• Basic Research •

Protective effects of lipoic acid-niacin dimers against blue light-induced oxidative damage to retinal pigment epithelium cells

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Received: 2018-06-17 Accepted: 2019-05-15

Abstract

• AIM: To evaluate the protective effects of lipoic acid-niacin (N2L) dimers against blue light (BL)-induced oxidative damage to human retinal pigment epithelium (hRPE) cells *in vitro*.

• METHODS: hRPE cells were divided into a control group (CG), a BL group, an N2L plus BL irradiation group, an α-lipoic acid (ALA) plus BL group, an ALA-only group, and an N2L-only group. hRPE cellular viability was detected by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assays, and apoptosis was evaluated by annexin-V-PE/7-AAD staining followed by flow cytometry. Ultrastructural changes in subcellular organelles were observed by transmission electron microscopy. Reactive oxygen species formation was assayed by flow cytometry. The expression levels of the apoptosis-related proteins BCL-2 associated X protein (BAX), B-cell leukmia/lymphoma 2 (BCL-2), and caspase-3 were quantified by Western blot analysis.

• RESULTS: BL exposure with a light density of 4±0.5 mW/cm^2 exceeding 6h caused hRPE toxicity, whereas treatment with a high dose of N2L (100 mol/L) or ALA (150 mol/L) maintained cell viability at control levels. BL exposure caused vacuole-like degeneration, mitochondrial swelling, and reduced microvillus formation; however, a high dose of N2L or ALA maintained the ultrastructure of hRPE cells and their organelles. High doses of N2L and ALA also protected hRPE cells from BL-induced apoptosis, which was confirmed by Western blot analysis: BCL-2 expression significantly increased, while BAX and caspase-3 expression slightly decreased compared to the CG.

• CONCLUSION: High-dose N2L treatment (>100 mol/L) can reduce oxidative damage in degenerating hRPE cells exposed to BL with an efficacy similar to ALA.

• KEYWORDS: lipoic acid-niacin dimers; retinal pigment epithelium cell; lipoic acid; oxidative stress; reactive oxygen species; apoptosis

DOI:10.18240/ijo.2019.08.05

Citation: Zou XL, Yu YZ, Yu HH, Wang GF, Pi RB, Xu Z, Zhang C, Zhou WJ, Li DD, Chen XG, Zou YP. Protective effects of lipoic acid-niacin dimers against blue light-induced oxidative damage to retinal pigment epithelium cells. *Int J Ophthalmol* 2019;12(8):1262-1271

INTRODUCTION

Among all eye diseases, age-related macular degeneration (AMD) is the main contributor to irreversible vision loss in elderly people, especially in the developing world[1]. Although the pathological mechanism of AMD has not yet been clearly elucidated, some evidence points to a critical role of oxidative stress in AMD progression. Human retinal pigment epithelial (hRPE) cells and photoreceptor cells are exposed to an environment of high oxygen pressure and high metabolism, and are therefore continuously subjected to oxidative stress[2]. Moreover, accumulating evidence indicates that mitochondria are the subcellular targets of oxidative

stress in hRPE cells and are closely associated with the hRPE cell aging process[3]. Accordingly, many recent studies have focused on investigating the relationships between hRPE cells and oxidative stress. Sreekumar *et al*[4] showed that humanin protected hRPE cells against oxidative stress-induced cell death and restored mitochondrial function. Lambros and Plafker[5] provided evidence indicating that nuclear factor-E2 related factor 2 (Nrf2), the master antioxidant transcription factor, played a significant role in hRPE cells during aging and the onset of AMD. Moreover, Liu *et al*[6] found that hydroxytyrosol was a mitochondria-targeting antioxidant nutrient that could protect against age-related hRPE degeneration. Despite this insight, it remains an arduous task to develop an effective strategy to protect hRPE cells from oxidative stress-induced damage to slow down or prevent AMD progression.

Alpha-lipoic acid (ALA) and nicotinic acid (niacin) are widely recognized as potent antioxidants that also exert neuroprotective effects. ALA functions in energy and metabolism, playing a primary role as a mitochondrial redox sensor, and is thus a powerful antioxidant[7] that has been shown to be effective for anti-aging, slowing the progression of vision loss, and improving diabetes retinopathy and its complications. However, the application of ALA is limited because it is only found in minute quantities in tissues and, thus, only a small amount of ALA can be absorbed from food. In addition to its antioxidant property, niacin is an important drug for reducing blood lipid levels, including low-density lipoprotein-cholesterols, very low-density lipoprotein-cholesterols, and lipoprotein(a), while raising high-density lipoprotein cholesterol when administered at a pharmacological dose[8]. However, like ALA, its clinical application is currently limited owing to adverse side effects such as flushing owing to blood vessel dilation, nausea or diarrhea, urethritis, and low lipid solubility[9]. Nevertheless, one study demonstrated that the pharmaceutical activity of niacin could be strengthened and its side effects weakened when combined with other medications[10].

The novel complex formed between niacin and ALA, designated as the lipoic acid-niacin dimers (N2L), was developed in Dr. Pi RB. Pi's laboratory at the School of Pharmaceutical Sciences, Sun Yat-sen University, under the chemical name of 5-[1,2-dithiolan-3-yl]-N-(2-{[(3-pyridyl) formyl]amino}ethyl) pentanamide[11-12]. Unlike ALA and niacin alone, N2L is a multi-functional medication that exhibits stronger anti-oxidative and anti-inflammatory properties and superior blood-lipid regulation. This complex further enables higher drug bioavailability, with easier crossing of the blood-brain barrier and faster drug metabolism. In addition, the side effects of niacin and ALA mentioned above are reduced in N2L[11-12]. Recently, Pi *et al*[12] evaluated the potential of N2L as a neuroprotective drug. They reported that N2L was not toxic to hippocampal mouse neurons, but efficiently protected neurons against cell toxicity due to L-glutamate, and they demonstrated the superior property of the niacin-like unit in the context of N2L.

