FITC and PI. Apoptotic cells were separated and quantified by a FACSCalibur Flow Cytometry System (Becton Dickinson, Calibur). Cells were treated with 10 μg/ml JC-1 staining solution for 20 min at 37°C (CellROX®, Life Technologies Corporation C10443). Changes in the relative proportion of red and green fluorescence on behalf of membrane potential. Cells were treated with probe solution to the desired concentration for 30 min at 37°C, requirement to avoid light. Cells were treated with Fluo 3-AM solution for 30-60 min at 37°C, and covered by the solution. Remove Fluo 3-AM working fluid, HBSS solution was added to cover the cells for about 20-30 min at 37°C, and the cell was detected by flow cytometry.

### *Western blot analysis*

Different treated cells were cultured in 6-well plates at a density of 1×10<sup>6</sup> cells, cells were collected in lysis buffer, and the total protein was extracted. Approximately 50 μg of protein were electrophoresed by 12% SDS-PAGE and transferred to nitrocellulose membranes and incubated with rabbit anti-human monoclonal antibody against caspase-3, Bax, Bcl-2, CHOP, Bip, CaMKII and GAPDH (1:5000 dilution, Abcam, USA). HRP-labeled secondary antibodies (1:5000 dilution) were added for two hours at room temperature, followed by ECL, and the results were normalized to the expression of GAPDH.

### *Statistical analysis*

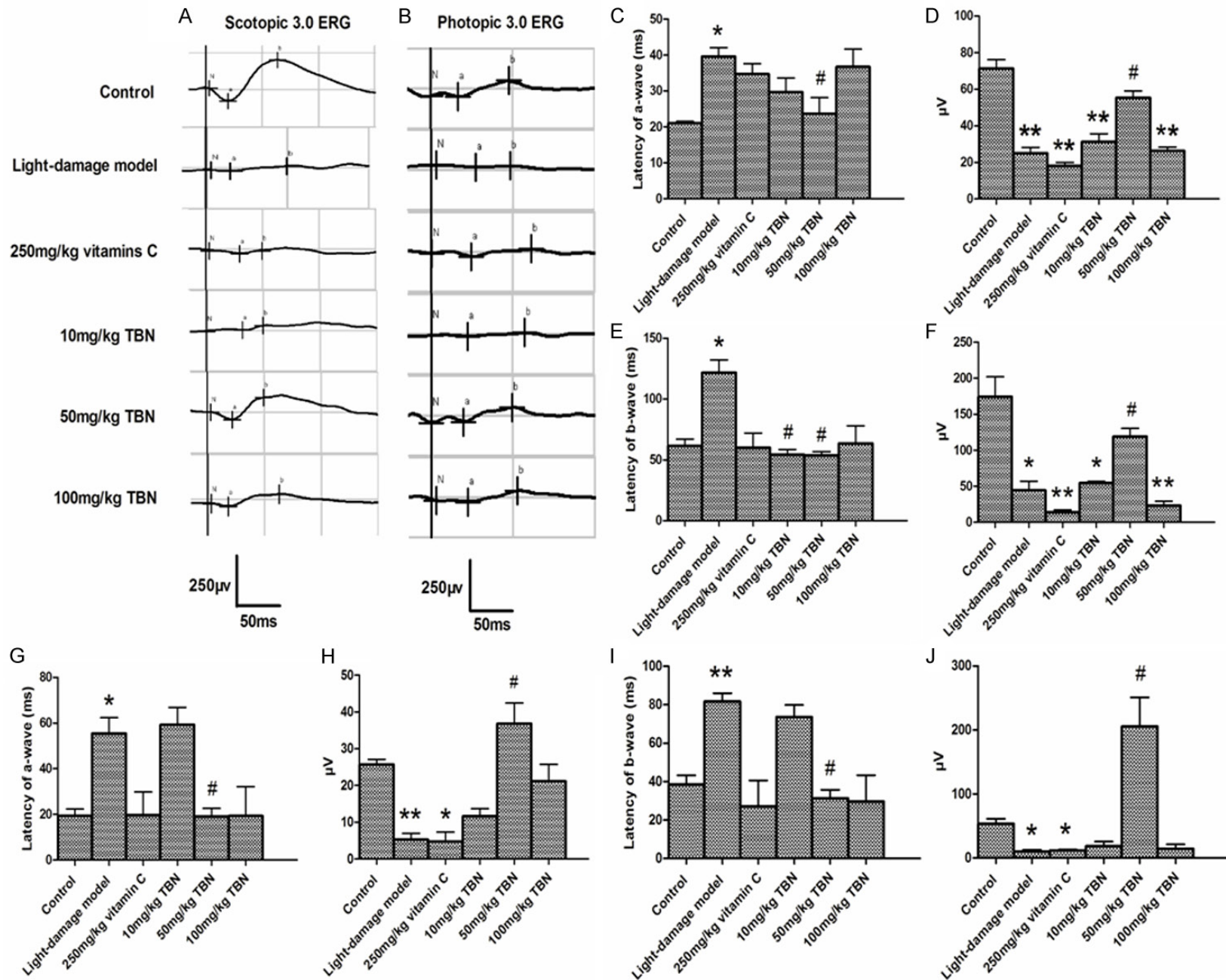
Statistical analysis was performed using SPSS 19.0 and GraphPad Prism 5 software. Statistical data represent mean ± sem and were determined using single factor analysis of variance. Comparisons between the two groups were performed using a student-test. *P*<0.05 was considered statistically significant.

