Thioredoxin is a potential therapy for light-induced photoreceptor degeneration in diabetic mice

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Abstract

BACKGROUND: Retina degeneration is a set of disease that can be characterized by progressive loss of rod or cone photoreceptors that can experience higher light exposure compared with other parts of our body.

MATERIAL AND METHODS: To investigate in diabetic mice the effects of different light on retinal photoreceptor and its related mechanisms, 4–6 weeks male Balb/c mices were randomly divided into control group, light treatment group (light 1 and light 2), light treatment with filter membrane group (LFM1 and LFM2), light treatment filter blue light group (LFB), diabetic group (DM), diabetic with light treatment group (DM+Light), diabetic light treatment with filter membrane group (DM+LFM1 and DM+LFM2), diabetic light treatment with filter blue light group (DM+LFB). Electroretinography (ERG) was used to assess retinal function. H&E staining was used to observe the morphology and measure the outer nuclear layer thickness in the retina. The related gene expression was detected by Real-time PCR.

RESULTS: Light can significantly reduce the retinal function, outer nuclear layer thickness, and Trx expression. After different kinds of membranes and blue light filter treatments, the outer nuclear layer thickness, Nrf2, Trx expression can be improved in diabetic mice. However, the expression of Txnip and ASK1 was decreased.

CONCLUSION: Light can increase the photoreceptor damage in diabetic mice. The reduction of light intensity and change in the visible light components can reduce the light-induced damage in diabetic mice, and the mechanism may be related to up-regulation the antioxidant protein Trx expression.

ORIGINAL ARTICLE

INTRODUCTION

There are several different types of cell in the retina organized in multiple layers which are affected by various diseases of the retina contributing to blindness around the world. Retina degeneration is a set of disorders that can be characterized by progressive loss of rod or cone photoreceptors which can experience higher light exposure compared with other parts of our body. Progressive dysfunction and degeneration of photoreceptors (PR) is a main reason leading to blindness (Mitra *et al.* 2014). Photoreceptor damage is irreversible and the main reason of blindness of many human retina degenerative diseases such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP). Among the damage models the light damage has been used in rodents as a model of photoreceptor degeneration for a long time (Song *et al.* 2016).There are many factors which can induce photoreceptor cell death in light damage model (Shimazawa *et al.* 2015) such as oxidative stress (Imai *et al.* 2010), endoplasmic reticulum (ER) stress and mitochondrial damage.

Diabetes mellitus is one of the most common diseases and can lead to many complications such as diabetic retinopathy (DR), kidney damage and heart disease. Although DR is considered primarily as a vascular complication with alteration of the blood retinal barrier in diabetes (Antonetti *et al.* 2012) and there are more and more studies suggesting that reduced function of neurons in retina starts earlier than vascular damage (Ren *et al.* 2017; Sohn *et al.* 2016) . Exposal to light exposure experience, oxidative stress and accumulation of reactive oxygen species (ROS) which affects visual function is meet by in almost every DR patient (Du *et al.* 2013), so seeking for some method or key molecular mechanism to protect from light damage in DR patient is very important.du

Thioredoxin (Trx) first identified in Escherichia coli in 1964 by Peter Reichard and his Coworkers (Laurent *et al.* 1964) is a 12kDa small protein with redox-active dithiolin the active site –Cys–Gly–Pro–Cys– that plays an important role in many processes such as proliferation, anti-apoptosis, anti-oxidative stress and regulation transcriptions. Trx, Thioredoxin reductase (TrxR) and NADPH comprise the Trx system, which contributes to maintaining the redox status balance in all cells (Xu *et al.* 2012). Trx can bind with its inhibitor Thioredoxin-interacting protein (Txnip) and Apoptosis signal-regulating kinase1 (ASK1) in the normal condition. However, when Trx is oxidized, ASK1 and Txnip are dissociated from Trx leading to stimulate the downstream related signaling pathway.