In this study, we focused on the potential benefits of the stronger anti-oxidative property of N2L in protecting hRPE cells from oxidative damage. We previously reported that N2L could protect hRPE cells from apoptosis and cell death induced by acrolein, suggesting that N2L could protect cells from the injury caused by oxidative stress[13]. In another study, we found that N2L could protect RPE-19 cells by up-regulating expression of the anti-apoptotic factor B-cell leukmiay lymphoma 2 (BCL-2) and inhibiting expression of the pro-apoptotic factor BCL-2 associated xprotein (BAX)[14].

The primary purpose of this study was to explore the potential effects of N2L in protecting hRPE cells from oxidative damage induced by blue light (BL) *in vitro*, which were compared to the effects of ALA. These results could serve as a valuable basis for developing N2L as an alternative therapeutic drug for preventing and treating AMD.

MATERIALS AND METHODS

Ethical Approval The experiments performed in this study complied with the tenets of the Declaration of Helsinki: all work was performed after approval from the Organ Procurement Organization and the Hospital Ethics Committees, and all donors signed informed consent forms.

Tissue Samples and Cell Culture hRPE cells were obtained from freshly isolated donor eyes from patients died by accident (*i.e.*, immediately post-operation) provided by the eye bank of the Department of Ophthalmology, General Hospital of Southern Theatre Command of PLA.

Each freshly isolated eye was soaked for 15min in 20 mL antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin; Gibco, USA) and D-Hanks medium (Gibco, USA), after which the retinal neuroepithelium layer was peeled away by carefully removing the anterior segment by cutting it out from the limbus. Then, the upside down, hanging eye cup was separated from the retinal neuroepithelium layer and the vitreous body. After placing the outer wall of the eye containing the RPE layer and sclera in the eye cup and washing with D-Hanks medium, 3.0 mL 1% trypsin (Gibco, USA) was added, and the specimen was incubated in a humidified 5% CO_2 atmosphere at 37℃ for 1h. Subsequently, 3 mL 10% fetal bovine serum (FBS; Gibco, USA) was added, and the hRPE cells were collected in 15-mL centrifuge tubes, and centrifuged twice at 1100 rpm for 8min after washing the cells with phosphate-buffered saline (PBS). Then, the cells were identified by immunofluorescence with the RPE65 biomarker[15], and the cells were plated in culture dishes and cultured in Dulbecco's

modified Eagle's medium-low glucose (DMEM-LG; Gibco, USA) supplemented with 10% FBS and antibiotics, and incubated at 37℃ for 72h. Finally, the growth of the hRPE cells was observed by IX70 Inverted Microscope (OLYMPUS, Japan). After reaching confluence, the cells were detached in trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco, USA) solution. hRPE cells in logarithmic growth phase at passages 4-6 were used for the experiments.

Establishment of the BL-Induced hRPE Cell Damage Model A humidified 5% CO_2 incubator was converted into a BL incubator box. Non-reflective paper was adhered to the inside walls of the box, and five light-emitting diodes producing BL (Yuelong Factory, Zhongshan, Guangdong Province, China) were fixed on the inside top of the incubator, ensuring that the lights were well distributed within the incubator. The distance between the BL, and cell culture plane was adjusted so that the BL density was 4±0.5 mW/cm^2 with FL-1D BL irradiation (Photoelectric Instrument Factory of Beijing Normal University). The wave length of the BL was approximately 450 nm. In previous experiments, we found that the hRPE cells were clearly injured when exposed to 4±0.5 mW/cm^2 BL for at least 6h.

Experimental Groups The hRPE cells were cultured in 10% FBS-supplemented DMEM-LG until to 50% confluency, then the hRPE cells were grouped into the control group (CG), cultured in cultured in serum-free DMEM without any treatment (*i.e.*, no N2L or ALA); the BL-irradiation group, exposed to BL and cultured in cultured in serum-free DMEM without N2L and ALA; the N2L plus BL-irradiation group, exposed to BL and cultured in serum-free medium with 50, 100, 150, or 200 μmol/L N2L; the ALA plus BL-irradiation group, exposed to BL and cultured in serum-free medium with 50, 100, 150, and 200 μmol/L ALA. N2L and ALA (School of Pharmacy, Sun Yat-sen University, China) were dissolved in DMEM-LG without FBS (Serum starvation therapy) and the drug concentration was adjusted to the indicated concentration in the culture medium.

As the hRPE cells were divided into CG, the BL group, the N2L plus BL-irradiation group and the ALA plus BL-irradiation group, all the cells were cultured for a further 24h (drug incubation time) in medium containing different concentrations of ALA or N2L (0-200 μmol/L) until the cells reached confluence almost at the same time. Finally, the treatment hRPE cells groups were exposed to BL at 4±0.5 mW/cm^2 for 0-24h in different experiment groups (including CG).