## **Results**

### *The properties of scotopic and photopic flicker ERGs*

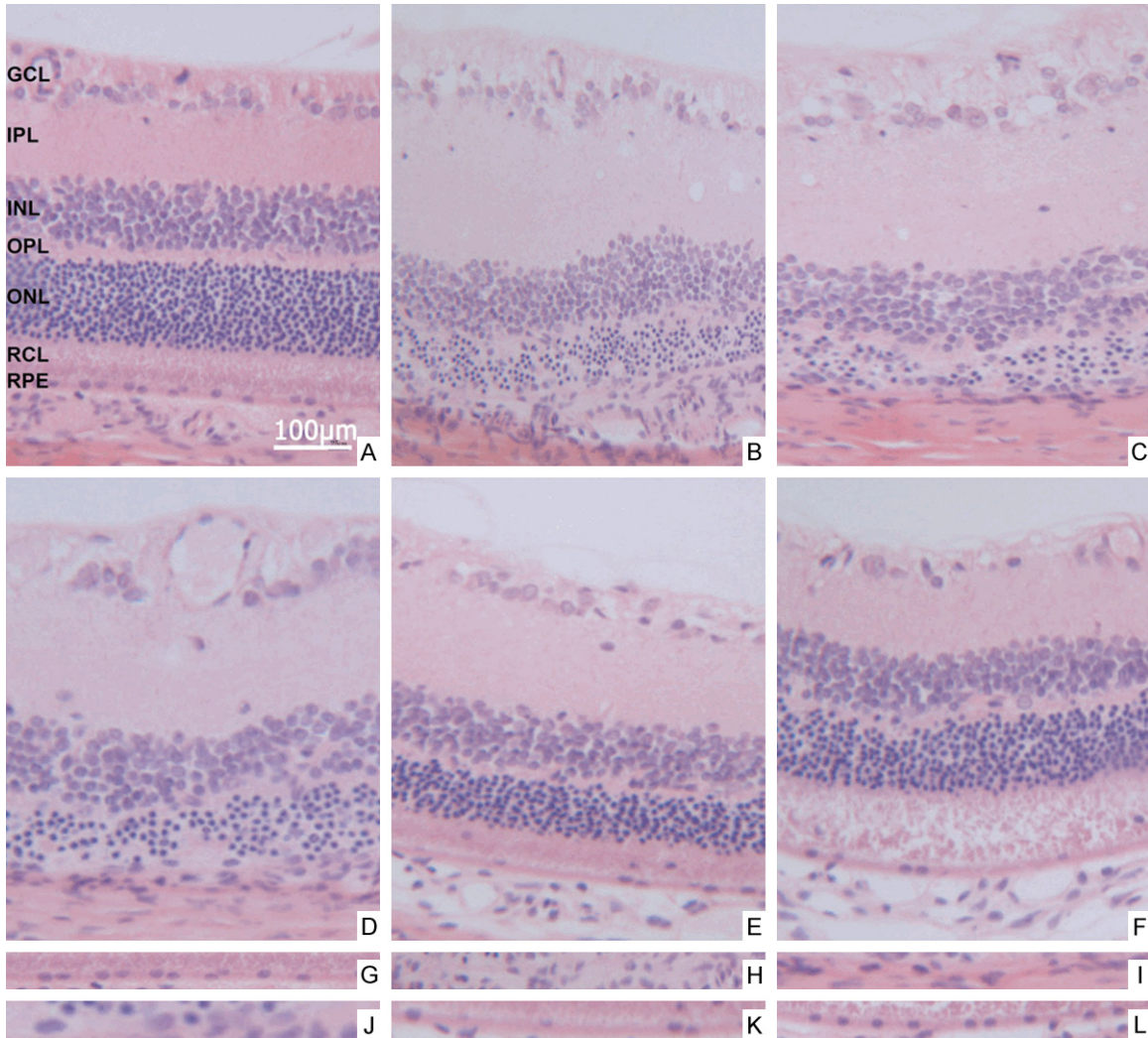
During dark and light adaptation, the representative Flicker ERGs responses obtained from different group SD rats are shown in (**Figure 1A, 1B**). The flicker response of light-damage