In this research, we used different methods comprising two kinds of membranes and filter blue light to treatment the light damage in diabetic mice, aiming to seek for some targets to prevent the damage induced light in diabetic mice and to provide some evidence for treatment of light damage in diabetic retinopathy.

MATERIALS AND METHODS

Animal care

All the experimental procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committees of Dalian Medical University Laboratory Animal Center. Six-week-old male inbred Balb/c mice weighing 20–25 g (Dalian Medical University Laboratory Animal Center) were housed 6 per cage in an animal colony facility for 2 weeks. The animals were maintained in a room with a constant temperature (22±2℃). All the animals were born and raised in a 12-h-light/12-h-dark environment with an average illumination of 100 lx. Tap water and food pellets were provided.

Diabetes model and light exposure

Mice were randomly divided into the DM group and the non-diabetes group and maintained on a high-fat and sugar diet composed of (by mg) 10% sugar, 10% lard, 5% yolk, 1% cholesterol, and 0.2% bile salt and a standard chow diet, respectively. Each mouse remained on the assigned diet throughout the whole experiment. After eight weeks, the DM group was intraperitoneally injected with 80 mg/kg STZ. The tail vein blood glucose was measured every three days two times after the injection, and those with blood glucose ≥16.7 mmol/L were considered DM mice (Zhang *et al.* 2013; Xu *et al.* 2014; Lu *et al.* 2012). The experiments were conducted between 10:00 and 14:00. STZ (Sigma) was dissolved in cold 50 mM citric acid buffer (pH 4.5).

The light exposure in mice was performed as previously described (Kong *et al.* 2016). In brief, the mice were dark adapted for 24 h before the experiments and were exposed to 5000 lux of diffuse, cool, white fluorescent light for 24h to determine an acute light-induced retinal degeneration model for subsequent experiments. Mice were randomly divided into the control group, light treatment group (light 1 and light 2), light treatment with filter membrane group (LFM1 and LFM2), light treatment filter blue light group (LFB), diabetic group (DM), diabetic with light treatment group (DM + Light), diabetic light treatment with filter membrane group (DM + LFM1 and DM + LFM2), diabetic light treatment with filter blue light group (DM + LFB).

Electroretinography (ERG)

ERG (GOTEC, China) was performed as previously described (Kong *et al.* 2011). Briefly, the mice were kept in total darkness overnight after different treatments (Control group, light 1, light 2, LFM1, LFM2 and LFB) before the ERG recording. After the mice were anesthetized and the pupils were dilated, full-field ERG at a light intensity of 10 cd•s/m² and a band pass of 1–300 Hz was performed using a GUOTE ERG system. For the quantitative analysis, the A- and B-wave amplitudes

were measured. The ERG waveforms of both eyes in the same animal were recorded simultaneously.

Histological analysis

Quantitative histology was performed as described (Kong *et al.* 2007). After sacrificing the mice via CO_2 inhalation, the mouse eyes were enucleated at the indicated time points. The enucleated eyes were immersed in 4% paraformaldehyde containing 20% isopropanol, 2% trichloroacetic acid, and 2% zinc chloride for 24 h and then in 70% ethanol for 24–60 h. After alcohol dehydration, the eyes were embedded in paraffin, and 5-µm-thick sagittal sections containing the entire retina, including the optic disc, were sliced. The retinal sections were stained with hematoxylin-eosin (HE). In each of the superior and inferior hemispheres, we measured the thickness of the outer nuclear layer (ONL) in the retina by the software (Nikon).

