Silibinin declines blue light-induced apoptosis and inflammation through MEK/ERK/CREB of retinal ganglion cells

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ABSTRACT

Purpose: This study aimed to assess the protective effects of silibinin on blue light-emitting diode (LED)-induced retinal ganglion cells (RGCs) damage.

Methods: Silibinin was applied in RGCs damage in vitro model to test its protective effects. Cell viability was assessed with the MTT method and cell apoptosis was evaluated by TUNEL and Annexin V/propidium iodide staining. The expressions of apoptosis related proteins and influenced signalling pathways were measured using western blotting and immunohistochemistry. Inflammatory factors induced by RGC damage were detected using ELISA method.

Results: It was found that silibinin in 50 and 100 μM treatment showed a significant protective effect in RGCs under blue light damage. Apoptosis assay showed that silibinin treatment could significantly improve the apoptotic status of RGCs. When the potentially affected signal pathway was considered, blue light would down-regulate the expression of MEK1/ERK/CREB. The levels of inflammatory factors (TNF-α, IL-1β, IL-6 and IL-10) were significantly regulated by silibinin treatment.

Conclusions: Silibinin pretreatment would demonstrate protective effect against blue light induced acute RGCs damage. Silibinin treatment has a direct suppression of apoptosis and inflammation through the activation of MEK/ERK/CREB pathway in vitro.

Introduction

Glaucoma is regarded as a group of disorders marked by progressive degeneration and it is associated with the death of retinal ganglion cells (RGCs) and axons within the optic nerve or optic neuropathy [1]. Glaucoma is now one of the leading causes of blindness, with an increasing prevalence in the whole world [2] and an estimated 60 million people have optic neuropathy due to glaucoma [3]. The incidence of primary open-angle glaucoma (POAG) increases with age and elevated intra-ocular pressure (IOP) and other signs such as a change in retinal morphology or loss of visual function are sufficient for a glaucoma diagnosis without an elevated IOP [3]. The presently available marketed anti-glaucoma drugs formulations have issues of either difficulty in crossing the blood-retinal barrier or lower systemic bioavailability [4]. Hence, the drugs with lower therapeutic index were in urgent need, which would eventually lead to deposition of concentrated solutions at ocular site, producing toxic effects and cellular damage to the eye.

Nowadays, the pathophysiology of glaucoma remains unclear and it is often defined by the loss of RGCs. In contrast to the peripheral nervous system, the central nervous system loses its capability of regeneration after injury. It was reported that apoptosis and inflammation were involved in retinal degeneration and they were also related to the development of glaucoma [5]. According to medical studies of glaucoma, high IOP or retinal ischemia leads to constant RGC apoptosis and anti-apoptosis would help in the management of glaucoma [6]. Mounting evidence suggests that neuroinflammation is a key process in glaucoma, yet the precise roles of neuroinflammation in glaucoma are still unknown. Understanding these complex processes, which may also be a key in other common neurodegenerations will lead to targeted therapeutics for a disease that affects as many as 80 million people worldwide. A most recent study demonstrated that antagonists of adenosine A2A receptor control retinal inflammation and afford protection to rat retinal cells in glaucoma models [7]. Thus, innovative neuro-protective and -regenerative therapies are urgently required for potential glaucoma treatment. Thus, the idea of the study was to look for significantly effective drugs in the RGCs degeneration, which could alter the survival and regeneration of RGCs in glaucoma.

Silibinin, which is a flavonoid plant extract from the dried fruits of Silybum marianum L., is mainly composed of four flavonoids [8]. Silibinin is one of the widely used drugs in clinical practice, and its pharmacological action characteristics are recognized, including anti-free radical activity, anti-lipid peroxidation, anti-lipoxygenase action, anti-glutathione (GSH) emptying effect, anti-tumor function and hypolipidemic effect.