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**Figure 1.** Example of individual representative tracings of flash ERG performed in light induce retinal damage of SD rats before and after treatment. (A-J) Representative responses of photopic and scotopic 3.0 ERG recordings each subgroup using a series of temporal frequencies during dark and light adaptation, each waveform was recorded for 50 ms (A, B); each subgroup implite times (ms) and amplitudes ( $\mu\text{v}$ ) of a- and b-wave changes of flash ERG in scotopic (C-F) and photopic (G-J) conditions. All values are presented as mean  $\pm$  sem, \* $P < 0.05$ , and \*\* $P < 0.01$  compared to control or # $P < 0.05$  compared to model.



**Figure 2.** Histopathological observations. (A-L) Retina of normal control group (A, obj 10), GCL: ganglion cell layer, IPL: internal plexiform layer, INL: internal nuclear layer, EPL: external plexiform layer, ONL: outer nuclear layer, PRL: photoreceptor layer, RPE: retinal pigmented epithelium; HE staining of retina one week after SD rats model of light-induced retinal oxidative damage was established (B, obj 10); treatment groups of vitamin C at the indicated dose 250 mg/kg (C, obj 10) and TBN (10, 50, 100 mg/kg) (D-F, obj 10) pre-protection for one week; each subgroup of RPE cells morphological changes (G-L, obj 10). All values are presented as mean  $\pm$  sem.

model group had a negative waveform control normal group SD rats, both a-wave and b-wave implicit times slightly extended during rod- (Figure 1C, 1E) and cone-driven ERGs (Figure 1G, 1I), amplitudes of the a-wave and b-wave were significantly lost during rod- (Figure 1D, 1F) and cone-driven ERGs (Figure 1H, 1J).

In the treatment groups, both a-wave and b-wave implicit times of the recordings from the TBN (50 mg/kg for 7 d) pre-protection groups was shorter than that of light-damage model group during rod- (Figure 1C, 1E) and cone-driven ERGs (Figure 1G, 1I), but there was no statistical significance; amplitudes of the a-wave

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**Table 1.** Morphological analyses of the retinas

Groups	Number of ONL (Standard deviation)	Thickness of the ONL (Standard deviation)
Control	855.67 (21.08)	0.167 (0.38)
Light-damage group	450.33 (20.50)	0.1 (0.23)
250 mg/kg vitamin C	274 (25.24)	0.12 (0.46)
10 mg/kg TBN	310.33 (9.5)	0.11 (0.55)
50 mg/kg TBN	554.33 (10.21)	0.1 (0.24)
100 mg/kg TBN	484.67 (26.76)	0.1 (0.17)
P value	P<0.0001	P>0.05

and b-wave were significantly improved control light-damage model group during rod-driven ERGs (**Figure 1D, 1F**), however, it did not reach to normal values; amplitudes of the a-wave and b-wave of cone-driven ERGs (**Figure 1H, 1J**) were similar to that of rod-driven ERGs, even slightly higher than the normal group.

Compared with light-damage model group, the representative Flicker ERGs waveform was not significantly changed by vitamin C pre-protection. From the above results we identify that TBN can partly protect effect on ERG reaction of light induced in retina oxidative damage of SD rats, but vitamin C have no effect on light-damage model group.

### *Effect of vitamin C and TBN on light-induced retinal damage of SD rats*

On normal control group, each layer of retina was organized and cellular stratification neat (**Figure 2A, 2G**). Compared with normal control group, light-damage group retina, whose cell layers morphological obviously disorder. Thickness reduction was not seen in ONL. Nuclei number of ONL was counted. Significant reduction of nuclei number of ONL was observed in light-damage group compared to the control. RPE layers were discontinuous (**Figure 2H**).