Quantitative real-time PCR

Total RNA was obtained from each cell preparation using TRIzol (Invitrogen). Reverse transcription was performed using a Perfect for Real-Time PCR Kit (Takara). Real-time PCR was conducted to measure Txnip expression using a SYBR-Green mixture (Takara) and reverse-transcribed cDNA as the template. PCR was performed in a final volume of 20 µl using an ABI Prism 7000 Sequence Detection System under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 3 s, 72°C for 30 s, and 55°C for 30 s. GAPDH was used as an internal control. The following primers were used: ASK1(mouse), forward: 5′-TTT-GTTTCGTGAGACTGCGTACC-3′, reverse: 5′-AGA-CACTTGGGCACACTACACA-3′; and Trx (mouse), forward: 5′-CAAATGCATGCCGACCTTCCAGTT-3′, reverse: 5′-TGGCAGTTGGGTATAGACTCTCCA-3′. The primer sequences for GAPDH (mouse) and Txnip (mouse) were previously reported (Ren *et al.* 2015).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed as described (Zhou *et al.* 2005). After euthanasia by CO_2 inhalation, the eyes were immediately enucleated, and the neural retinas were separated from the eyes under a microscope. Total mRNA was extracted from the retinas using an RNA isolation reagent (TRIZOL, TaKaRa), according to the manufacturer's instructions and treated with RNase-free DNase (TaKaRa, Dalian, China). The concentration of RNA was quantified by reading at 260 nm on a spectrophotometer. The cDNA was synthesized using the First-Strand Synthesis System for RT-PCR kit (TaKaRa), according to the manufacturer's protocol, using 1ug of total RNA. The PCR conditions consisted of a denaturation step at 94°C for 2 min, then 35 cycles of 94°C for 30 s, 56 °C for 30 s, 72°C for 15 s, and a final extension at 72°C for 10 min. The PCR products were visualized by electrophoresis on 1% agarose gel (Invitrogen Corp, Carlsbad, CA, USA), stained with 0.5 mg/ml ethidium bromide, and photographed under UV light. As an internal control, RT-PCR for GAPDH was performed simultaneously. The primers for the target genes are: Nrf2, forward primer, 5-AGTTCTCGCTGCTCGGACTA-3'and reverse primer, 5-AGGCATCTTGTTTGGGAATG-3; GAPDH, forward primer, 5-TGTGATGGGT-GTGAACCACGAGAA-3 and reverse primer, 5-GAGCCCTTCCACAATGCCAAAGTT-3.

Statistical analysis

The data are presented as the mean ±SEM. The statistical analyses were performed using one-way analysis of variance (ANOVA) for continuous variables. SPSS 17.0 was used for all of the statistical analyses. Statistical significance was defined as $p<0.05$.

RESULTS

Functional evaluation of photoreceptor degeneration by electroretinography (ERG)

By the ERG recordings from each group, the data showed in Fig. 1A&B, the amplitudes of A-wave and B-wave were significantly decreased in light damage group compared with control group. However, the amplitudes of A-wave and B-wave were significantly increased with different kind light filter membranes and light filtration treatment. These results clearly indicated that pretreatment with light filter membrane and light filtration preserved the visual function of light-damaged retinas effectively.

Morphological evaluation of photoreceptor degeneration by quantitative histology

It was possible to document the photoreceptor degeneration by measuring thickness across the retina section H&E staining. The results of this quantitative histology showed in Fig. 1C and demonstrated that exposure to 5000 lux light for 24h reduced the thickness and induced significant photoreceptor degeneration in lux group compared with control group ($p<0.01$). Loss of photoreceptor cell nuclei in the ONL was remarkably observed in mice after exposure to 5000lux light for 24 h. However, it can inhibit the ONL layer thickness reducing after pretreatment with light filter membrane and light filtration. However, the ONL layer thickness reduces were inhibited after pretreatment with different kind of light filter membranes and pretreatment with light filtration. The results of this quantitative histology showed in Fig. 2A&3A.