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In another study, it was also found that silibinin is a protective drug in retinal diseases. In a recent study, silibinin pretreatment of RPE cells up-regulated proline hydroxylase-2 expression, inhibited HIF-1α subunit accumulation, and inhibited VEGF secretion. Silibinin-induced HIF-1α and VEGF down-regulation required suppression of hypoxia-induced phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway [10]. And according to a recent study, silibinin might play a role in the onset of diabetic peripheral neuropathy [11]. Therefore, based on the aforementioned information, we hypothesized that silibinin could regulate blue light induced apoptosis and inflammation of RGCs and then demonstrate a significant protective effect.

Materials and methods

Cell culture of RGC

RGCs were isolated from retinas of postnatal day 6 neonatal mice according to the two-step immunopanning method following a published protocol [12]. Subsequently, collected tissues were washed with phosphate-buffered saline (PBS) containing 100 U/mL penicillin and 50 μg/mL streptomycin for four times. Tissues were then digested with 0.25% trypsin containing 100 U/mL penicillin and 50 μg/mL streptomycin at 37°C for 25 min. Isolated cells were seeded at 2500 cells/cm² on a PDL (Poly-D-lysine) and laminin-coated dish and cultured in RGC medium [serum-free B27-supplemented Neurobasal® Medium, minus phenol red. The culture medium was replaced every 10 min, for a total of three times. The unadherent cells were removed by washing with PBS for three times. As previously described, primary RGCs were induced with 50 mmol/L glucose for subsequent experiments. Animals were handled according to the institutional guidelines on animal care of the University of Inner Mongolia Medical University.

In addition, the cultured RGCs were incubated for 24 h in either darkness or blue light (530 nm with a peak of 470 nm, 12.08 W/m²). Blue light was delivered by LEDs (Electro DH SL, Barcelona, Spain) and the temperature maintained at 37°C throughout. The intensity of light emitted from LEDs was checked by use of a spectrophotometer bought from Konica Minolta (CL-70F).

Cell viability detection

Cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA) to assess the cell viability of RGCs. Briefly, confluent monolayers of RGCs in different groups were used and the media were removed at the end of the treatment period. After that, RGCs monolayers were then exposed to 200μl of MTT solution (0.5 mg/ml in PBS) for 2 h. After washing the cells with PBS, the formazan crystals were solubilized by DMSO (200 μl per well; Sigma-Aldrich Chimie, St. Louis, MO) before the plates were measured at 540 nm.

TUNEL assay

RGCs at 80% confluency were treated with blue light or blue light in combination with different concentrations of silibinin for 4 h, and then co-incubated with palmitate for another 24 h. DNA fragments were labelled using the In Situ Cell Death Detection TMR red kit (Roche Diagnostics Corp., Indianapolis, IN, USA) following the manufacturer’s instructions. Cells were mounted with anti-fade reagent containing 40,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA). Images were captured under an inverted fluorescence microscope (Olympus, Tokyo, Japan). The ratio of TUNEL-positive cells among all the RGCs was indicated as apoptotic status. This was evaluated at a 20× magnification with Image-Pro Plus 6.0 Imaging System (Silver Springs, MD).

Cell apoptosis

RGCs were treated with either blue light or blue light with 2 h before exposure to silibinin. After 24 h of incubation, the cells were subsequently washed with PBS and stained with the Annexin V-FITC Apoptosis Detection Kit Cell apoptosis was measured using an Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions by flow cytometry.