Cell Viability In order to find out whether N2L has toxicity, and the significant protected concentrations of N2L and ALA on hRPE cells, the grouped RPE cells were exposed to BL for 24h by comparing cell viability; then the hRPE cells at a certain drug concentration were exposed to BL for 3, 6, 12, and 24h, to study the relationship between cell viability and exposure time; finally study the relationship between hRPE cell viability and drug-treatment time (6, 12, or 24h) before BL illumination with N2L or ALA at a certain concentration. As grouped and after treatment, hRPE cell viabilities were measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay (Gibco, USA). Briefly, the cells were plated in triplicate wells of 96-well microplates at a density of 5×10^4 cells per well; three wells also served as the CG. All hRPE cells were cultured according to the experimental groups, CG were covered with tinfoil when the 96-well microplates exposed to BL. Twenty microliters of MTT (5 mg/mL) solution was added to each microwell, and the cells were incubated in a 5% CO_2 incubator for 4h at 37℃. After carefully removing the medium from each well, 150 μL dimethyl sulfoxide (Sigma, St. Louis, MO, USA) was added, and the 96-well microplates were placed on a table concentrator for 10min to allow time for sufficient mixing. The optical density was read on a microplate reader at 490 nm.

Transmission Electron Microscopy Experiments Different hRPE cell treatment groups (including drug levels of 50-200 μm N2L and ALA with 24h drug incubation time) were exposed to BL for 6h. Transmission electron microscopy (TEM; HITACHI H-7650, Japan) was used to evaluate changes in the ultrastructure of hRPE cells after the various treatments. The cells were treated in 100-mm culture dishes, after which the medium was removed. The grouped cells were collected in 1.5-mL centrifuge tubes and centrifuged at 3500 rpm for 25min. The medium was removed, and the cells were fixed in 0.8 mL of 2.5% glutaraldehyde for more than 2h at 4℃, followed by sequential dehydration, soaking, embedding, sectioning in an ultramicrotome, and lead/uranium double-staining. Finally, the ultrastructures of hRPE cells were observed and photographs were acquired by TEM.

Cell Apoptosis Different hRPE cell treatment groups (including drug levels of 50-200 μm N2L and ALA with 24h drug incubation time) were exposed to BL for 6h. The apoptosis rate of hRPE cells was detected with an Annexin-V-PE/7-AAD Cell Apoptosis Kit (Merck Millipore, Germany) according to the manufacturer instructions. Briefly, the cells of each group were plated in 6-well plates at 1×10^5 cells per well and treated with the indicated factors. The cells were then digested with 0.25% trypsin-EDTA and gathered into 15-mL centrifuge tubes. After centrifugation at 1000 rpm for 5min and resuspending the cells, 100 μL of each cell suspension was added to a 96-well microplate and 100 μL Annexin-V-P E/7-AAD solution was added to each well. The samples were stained for 20min at room temperature in the dark and detected with a Guava flow cytometry system.

Measurement of Reactive Oxygen Species Production in hRPE Cells Different hRPE cell treatment groups (including drug levels of 50-200 μm N2L and ALA with 24h drug incubation time) were exposed to BL from 0.5h to 6h. To detect the extent of oxidative damage induced by BL and the influence of the respective treatments on this damage, the amount of reactive oxygen species (ROS) produced in hRPE cells was evaluated based on the fluorescence intensity of the fluorescent probe 2', 7'-dichlorofluorescein-diacetate (DCFH-DA; Sigma, USA). The cells were incubated with 1 μL of 10 μmol/L DCFH-DA for 30min in the dark at 37℃, and then the plates were washed twice with serum-free DMEM-LG and then twice with PBS, and resuspended in DMEM-LG at 2×10^4 cells/mL. The cells were analyzed within 30min by flow cytometry using excitation and emission wavelengths of 488 nm and 525 nm, respectively. The results are expressed as the fluorescence intensity of DCFH.

Expression of Apoptosis-Related Proteins Different hRPE cell treatment groups (including drug levels of 50-200 μmol/L N2L and ALA with 24h drug incubation time) were exposed to BL for 6h. hRPE cells were homogenized in lysis buffer (30 mmol/L Tris-HCl, 10 mmol/L EGTA, 5 mmol/L EDTA, 1% Triton X-100, 250 mmol/L sucrose) containing 1 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 15 mg/mL aprotinin, 5 mg/mL leupeptin, 5 mg/mL pepstatin, and 1 mmol/L Na_3VO_4. The homogenate was sonicated three times for 2s each and centrifuged at 16 000 g for 15min at 4℃. The protein concentration in the cells was detected with a BCA protein assay kit (Beyotime, China). Then, the protein was collected and stored at -80℃. For each sample, an equal amount of protein was loaded onto a 12% sodium dodecyl sulfate gel and run for 2-3h, after which the proteins were transferred to polyvinylidene fluoride membranes with an electrophoresis apparatus run at 200 mA for 1h. The membranes were incubated overnight at 4℃ with primary antibodies against β-actin (1:1000; Beyotime, China), cleaved caspase-3 [1:200; Cell Signaling Technology (CST), USA], BAX (1:200; CST, USA), and BCL-2 (1:200; CST, USA). The membranes were then washed three times with PBS (10 min/wash) and incubated for 1h at 37℃ with a secondary goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (1:3000; CST, USA) or a secondary goat anti-mouse IgG HRP-conjugated antibody (1:3000; CST, USA). The membranes were processed using the CEL-plus kit (Merck Millipore, Germany) to visualize the immune-blotting signals, after which the membranes were washed and the bands were analyzed with Image J software.