On treatment groups, there were no morphological improvement in the vitamin C (250 mg/kg for one week) pre-protection group of light-induced retinal damage, in contrast, the degree of cell layer damage increased slightly (**Figure 2C, 2I**). Similarly, there was no obvious change in light-induced retinal damage of TBN (10 mg/kg for one week) pre-protection group (**Figure 2D**), no significant improvement in RPE layer was found (**Figure 2J**). These results suggest that, the light damage of the retina is continuously carried out with time. When the concen-

tration of TBN increased to 50, 100 mg/kg for one week, there was morphological improvement in light-induced retinal layer damage (**Figure 2E, 2F, 2K, 2L**), but they did not reach to morphological of retinal layers in normal control group.

Morphological analyses concerning the number and thickness of ONL, did not show any statistically significant changes in TBN at the indicated dose 50 and 100 mg/kg for one week (**Table 1**). From the above results we identify that TBN can partly protect effect on morphological changes of light induced in retina oxidative damage of SD rats, and to delay light damage of retina in SD rats, but vitamin C do not have the above protective effect.

### *Oxidative damage and protection of ARPE-19 cells*

To investigate the effect of t-BHP on cell survival, ARPE-19 cells were treated with t-BHP at 10, 100, 300 or 500  $\mu$ M concentrations, each for 2, 6, 12 or 24 h. t-BHP began to induce about 50% cell death at 300  $\mu$ M for 6 h (**Figure 3A**); 500  $\mu$ M produced a similar effect. We used a 300- $\mu$ M for 6 h treatment for the following tests.

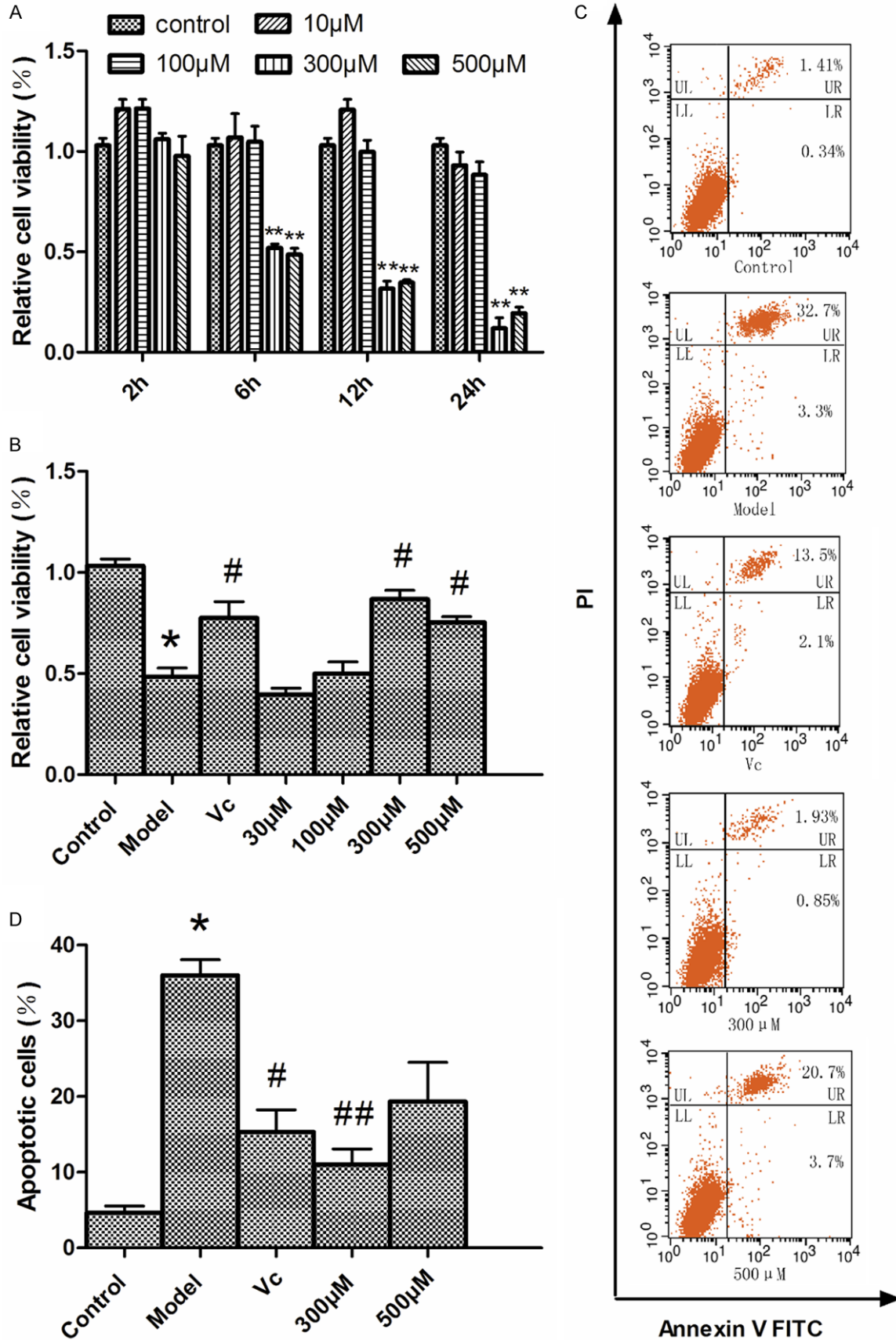
The concentration-dependent effects of vitamin C and TBN in the oxidative damage model of ARPE-19 cells were shown in (**Figure 3B**). After vitamin C pre-protection, relative cell viability significantly increased. TBN was markedly effective at increasing cell viability when administered 300, 500  $\mu$ M for 4 h pre-protection oxidative stress damage of ARPE-19 cells.