Western blot analysis

To determine the expression of cleaved caspase-3, cleaved caspase-9, Bax, Bcl-2 and phosphorylation of MEK1, ERK1/2 and CREB, protein isolated from RGCs were subjected to WB analysis. Similarly, RGCs from different groups were lysed in buffer containing 10 mM Tris (Sigma-Aldrich Corp., St. Louis, MO), 1 mM EDTA (Sigma-Aldrich Corp., St. Louis, MO, USA), and 0.1% Triton X-100 (Sigma-Aldrich Corp, St. Louis, MO, USA) to yield total protein. Lysates were centrifuged at 13,000 g for 20 min at 4°C. After quantitation of the total protein in all the groups, an equal amount of protein (20 μg) was loaded in each lane and electrophoresed together with molecular weight standards (BioRad, Hercules, CA, USA) in separate lanes on a 10% SDS polyacrylamide gel. After electrophoresis, the proteins were transferred onto PVDF membranes (Millipore, Billerica, MA). The membrane was blocked with 5% nonfat dry milk for 2 h and after that incubated overnight at 4°C with primary antibody in 1:1000 dilution. Image-Pro Plus 6.0 Imaging System was used for analyzing the blot results.

Inflammatory factors detection

The supernatant of culture media was collected from cell culture media and stored in anticoagulant-free tubes. The levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) were measured by enzyme-linked immunosorbent assay (ELISA) kits (Proteintech, Beijing, China) according to the manufacturer’s protocols.
Statistical analyses

Statistical analyses were performed using SPSS13.0 software (SPSS Inc., Chicago, IL, USA). Wilcoxon’s test was used to compare the mean value. The results were considered statistically significant when p values were less than .05.

Results

Effect of silibinin on cell viability of RGCs under blue light

Silibinin, which is a flavonoid plant extract from *Silybum marianum* L (Figure 1(A)), has been reported to demonstrate a significant effect in different disorders. First, we examined the effect of silibinin on cell viability in RGCs cultured under blue light with silibinin in different concentrations for 24 h. The results of MTT assay were used for the detection of the cell viability. The silibinin at the concentration of 5, 25, 50 and 100 µM were chosen to detect its protective effect. In this study, it was found that silibinin less than in 50 µM did not protect the RGCs cellular viability under blue light, while silibinin in 50 and 100 µM demonstrated a significant protective effect under the blue light condition (Figure 1(B)). In the following experiment, 100 µM of silibinin would be used in the following experiments.

Silibinin regulates apoptosis-related protein expressions in RGCs

Caspase cascade and Bcl2/Bax pathways play important roles in intrinsic apoptotic pathways. When apoptosis is triggered, Bax moves to the mitochondrial outer membrane to promote the opening of the permeability transition (PT) pore. Sequentially, cytochrome C will be released to the cytoplasm and further activates the caspase cascade to induce apoptosis [13]. Here through western blotting, it was demonstrated that silibinin treatment would reduce the expression of Bax, cleaved caspase-3 and cleaved caspase-9, while it up-regulated the expression of Bcl2 (Figure 2). Since the ratio of Bcl-2 and Bax is important in the apoptosis process, it was found that Bcl2/Bax ratio increased after silibinin treatment. These data suggest that silibinin application would activate an anti-apoptosis effect in blue-light damage RGCs model.

Effect of silibinin on the apoptosis of RGCs induced by blue light damage

To explore the specific role of silibinin in the apoptosis of RGCs after blue light and blue light in combination with silibinin treatment, we detected the apoptotic rate by Annexin-VFITC/PI staining and TUNEL assay, respectively. TUNEL assay showed that less apoptotic RGCs were found in the silibinin treatment group than the blue light treated group (p < .05, Figure 3(A,B)). Through Annexin-VFITC/PI staining, it was found that the apoptotic rate (Figure 4(A,B)) in the silibinin group were remarkably decreased when compared with
Figure 3. The protective effect of silibinin on blue light induced apoptosis in RGCs. (A) Representative images of TUNEL-positive cells (red) and total cells (blue). (B) Quantification of TUNEL positive cells, presented as percentages of total cells. The bars indicate the SD; $n = 6$, in terms of independent wells; $**p < .001$.

Figure 4. Effects of silibinin on apoptosis rate in blue light induced RGCs. (A) Flow cytometry analysis of apoptosis in cultured podocytes in different groups and quantitation of these results ($n = 5$). (B) Cell apoptosis detected using flow cytometry. The bars indicate the SD; $n = 6$, in terms of independent wells; $**p < .001$. 
those of the blue light group \((p < .05)\). Therefore, we believed that silibinin treatment could significantly decrease the apoptotic rate of RGCs induced by blue light damage.