Statistical Analysis Data are expressed as mean±standard deviation, and were processed with IBM SPSS 20.0 software (USA). Comparisons of data among groups were evaluated by analysis of variance, followed by the Dunnett-T_3 test. A P value <0.05 was considered statistically significant.

RESULTS

N2L Protects hRPE Cells from BL-Induced Damage N2L was not toxic to hRPE cells at the tested concentrations, similar to ALA. The cell viability decreased significantly to 61.10%±3.69% after BL irradiation compared to the CG reference level (set to 100%; Figure 1A). However, the addition of 100 μmol/L N2L and 150 μmol/L ALA significantly protected the hRPE cells from subsequent BL-induced damage (BL irradiation for 24h; Figure 1B). Furthermore, the cell viability decreased in proportion to the time of exposure to BL irradiation. At 3h of irradiation, the cell viability in the BL group decreased slightly, whereas the cell viability decreased significantly after irradiation for 24h, even with 100 μmol/L N2L and 150 μmol/L ALA treatment (Figure 1C). In addition, the cell viability increased in proportion to the incubation time with the drug. At 24h, no differences were found in the viabilities between cells treated with 100 μmol/L N2L or 150 μmol/L ALA, versus the CG (Figure 1D).

TEM Observations of the hRPE Cell Ultrastructure The structures of cells in the CG were clearly visualized, revealing several cells with slender microvilli, melanin granules, and abundant cytoplasmic organelles. The chromatin was distributed homogeneously in the nucleus (Figure 2A, 2A1). In contrast, obvious damage was clearly induced by exposure to BL. For example, the cell microvilli decreased in number or disappeared completely. The cell bodies were diminished or distorted. The mitochondria were enlarged in the cytoplasm, and the mitochondrial crest clearance became wider. Chromatin pyknosis was observed in the cell nucleus (Figure 2B, 2B1). Compared to these untreated cells, the BL-induced damage was far less obvious for hRPE cells pre-treated with 50 μmol/L ALA (Figure 2C, 2C1) and 50 μmol/L N2L (Figure 2G, 2G1). Although the cell microvilli also decreased or detached from the cell membrane, minimal vacuole-like denaturation was found in the cytoplasm. Similar to the BL group, the mitochondria were swollen and the cell nuclei were more rounded with some speckled chromatins detected. Cell damage was noticeably reduced in the presence of increasing concentrations of ALA or N2L. The structure of BL-exposed hRPE cells treated with 100 μmol/L ALA (Figure 2D, 2D1) was generally normal, with only slight changes detected. Slender microvilli were abundant, the mitochondria and other organelles were swollen or only slightly enlarged, and the chromatin was homogeneously distributed in the nucleus. The cells showed similar normal structures in groups treated with higher concentrations of ALA (150 μmol/L, Figure 2E, 2E1; 200 μmol/L, Figure 2F, 2F1) or N2L (100 μmol/L, Figure 2H, 2H1; 150 μmol/L, Figure 2I, 2I1, 200 μmol/L, Figure 2J,

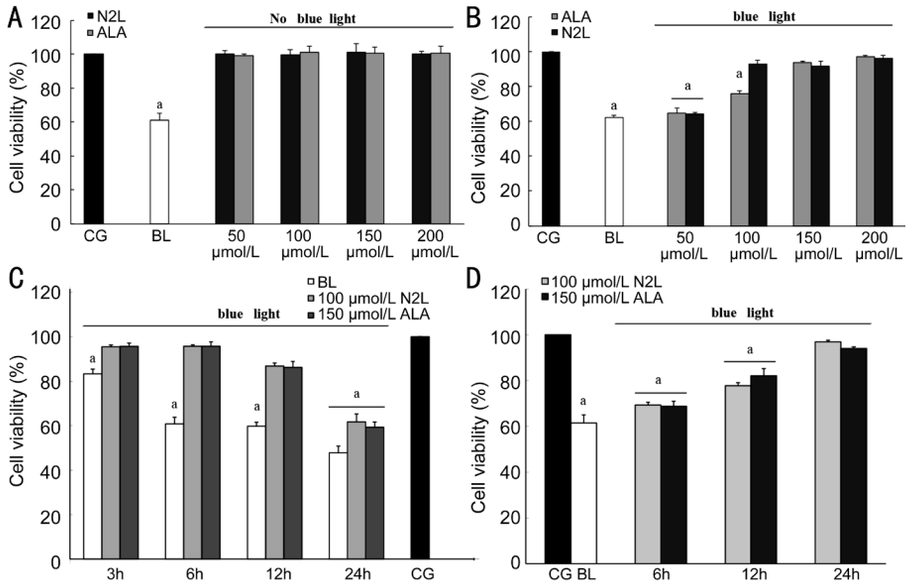

Figure 1 Cell viabilities tested by the MTT assay A: No effects on hRPE cell viability were observed with N2L and ALA at any concentration tested, compared to the CG; B: Effects of different levels of N2L, ALA, and BL alone on cell viability after BL irradiation for 24h compared to the CG; C: Influence of BL on the cell viability over time (3 , 6, 12, or 24h), and the protective effects of 100 μmol/L N2L and 150 μmol/L ALA against this damage; D: hRPE cell viability in relation to drug-treatment time (6, 12, or 24h) before BL illumination. All values are relative to the CG cell viability set to 100%. Data are shown as means±standard deviation (n=3). [a]P<0.05 versus control. CG: Control group; BL: Blue light group; N2L: N2L group alone; ALA: ALA group alone.