Treatment with 300  $\mu$ M t-BHP for 6 h induced late apoptosis in 32.7% of cells, indicating that apoptosis is the major death process induced by t-BHP. However, late apoptosis respectively was 13.5%, 1.93% and 20.7% when pre-protection with 300 and 500  $\mu$ M for 4 h (**Figure 3C, 3D**). These findings demonstrated that compared with the oxidative damage model group, TBN had the anti-apoptotic effect, and the inhibition of apoptosis was slightly stronger than vitamin C.

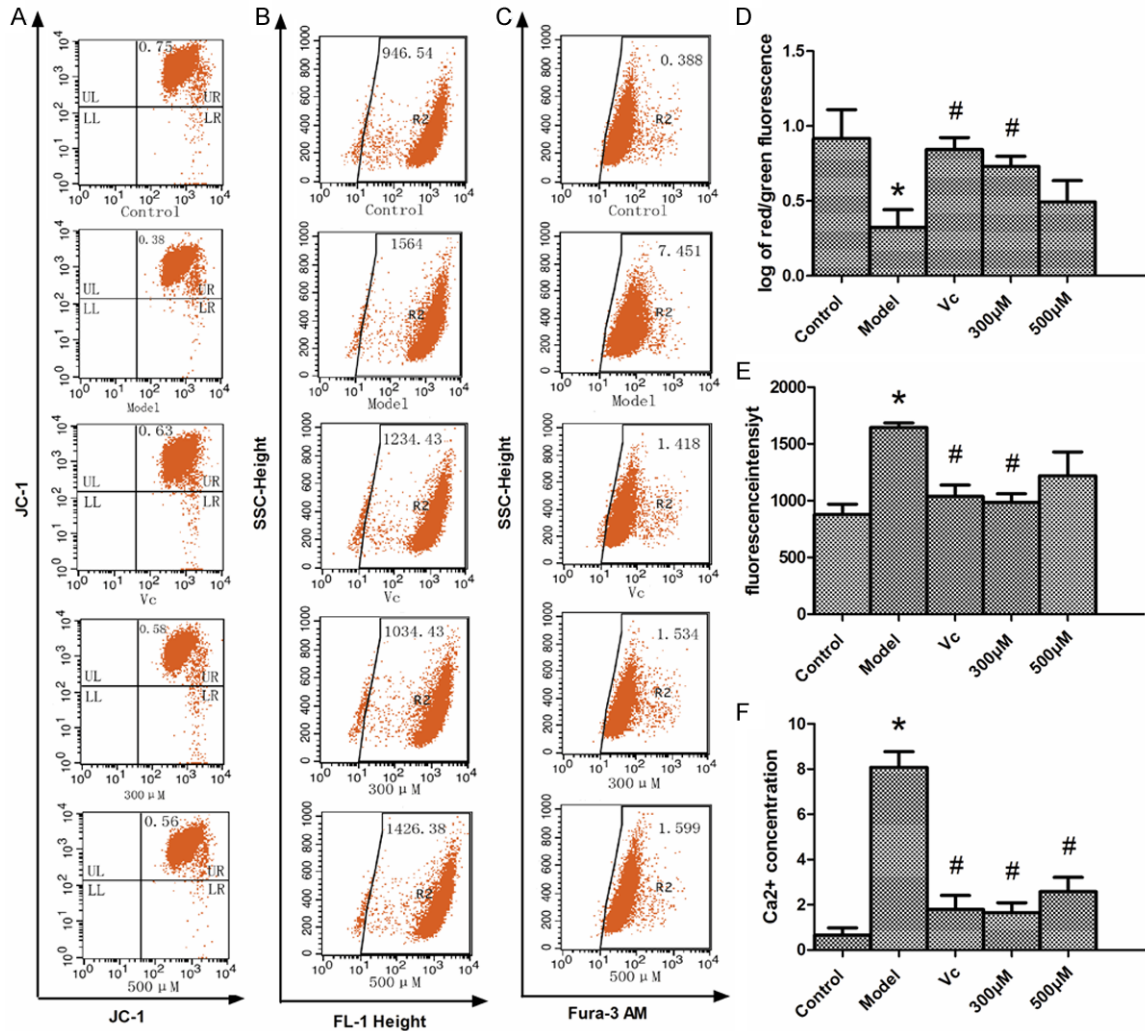
### *TBN inhibited apoptosis partly via modulating $\Delta\Psi_m$ , ROS and $Ca^{2+}$*