Decreased expression of Trx and Nrf2 in light-damaged mice

The retinal levels of Trx and Nrf2 mRNA were analyzed by RT-PCR in light damage mice. The results showed that the expression of Trx and Nrf2 in the retina was decreased significantly after light exposure ($p<0.01$), However, they are increased after pretreatment with light filter membrane and light filtration in normal and
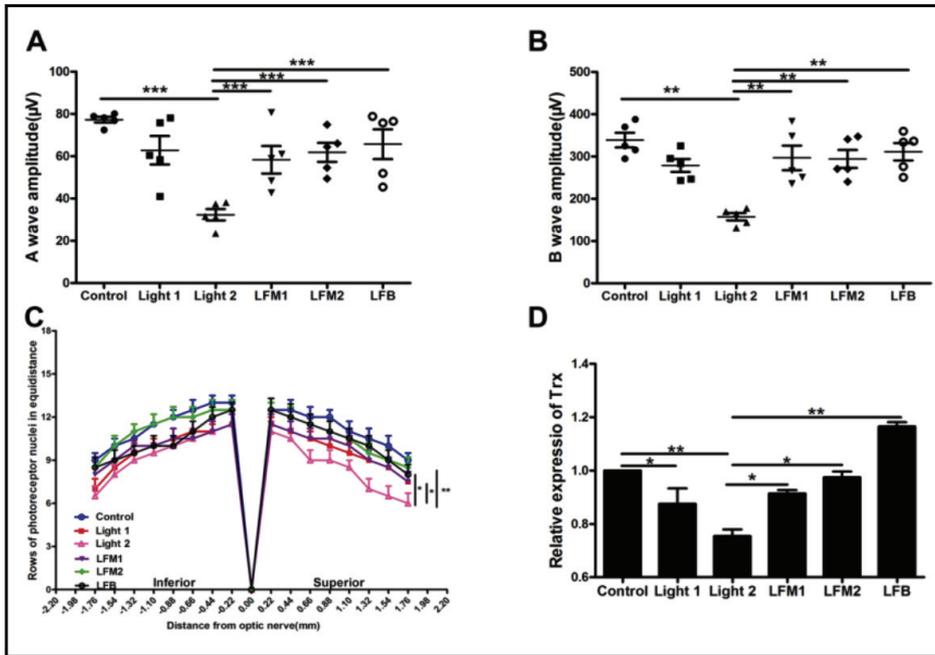
Fig. 1. Light damage induced photoreceptor degeneration
(A) - (B)the amplitudes of A-wave and B-wave were recorded by ERG in light damage mice.
(C) The counting of nuclei in the outer nuclear layer.
(D) The expression of Trx in light damage mice.
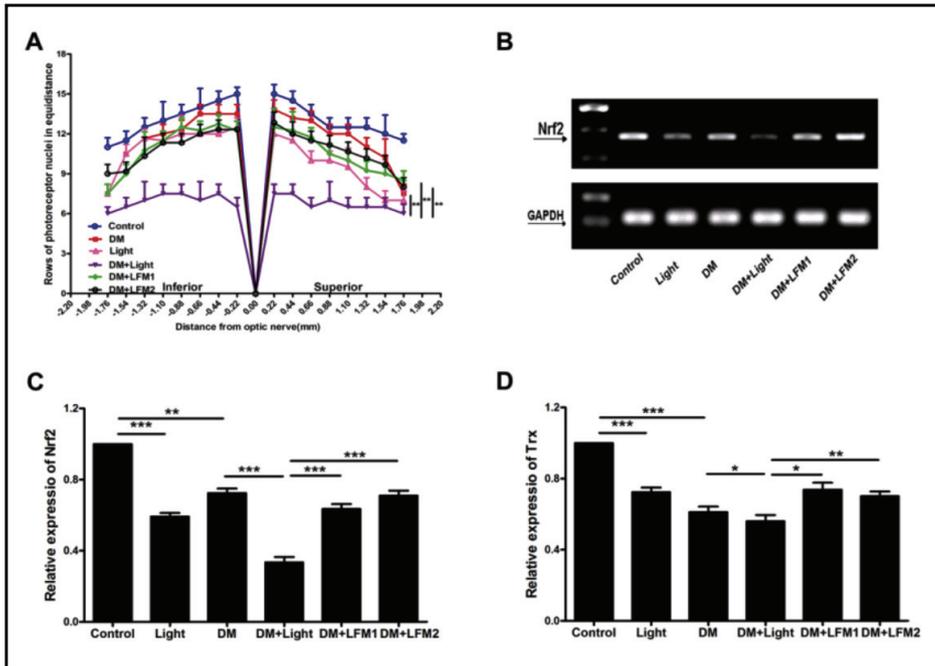*p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 2. The effect of light filter membrane on the light-induced photoreceptor degeneration in diabetic mice
(A) The counting of nuclei in the outer nuclear layer in light-damage diabetic mice with a different kind of light filter membrane.
(B) - (C) The expression of Nrf2 in light-damage diabetic mice with a different kind of light filter membrane.
(D) The expression of Trx in light-damage diabetic mice with a different kind of light filter membrane.
*p < 0.05, **p < 0.01, ***p < 0.001.