2J1). In these cases, slender microvilli were abundant and the mitochondria were not swollen or enlarged. Moreover, melanin granules and other organelles were clearly seen, and the chromatin was homogeneously distributed in the nucleus.

hRPE Cell Apoptosis The percentage of apoptotic hRPE cells in the different groups was detected by flow cytometry, which revealed that a high dose N2L or ALA could inhibit apoptosis (Figure 3). By comparing the rates of hRPE cell apoptosis between the CG with cell apoptosis of 7.57%±0.84% and the BL group with cell apoptosis of 25.4%±3.13%, we found that apoptosis increased significantly in the BL group (P<0.05). As shown, a high dose of N2L (150 μmol/L) or ALA (150 μmol/L) significantly inhibited BL-induced hRPE cell apoptosis, compared to that in the BL group (all P<0.05, compared with the CG. While there is no statistical significance was observed between 100 μmol/L N2L with cell apoptosis of 6.77%±0.55% and 100 μmol/L ALA with cell apoptosis of 11.17%±0.9% compared with the CG.

Reactive Oxygen Species Production To assess the mitochondrial function in hRPE cells, we detected the contents of intracellular ROS by flow cytometry after staining with the fluorescent probe DCFH-DA (10 μmol/L; Figure 4). Pre-treatment with 100 μmol/L N2L or 150 μmol/L ALA resulted in a time-independent inhibition of ROS production after exposure to BL for various durations [0 (CG), 0.5, 1, 2, 3, 4, or 5h]. After BL illumination for 0.5h, a slight reduction in the ROS fluorescence was noted in the groups treated with 100 μmol/L N2L or 150 μmol/L ALA, but the difference compared to the BL group was not statistically significant. However, with irradiation for 1h or more, the intracellular ROS contents were significantly reduced in the 100 μmol/L N2L- and 150 μmol/L ALA-treatment groups.

Expression of Apoptosis-Related Proteins To examine changes in the expression of apoptosis-related proteins due to BL, we quantified the protein-expression levels of the pro-apoptotic proteins BAX and caspase-3, and the anti-apoptotic protein BCL-2 by Western blot analysis. The apoptotic ratio of protein BAX, BCL-2 and caspase-3 in BL groups, ALA groups and N2L groups were compared to the CG in each classification. As shown in Figure 5, compared to the CG with protein apoptotic ratio of BAX was 0.1%±0.01%, of caspase-3 was 0.25%±0.04%, the BL group showed significantly up-regulated expression of both BAX with protein apoptotic ratio was 0.23%±0.03% and caspase-3 with protein apoptotic ratio was 0.57%±0.06% (P<0.05), but down-regulated expression of BCL-2 in BL group with protein apoptotic ratio was 0.21%±0.14% compared to CG with protein apoptotic ratio was 0.32%±0.38% (P<0.05). In contrast, treatment with 100 μmol/L N2L and 150 μmol/L ALA significantly up-regulated BCL-2 expression, with apoptotic ratio was 0.49%±0.05% in N2L group, apoptotic ratio was 0.51%±0.07% in ALA group, which compared to CG with apoptotic ratio was 0.32±0.38% (P<0.05), and slightly down-regulated BAX and caspase-3 expression, with BAX apoptotic ratio was 0.04%±0.001% in N2L group. BAX apoptotic ratio was 0.06%±0.01% in ALA