We investigated  $\Delta\Psi_m$  status as a function of TBN. Compared with oxidative damage model group,  $\Delta\Psi_m$  was significantly increased by a 4 h

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**Figure 3.** Oxidative stress injury and protective effect in ARPE-19 cells model. (A-D) ARPE-19 cells were treated with t-BHP at the indicated concentrations (10, 100, 300, 500  $\mu\text{M}$ ) for 2, 6, 12 or 24 h and cell viability was then analyzed by cell proliferation assay using CCK-8 as described under 'Materials and Methods' (A); pre-injury (300  $\mu\text{M}$  t-BHP for 6 h) in ARPE-19 cells were pre-protected with vitamin C at the indicated dose (100  $\mu\text{M}$ ), TBN at the indicated concentrations (30, 100, 300, 500  $\mu\text{M}$ ) for 4 h and cell viability was then analyzed by cell proliferation assay using CCK-8 Method (B); the percentage of different groups apoptotic cells was detected by Annexin V/PI dual staining with flow cytometry (C, D). All values are presented as mean  $\pm$  sem, \* $P$ <0.05, and \*\* $P$ <0.01 compared to control or # $P$ <0.05 and ## $P$ <0.01 compared to model.



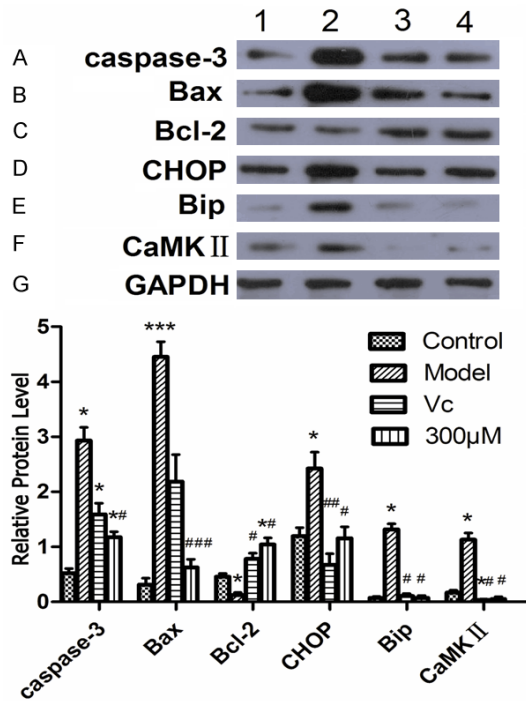
**Figure 4.** Effects of Vc and TBN on oxidative stress. (A-F) ARPE-19 cells were treated with t-BHP at the indicated concentrations 300  $\mu\text{M}$  for 6 h, vitamin C at the indicated dose 100  $\mu\text{M}$  and TBN (300, 500  $\mu\text{M}$ ) for 4 h, cellular mitochondrial membrane potential ( $\Delta\Psi\text{m}$ ) was detected with the JC-1 probe (A, D); intracellular ROS levels and Ca<sup>2+</sup> concentration respectively determined by DCF-DA (B, E) and Fura-3 AM (C, F). All values are presented as mean  $\pm$  sem. From three separate experiments, \* $P$ <0.05 compared to control or # $P$ <0.05 compared to model.

pre-protection of 300 Mm TBN, from 0.38 to 0.58, however, slightly lower than vitamin C, while pre-protection 4 h, even 500  $\mu\text{M}$  TBN decreased  $\Delta\Psi\text{m}$  slightly (Figure 4A, 4D).