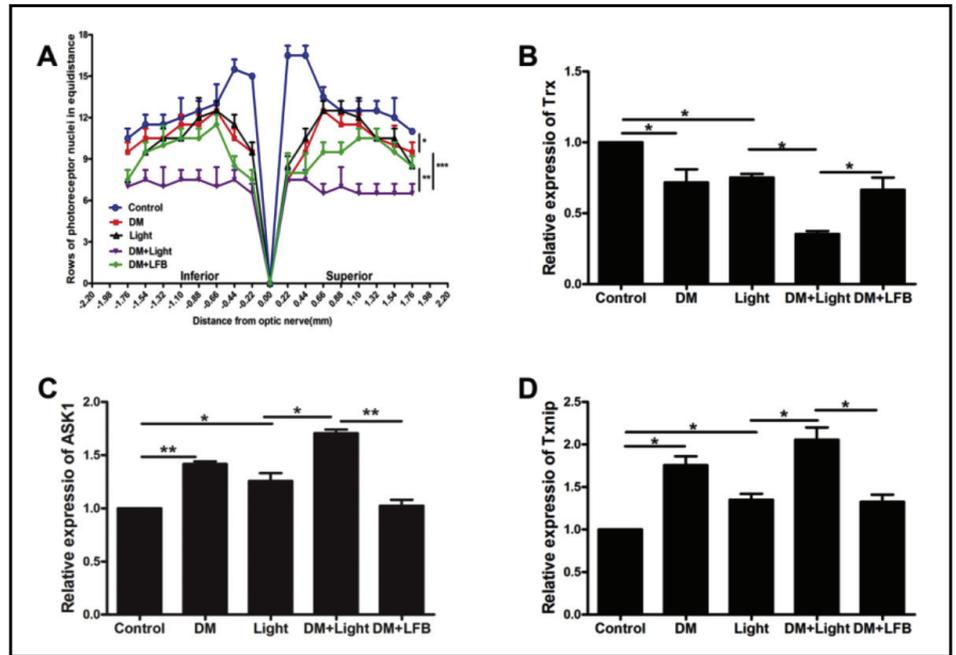diabetic mice compared with control group, the data was shown in Fig. 1D, Fig.2 B&C and Fig.3 B.

Increased expression of ASK1 and Txnip in light-damaged mice

To detect the expression of ASK1 and Txnip on mRNA level was analyzed by real-time PCR in light damage mice. The results showed that the expression of ASK1 and Txnip in the retina was increased significantly after light exposure (p<0.01). However, they are decreased after pretreatment with light filtration in diabetic mice compared with light-damaged diabetic mice, data was shown in Fig.3 C&D.

DISCUSSION

Excessive exposure to light can cause two irreversible types retina damage which are thermal damage and photochemical damage. The thermal damage happens when the retina is exposes to extremely bright light for a short time. However, the photochemical damage occurs when the retina is exposed to light for an extended period of time (Noell *et al.* 1966). Moreover, some research suggests a complicated association between photochemical damage and light-induced retinal degeneration recently.

Fig. 3. The effect of blue light on the light-induced photoreceptor degeneration in diabetic mice (A) The counting of nuclei in the outer nuclear layer in light-damage diabetic mice with blue light filtration. (B) The expression of Trx in light-damage diabetic mice with blue light filtration. (C) The expression of ASK1in light-damage diabetic mice with blue light filtration. (D)The expression of Txnip in light-damage diabetic mice with blue light filtration. *$p < 0.05$, **$p < 0.01$.