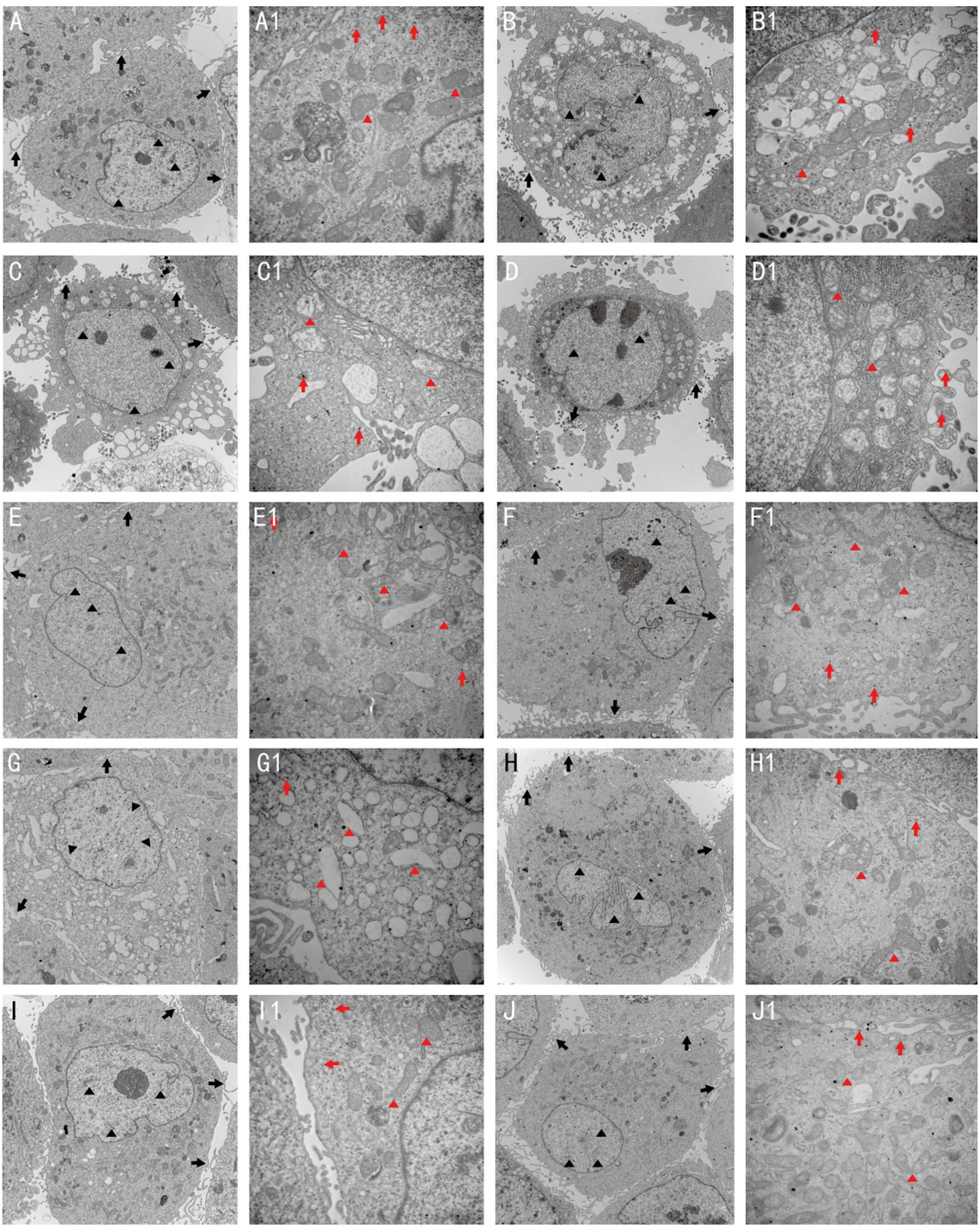

Figure 2 Ultrastructural changes in hRPE cells treated with different concentrations of N2L and ALA after BL exposure, as determined by TEM A, A1: CG; B, B1: BL group; C, C1: 50 μmol/L ALA with BL; D, D1: 100 μmol/L ALA with BL; E, E1: 150 μmol/L ALA with BL; F, F1: 200 μmol/L ALA with BL; G, G1: 50 μmol/L N2L with BL; H, H1: 100 μmol/L N2L with BL; I, I1: 150 μmol/L N2L with BL; J, J1: 200 μmol/L N2L with BL. Black triangles pointed to chromatin, black arrows pointed to slender microvilli, red triangles pointed to mitochondria, and red arrows pointed to melanin granules. A-J: ×8000 magnification; A1-J1: ×25 000 magnification. CG: Control group; BL: Blue light group; N2L: N2L plus BL-irradiation group; ALA: ALA plus BL-irradiation group.

group, which compared to CG with BAX apoptotic ratio was 0.1%±0.01%; and with caspase-3 apoptotic ratio was 0.17%±0.05% in N2L group, caspase-3 apoptotic ratio was 0.26%±0.05% in ALA group, which compared to GC with caspase-3 apoptotic ratio was 0.25%±0.04%, although these differences were not statistically significant.

DISCUSSION

N2L is a newly derived compound that shows superior properties compared to its constituent components, including a potent anti-oxidative effect, a neuroprotective effect, and higher bioavailability. However, few studies have been conducted with this novel complex to evaluate its potential for drug

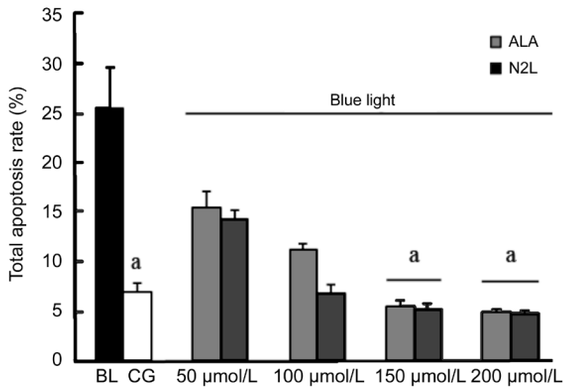

Figure 3 Apoptosis rates detected by flow cytometry after annexin V-PE/7-AAD fluorescent staining hRPE cells were incubated in DMEM-LG FBS-free medium and not otherwise treated (CG group), exposed to BL (BL group), or treated with increasing concentrations of N2L or ALA (50, 100, 150, and 200 μmol/L) prior to BL exposure. Data are shown as means±standard deviation (n=3). ^aP<0.05, versus the GC group. CG: Control group; BL: Blue light group; N2L: N2L plus BL-irradiation group; ALA: ALA plus BL-irradiation group.

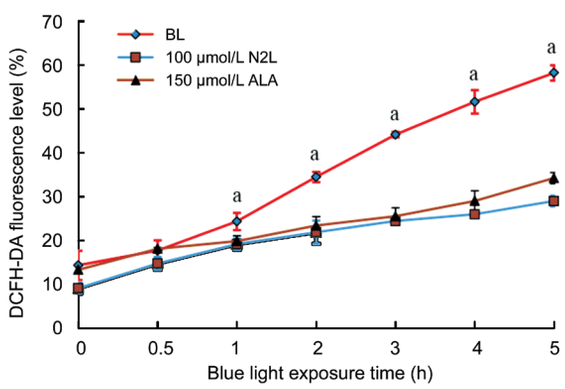

Figure 4 Intracellular ROS levels measured by flow cytometry after staining with the fluorescent probe DCFH-DA Data are shown as the means±standard deviation (n=3). ^aP<0.05 versus BL group (0-h group). CG: Control group; BL: Blue light group; N2L: N2L plus BL-irradiation group; ALA: ALA plus BL-irradiation group.

development. Thus, we conducted the first study to evaluate the protective effects of N2L on BL-induced oxidative damage in hRPE cells. Previous findings demonstrated that ALA could protect RPE cells, ganglion cells, and Müller cells from injury, and against neuropathic diseases in animal models of AMD, diabetic retinopathy or optic neuropathy[16]. Zhao *et al*[17] provided the first demonstration that ALA could protect the rat retina from light-induced damage and degeneration. Other studies showed that ALA was more readily accessible when its concentration reached 100-400 μmol/L in vegetables, fruits, or other foods[18-19]. Therefore, we used ALA as a reference to evaluate the protective effect of N2L in hRPE cells.