We then tested whether oxidative damage and protection were the cause of  $\Delta\Psi\text{m}$  loss and

improve by measuring intracellular ROS and Ca<sup>2+</sup> levels alterations. There were resulted in an increase in ROS levels and the intracellular Ca<sup>2+</sup> concentration associated with a 6 h induced of 300  $\mu\text{M}$  t-BHP, while 300  $\mu\text{M}$  TBN pre-protection for 4 h produced about 13.18% and more than fourfold decrease, however, the





**Figure 5.** Proteins expression levels of CHOP, Bip, Bax, Bcl-2, caspase-3 and CaMKII of each subgroup. ARPE-19 cells were treated with t-BHP at the indicated concentrations 300  $\mu$ M for 6 h, vitamin C at the indicated dose 100  $\mu$ M and TBN 300  $\mu$ M for 4 h. GAPDH was used as the internal control. All values are presented as mean  $\pm$  sem from three separate experiments, \* $P$ <0.05 and \*\*\* $P$ <0.001 compared to control or # $P$ <0.05, ## $P$ <0.01 and ### $P$ <0.001 compared to model.

effect is still lower than vitamin C (Figure 4B, 4C, 4E, 4F). These results indicate that TBN can protect ARPE-19 cells oxidative damage induce apoptosis a portion of via increasing  $\Delta\Psi$ m and removing intracellular excess ROS and  $Ca^{2+}$ , we used a 300- $\mu$ M TBN pre-protection for 4 h treatment for the following tests.

*Expression of oxidative stress proteins in mitochondria and endoplasmic reticulum*

The expression of mitochondrial, ER stress and apoptosis-related proteins (caspase-3, Bax, Bcl-2, CaMKII, CHOP and Bip) had been detected with western blotting. The protein caspase-3 was found to be significantly increased in t-BHP treatment model group compared to normal control, while caspase-3 of TBN pre-protection group showed downregulation compared to model group, but the expression of protein was significantly higher than that of nor-

mal control group. However, the inhibition of vitamin C on caspase-3 was not obvious compared to TBN (Figure 5A). Further, the protein level of Bax was significantly upregulated in t-BHP of treated cells with respect to normal control, while significantly downregulated in TBN pre-protection group compared to model group (Figure 5B). The protein level of Bcl-2 of model group showed downregulation compared to normal control, the expression of protein-Bcl-2 in vitamin C and TBN groups were significantly improved compared to model group, and treatment of TBN group was significantly higher than normal control group (Figure 5C).

Compared with normal control, the expression of ER stress-related marker CHOP and Bip were significantly increased in t-BHP treatment model group, and the expression of protein CHOP and Bip in vitamin C and TBN groups were obviously upgraded compared to model group (Figure 5D, 5E). Since one possible apoptotic pathway has been thought that  $Ca^{2+}$  released from mitochondria could trigger the expression of protein CaMKII, we evaluated the CaMKII activity in ARPE-19 cells with before and after of pre-protection. The expression of protein CaMKII significantly increased compared with normal control, while the expression of protein CaMKII in vitamin C and TBN groups were significantly improved compared to model group (Figure 5F). These findings confirm that TBN inhibited t-BHP induced in the apoptosis of ARPE-19 cells via regulated the expression of oxidative stress related proteins in mitochondria and ER.

**Discussion**

Traditional therapeutic products targeting degenerative diseases have largely focused on palliative forms of treatment that mainly ameliorate or control the symptoms of a disease [24]. There is currently no cure available for light-induced retinal damage, and even palliative treatments are rare. light-induced retinal damage is one such retinal degenerative disorder which starts with the progression of age. Metabolism plays an important role in initiation of such diseases of ageing. Slow metabolism leads to the occurrence of mitochondria oxidative stress [25]. Treatment options span a broad range of therapeutic approaches, including thermal laser photocoagulation, surgical

approaches (excision, displacement, or transplantation), and new treatments targeting the choroidal neovascularization (CNV) component and its pathogenic cascade, such as verteporfin with photodynamic therapy (vPDT) and more recently anti-vascular endothelial growth factor (VEGF) therapies.