**Analysis of the expression of inflammatory factors in RGCs treated with blue light with/without silibinin**

There is a well-known association between inflammation and light damage [14], which contributes to the injury and repair of RGCs during damaging conditions. In this study, we focused on the expression of some inflammatory factors in RGCs under blue light damage with/without silibinin. The cell culture medium in different groups was harvested and used for advanced studies. The levels of TNF-\(\alpha\) (Figure 5(A)), IL-1\(\beta\) (Figure 5(B)), IL-6 (Figure 5(C)) and IL-10 (Figure 5(D)) were measured by ELISA. The level of all these inflammatory factors was significantly different with the control group, while no difference was detected in TNF-\(\alpha\), IL-1\(\beta\), IL-6 and IL-10 expression level after treatment of silibinin comparing with the control group. The protective effects of silibinin in blue light damaged RGCs involved in different inflammatory factors.

**Treatment of silibinin on MEK1/ERK/CREB signalling on blue light damage of RGCs**

To investigate whether silibinin protects inflammation and apoptosis through up-regulation of MEK1/ERK/CREB signalling, we detected the expression of pMEK1, ERK1/2 and pCREB in control, blue light and blue light in combination with silibinin groups. It was found that blue light would down-regulate the expression of MEK1/ERK/CREB. After the treatment with silibinin, the expression of pMEK1, ERK1/2 and pCREB were significantly upregulated (Figure 6). Overall, these results suggest that silibinin protects against blue light induced oxidative damage by overexpression of MEK1/ERK/CREB pathway.

**Discussion**

Glaucoma is still one of the leading causes of blindness in the whole world. RGCs death is the most important pathologic change in the development of glaucoma. A convenient strategy that is commonly used at the first stage of research in the field of neuroprotection for different ocular pathologies, including glaucoma, is to develop an *in-vitro* model and
it is related events that occur in vivo. Related to this, the development of neuroprotective approaches capable of preserving the cells after the aggression and the damage generated by blue light is an important advance in the development of long-term treatments for glaucoma.

Apoptosis is an intracellular signalling cascade that causes programmed cell death. This process is necessary for normal growth and development; however, apoptosis also occurs in the major pathologic pathways of numerous diseases, including glaucoma. Apoptosis is triggered through both the intrinsic and extrinsic apoptotic pathways. As glaucoma is usually viewed as a neurodegenerative disease, the resistance against the apoptosis therein emerges as a pivotal strategy to save RGC in terms of glaucoma treatment [15]. There exist two primary apoptotic pathways, the intrinsic pathway and the extrinsic pathway, both of which depend on the activation of the cascade of cysteine proteinases as caspases. In this investigation, to explore whether blue light damages RGCs via apoptosis, we used TUNEL and western-blot for apoptosis-related proteins. TUNEL detects DNA fragmentation by labeling the terminal end of nucleic acids, while the Bcl-2 family proteins regulate the intrinsic pathway of apoptosis by controlling mitochondrial outer membrane permeability. In this result of this study, it was found that blue light significantly increased the protein level of pro-apoptotic proteins (cleaved caspase-3, cleaved caspase-9 and Bax) and reduce the expression anti-apoptotic of Bcl-2. In advanced study, it was found that the MEK/ERK/CREB pathway regulated by silibinin and thus plays an important role in the regulation of apoptosis and inflammation of the RGCs.

Conclusion
In conclusion, the effects of silibinin pretreatment against blue light induced acute RGCs damage was demonstrated, and the underlying mechanism was investigated in this study. The data indicated that direct suppression of apoptosis and inflammation through the activation of MEK/ERK/CREB pathway in vitro. Our study supported that silibinin may be used as a potential candidate for glaucoma treatment.

Disclosure statement
No potential conflict of interest was reported by the authors.

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