Several studies have demonstrated that ultraviolet or BL can injure the RPE or retinal ganglion cells, mainly *via* apoptosis or a cell death mechanism owing to oxidative stress. When BL reached 3-7 mW/cm^2 after irradiation for 3-24h, clear damage

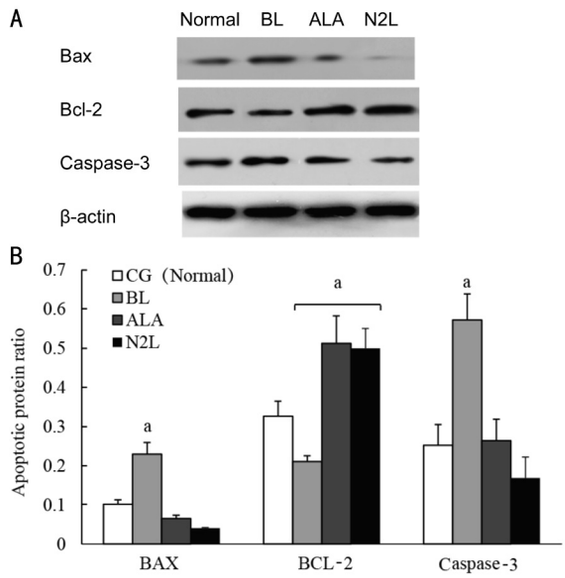

Figure 5 Effects of 100 μmol/L N2L and 150 μmol/L ALA on the protein expression levels of BAX, caspase-3, and BCL-2 A: hRPE cells were grouped into the CG, the BL group, and BL groups treated with 100 μmol/L N2L or 150 μmol/L ALA, and incubated accordingly for 24h. The cells were then exposed to 4±0.5 mW/cm^2 BL for 6h. The expression levels of apoptosis-related proteins were assessed *via* Western blotting. B: Quantitative analysis of the expression of apoptosis-related proteins in the different groups of hRPE cells. Data are shown as the mean±standard deviation (n=3). ^aP<0.05 versus control group (CG). CG: Control group; BL: Blue light group; N2L: N2L plus BL-irradiation group; ALA: ALA plus BL-irradiation group.

to the RPE or ganglion cells was observed, and the degree of cell damage depended on the BL density and irradiation time[20-22]. We previously found that the RPE was obviously damaged when exposed to BL at 4±0.5 mW/cm^2 for 3h, with the most evident damage detected after 6h of irradiation[23-24]. Therefore, we selected 4±0.5 mW/cm^2 as the parameters for BL treatment with hRPE cells in the experiments in this study, and 6h light irradiation time were selected in majority experiments.

As previously studies has showed and mentioned before, N2L could protect hRPE cells from apoptosis and cell death induced by acrolein[14], and N2L could protect RPE-19 cells by up-regulating expression of the anti-apoptotic factor BCL-2 and inhibiting expression of the pro-apoptotic factor BAX[15]. In the study we found that 150 μmol/L ALA or 100 μmol/L N2L treatment alone exerted no cytotoxic effects on hRPE cells by comparing cell viability, and thus these concentrations were used for subsequent experiments. At these levels, both drugs showed protective effects against the BL-induced damage in relation to both the incubation and BL-exposure times.

Organelles such as mitochondria, lipofuscins, and lysosomes play a critical role in the hRPE cell-damage mechanism induced by BL[25-26]. The mitochondrion is the only organelle that provides energy to living cells and functions as the site of

ROS generation (*via* the electron transport respiratory chain and ATP synthesis) upon BL stimulation in RPE cells[22,27]. Moreover, N-retinylidene-N-retiny-thanolenrine (A2E) is an autofluorescent component of lipofuscins in the RPE, which can itself produce ROS when it absorbs BL, thereby inducing lipid peroxidation and causing mitochondria to generate even more ROS. These ROS (along with A2E) may damage the mitochondria, lysosomes, or DNA in hRPE cells[25,28]. In turn, ROS formation triggers the apoptosis cascade through the mitochondrial pathway, and A2E can specifically target cytochrome C oxidase to activate the apoptosis pathway[20,26]. Sparrow *et al*[25,28] found that the oxidative stress reaction could decrease the mitochondrial membrane potential and open the mitochondrial permeability transition pore, thereby releasing mitochondrial cytochrome C to promote apoptosis in RPE cells.

Accordingly, we assessed the effects of BL, N2L, and ALA on the ultrastructural changes of hRPE cells, especially in the mitochondria, and detected the total apoptosis rate and degree of ROS production. We found that BL decreased or eliminated the cell microvilli, resulted in swelling of the mitochondria, and led to chromatin pyknosis in the nucleus of hRPE cells. However, treatment with 100 μmol/L N2L or 150 μmol/L ALA could maintain the integrity of the cell ultrastructure under BL exposure, demonstrating the protective effect of N2L at certain extent. The most obvious changes were noted in the mitochondria, and both N2L and ALA suppressed the marked elevation in apoptosis after BL irradiation in a dose-dependent manner.