There are two parts of our study. In vivo, we used intense light of no mechanical injury exposure to the eyes of SD rats, induced retinal light damage model. That is, the normal SD rats were dark adapted after 24 hours, 3500±200 Lux white light continuous exposure to the eyes of SD rats. In vitro, we used t-BHP to induce ARPE-19 cell oxidative damage model. To investigate the effect of TBN on retinal light damage in SD rats and its mechanism. Although there is no unified modeling method for retinal light injury animal models, it can be concluded from the results of ERG and HE staining, after 3500±200 Lux white light continuous exposure to the eyes of SD rats, and it has resulted in changes in the function and structure of the retina layer cells. The F-ERG reflects the electrical response of before retinal ganglion cells to the light stimulus. The latency and amplitude of a-wave and b-wave of ERG mainly reflect functional changes of photoreceptor cells and Müller cells. The a-wave and b-wave in the light-induced damage group were almost disappeared, under the condition of dark and light adaptation, after light-injury damage retina of SD rats had no response to ERG 3.0 stimulation. Refer to relevant literatures, Müller cells are one of the main glial cells in retina of spinal animal, the radial structure of Müller cells specific throughout the whole layer of retina, it is important to maintain the stability of the retinal anatomy and function. Müller cells dysfunction in the early stage of the disease, it can delay thinning of retinal ONL layer [26-28].

Cells damage induced by oxidative stress is believed to be able to disrupt mitochondrial function, thereby leading to decreased  $\Delta\Psi_m$ , increased ROS,  $Ca^{2+}$  levels and induction of apoptosis [29-31]. Our results show that TBN effectively protects ARPE-19 cells from t-BHP-induced reductions in  $\Delta\Psi_m$ , increased apoptotic cells ratio, ROS,  $Ca^{2+}$  levels, all of which are important for cell survival. TBN treatment can increase  $\Delta\Psi_m$  from 0.38 to 0.58, decrease apoptotic cells ratio, ROS,  $Ca^{2+}$  levels by about 13.18% and more than fourfold, which we

assume to be the most important contributor to TBN's protective effect. In addition, the literature reports that ER stress can also lead to cell apoptosis. In recent years, we have come to understand that the effectors of apoptosis are represented by a family of intracellular cysteine proteases known as caspases [32, 33]. Although TBN can protect ARPE-19 cells from oxidative damage induced by t-BHP, but we still don't clear that which are signaling pathways of TBN's protective effect. Our results confirm that TBN can regulate the expression of Bcl-2 and Caspase-3 protein, this may be a pathway for TBN to protect mitochondrial oxidative damage in ARPE-19 cells. ER is the organelle for protein synthesis and kinetic balance in the plasma, and ER stress may be involved in the pathogenesis of neurodegenerative diseases [34]. Our results found that the expression of CHOP and Bip protein in the model group was significantly higher than that of the normal group. In treatment groups, after vitamin C and TBN pre-protection, the expression of CHOP and Bip protein was significantly decreased compared with the model group. These results confirm that TBN can regulate the expression of CHOP and Bip protein, this may be the other pathway for TBN to protect apoptosis of ARPE-19 cells. In addition,  $Ca^{2+}$  homeostasis is crucial for regulating mitochondrial and ER function and regulating activity of calcium-dependent enzymes. Disruption of intracellular  $Ca^{2+}$  homeostasis induces cell death in a variety of pathological conditions involving  $Ca^{2+}$  increase and oxidative damage [35-37]. Several lines of evidence indicate that many of these detrimental effects are mediated by  $Ca^{2+}$ /CaMKII, such as, ROS formation, cytochrome c release, activation of caspase-3 and so on. We hypothesized that TBN can protect caused by  $Ca^{2+}$ /CaMKII signaling pathway was involved in t-BHP induced in apoptosis of ARPE-19 cells. Our results showed that the expression of CaMKII protein was significantly up-regulated in the model group, while the expression of CaMKII protein was significantly down-regulated in treatment groups. This part of results confirms our hypothesis.

In conclusion, through integrated analysis in vivo and vitro experiments, TBN was only partially protected in light-induced retinal damage in SD rats, we identified TBN may be used as a candidate drug at the treatment of light-induced retinal damage in SD rats, with expression lev-

els significantly correlated with mitochondrion and ER stress in vitro. Our functional investigations revealed that oxidative stress plays multiple path phenotypic roles in light-induced retinal damage in SD rats, suggests that oxidative stress may represent a ubiquitous path to degenerative diseases progression. A multi-factorial approach targeting multiple pathways of disease should be considered, taking into account patients' genetic phenotypes and stages of disease. Combining several of different treatment options may increase the likelihood of patients recovering or maintaining their vision. The broader involvement of mitochondrion and ER stress in the pathogenesis of light-induced retinal damage in SD rats and the mechanisms underlying TBN protection effects will be the focus of future investigations.

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### Disclosure of conflict of interest

None.

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