There are many factors can induce retinal cell death after light exposure including an increase in intracellular calcium levels, nitric oxide (NO), rhodopsin mutation, and free radicals, including reactive oxygen species (Wenzel et al, 2005). Oxidative stress had received extensive attention as the pathogenic contributor to light-induced retina damage. We think that the photoreceptor degeneration in the rodent light-damage was associated with oxidative stress and ROS accumulation [1, Wenzel et al, 2005; Zhao et al, 2014). Nrf2, NF-E2-related factor 2, is a stress-response transcription factor which plays a role in many cellular protection processes. Nrf2 is also serving as an important regulator of protective mechanism against oxidative stress in many cell types (Chen et al, 2017). Trx is a 12kD small protein that plays an important role in anti-oxidative stress process, which was regulated by Nrf2.

In order to investigate the role of Trx in retinal damage after exposure to light in the present study we first analyzed the expression of Trx after light-induced retinal damage in diabetic mice. The expression of Nrf2 and Trx was decreased in the injured retina after light damage and the expression of Trx was increased after treatment with different thickness filter membrane in diabetic mice. Moreover, the thickness of retina reduce was prevented. It indicated that activation Nrf2 can regulate Trx expression leading to preventive retina thickness reduction.

Apoptosis signal-regulating kinase1 (ASK1) is a member of the mitogen-activated protein kinase (MAPK) kinase family (MKKK) and it activated apoptosis (Lin et al, 2013). Thioredoxin-interacting protein (Txnip) is an cellular endogenous Trx inhibitor and it can activate apoptosis also (Wei et al, 2013). In our research, we found that the expression of Trx was
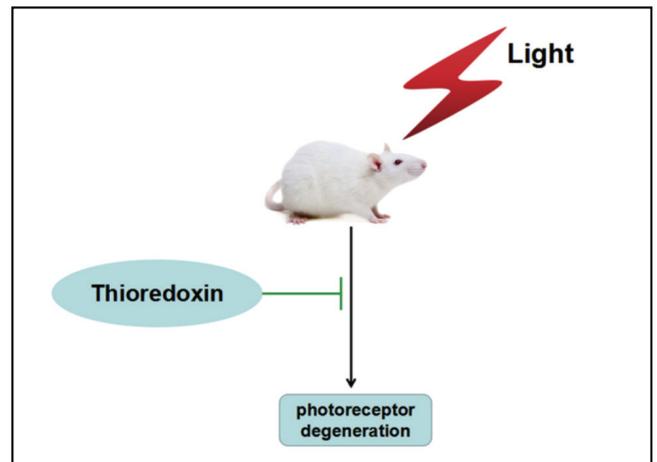

Fig. 4. The summary of related mechanism in the model.

decreased in injured retina after light damage and the expression of ASK1 and Txnip was increased. However, after treatment with filter blue light, the expression of Trx was increased and the expression of ASK1 and Txnip was decreased. The thickness of retina reduction was prevented as well. It indicated that up-regulated Trx can down-regulate ASK1 and Txnip expression leading to retina thickness reduction was prevented by light damage treatment with blue light filter.

In summary, Trx plays an important role in light-induced photoreceptor degeneration in diabetic mice. Treatment with different thickness filter membrane or blue light filter in diabetic mice can up-regulate Nrf2 activation and Trx expression. This could lead to the expression of ASK1 and decrease of Txnip. We have already summarized the related mechanism in figure 4. Finally, the retina thickness reduction after light

damage can be prevented in diabetic mice. It suggests a possible help for DR patients in protecting them from light damage in the future.

CONCLUSION

Light can increase the photoreceptor damage in diabetic mice. The reduction of the light intensity and change the visible light components can reduce such light-induced damage. The possible mechanism may be related to up-regulation of the antioxidant protein rx expression.

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