Liang and Godley[3] demonstrated that the ROS contents increased significantly after RPE cells were exposed to 2.8 mW/cm^2 BL from 1h to 6h, which was accompanied by mitochondria injury and a positive relationship observed between ROS production and the BL-irradiation time. We also found time-dependent effects for N2L and ALA, where the ROS contents decreased only slightly after 1h of illumination, but with more significant inhibitory effects detected after 2h of irradiation.

Several studies have already demonstrated a close relationship between ALA with mitochondrial ROS production, and both the reduced dihydrolipoic and oxidized forms of ALA quenched the formation of hydroxyl radicals (OH^-), singlet oxygen (O_2^-), and hydrogen peroxide (H_2O_2). As a natural antioxidant, ALA is a mitochondrial coenzyme that can reverse the age-associated decline in mitochondria function[19]. ALA can also reduce the oxidized forms of several important antioxidants, including vitamin C, glutathione, and coenzyme Q10[19,29]. Recently, Li *et al*[30] demonstrated that ALA could protect lens epithelial cells from apoptosis and activate anti-oxidative enzymes. Li *et al*[16] further explained that ALA can protect RPE cells from oxidative stress-induced injury through the protein kinase B (PKB)/BAX signaling pathway. Our data suggest that N2L may play a similar protective role with respect to ROS formation and oxidative stress.

Many researchers also have emphasized that apoptosis is the main mechanism of oxidative damage[27] and that the mitochondria are crucial in mediating apoptosis. Ji *et al*[31] found that ALA could protect ganglion cells from apoptosis by a mitochondrial pathway after light insults. The caspase family executes and participates in apoptotic activities in various cell types, and caspase-3 is the key trigger of the apoptosis cascade in hRPE cells[32-33]. The pro-apoptotic protein BAX and the anti-apoptotic protein BCL-2 are also crucial regulator of BL-induced apoptosis in hRPE cells[28,34]. Indeed, we inferred that N2L and ALA could prevent BL-induced apoptosis by re-balancing the expression of BAX, BCL-2, and caspase-3. The BCL-2 protein family is localized in the outer and inner mitochondrial membranes, along with other anti-apoptotic proteins such as BCL-X and BCL-w. Under normal circumstances, BCL-2 resides in the outer mitochondrial membrane can prevent calcium (Ca^{2+}) influx[26,35]. Therefore, the mitochondrial membrane potential can be stabilized and thus inhibit the mitochondria from releasing apoptosis-related cytokines such as cytochrome C, apoptosis-inducing factor, and pro-caspase-3[20] so as to prevent an apoptosis cascade. Once hRPE cells are exposed to BL, ROS production is induced and apoptosis is mediated by the responses of the mitochondria[27]. These changes can result in the swelling of cells and/or the mitochondria to release apoptotic cytokines accompanied by activating apoptotic-inducing factor and pro-caspase-3. Thus, elevated caspase-3 expression is a clear signal of activated cell apoptosis. Given that N2L and ALA significantly increased the expression of anti-apoptotic protein BCL-2, we inferred N2L and ALA could make the BCL-2 gathering together in the outer mitochondrial membrane, so as to enhance the mitochondrial membrane stabilization even under the circumstance of BL damage. At the same time, N2L and ALA reduced the expression of caspase-3 and BAX with BL damage, even made no statistical significance, which showed the reduced tendency, we suspected that the drug concentration decreased the sensitivity, and further experiments suggested to be taken. Although the result of the expressions of apoptotic related protein was not exactly as expected, combined all the other results above, we still inferred N2L could protect the hRPE cell mitochondria from BL-induced oxidative damage by acting as a mitochondrial cofactor, similar to ALA, and it maybe inhibit cell apoptosis via the mitochondrial pathway at some extent.

Overall, our findings inferred that N2L may protect hRPE cells against BL-induced oxidative stress, based on analyses of cell viability, mitochondrial morphological structures, ROS production, and apoptosis, exerting a similar effect to the well-known antioxidant ALA. We inferred that this protective effect

of N2L may be mediated by one or more of the following mechanisms: 1) direct scavenging of ROS; 2) activation of antioxidant defenses; 3) acting as a cofactor to protect, repair, and stimulate mitochondrial enzymes; and/or 4) most probably inhibiting apoptosis *via* the mitochondrial pathway. However, the protective mechanism remains unclear and should be the focus of further detailed studies, especially the probably inhibition effect of cell apoptosis. Besides it would be much more persuasive with sample enlargement in the experiments. The antioxidant effects of ALA have been studied in several clinical trials for the treatment of cataracts, glaucoma, diabetic retinopathy, cardiovascular diseases, and AMD. Given some of the limitations of ALA, our results inferred that N2L maybe can be a selective antioxidant therapeutic candidate for treating AMD and slowing down the progression of the vision impairment caused by AMD, of course there is still much work to do.

ACKNOWLEDGEMENTS

Foundations: Supported by the Guangzhou Science and Technology Foundation of Guangdong Province (No.2014J4100035; No.2014KP000071).

Conflicts of Interest: Zou XL, None; **Yu YZ,** None; **Yu HH,** None; **Wang GF,** None; **Pi RB,** None; **Xu Z,** None; **Zhang C,** None; **Zhou WJ,** None; **Li DD,** None; **Chen XG,** None; **Zou YP,** None.

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