Original Paper

Novel Protective Effects of Cistanche Tubulosa Extract Against Low-Luminance Blue Light-Induced Degenerative Retinopathy

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Key Words

Cistanche tubulosa extract • Blue light • Apoptosis • Retinal pigment epithelial cells

Abstract

Background/Aims: Blue light-emitting diode light (BLL)-induced phototoxicity plays an important role in ocular diseases and causes retinal degeneration and apoptosis in human retinal pigment epithelial (RPE) cells. *Cistanche tubulosa* extract (CTE) is a traditional Chinese medicine with many beneficial protective properties; however, few studies have examined the ocular protective roles of CTE. In this study, we investigated the mechanisms underlying the effects of CTE on BLL-induced apoptosis *in vitro* and *in vivo*. **Methods:** RPE cells were applied in the current *in vitro* study and cell viability was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis-related protein expression was determined by western blot analysis and immunofluorescence staining. Brown Norway rats were used to examine exposure to commercially available BLL *in vivo*. Hematoxylin and eosin staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and western blot assays were used to examine retinal morphological deformation. **Results:** CTE significantly inhibited hydrogen peroxide-, tert-butyl hydroperoxide-, sodium azide-, and BLL-induced RPE damage. Further, CTE reduced the expression of apoptotic markers such as cleaved caspase-3 and TUNEL staining after BLL exposure by inactivating apoptotic pathways, as shown via immunofluorescent staining. In addition, CTE inhibited the BLL-induced phosphorylation of c-Jun N-terminal kinase, extra signal-related kinases 1/2, and p38 in RPE cells. *In vivo*, the oral

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administration of CTE rescued 60-day periodic BLL exposure-induced decrements in retinal thickness and reduced the number of TUNEL-positive cells in the brown Norway rat model.
Conclusion: CTE is a potential prophylactic agent against BLL-induced phototoxicity.

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Introduction

Studies of age-related macular degeneration (AMD) have reported that 8.7% of the world's population suffers from this disease [1]. AMD is a neuro-degenerative disease that causes photoreceptor deformation, retinal pigment epithelial (RPE) atrophy, ganglion cell apoptosis, and central vision loss in the elderly population, and is becoming a global social burden. AMD is classified into either a dry or wet form [2, 3]. Improvements in technology have resulted in people spending more time using modern digital devices, and consequently, people are exposed to blue light (BL) emissions over long periods. However, the issue of BL-induced phototoxicity in the retina is not well-investigated. Overexposure to short-wavelength BL (450–495 nm) results in the generation of reactive oxygen species (ROS), such as superoxide and hydroxyl radicals, which cause increases in oxygen consumption and induce mitochondrial DNA damage. The accumulation of ROS results in oxidative stress and the extensive accumulation of retinoid adducts, which damage the retina further [4]. Research has shown that oxidative stress is a leading factor in the pathogenesis of dry AMD [5]. Furthermore, BL irradiation decreases the expression of retinal antioxidant enzymes, such as superoxide dismutase and catalase, by mediating Bax/Bcl-2 protein interactions and promoting endogenous ROS production. As such, RPE cells and the outer segment mitochondria of photoreceptors suffer from BL irradiation-induced injury [6, 7]. In addition to ROS generation, BL irradiation activates mitogen-activated protein kinases (MAPKs), which play an important role in cell survival and apoptosis. Mitochondria-dependent apoptosis-related pro-apoptotic proteins, such as Bax and caspase-3, are activated by BL-emitting diode light (BLL) irradiation via the c-Jun N-terminal kinase (JNK) and p38 pathways in RPE and retinal ganglion cells [8]. Moreover, the phosphatidylinositol 3-kinase (PI3K)/Akt and nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent anti-oxidative pathways are components of the defense system of RPE cells [9–12].

Cistanche tubulosa extract (CTE) contains phenylethanoid glycoside derivatives and has been used as a traditional Chinese medicine for decades. Echinacoside, acteoside, and isoacteoside are three major active compounds of CTE that are characterized as having neuroprotective, memory-enhancing, immune-regulating, and anti-liver fibrosis properties [13, 14]. Echinacoside is a ROS scavenger and protects against 1-methyl-4-phenylpyridinium ion (MPP⁺)-induced apoptosis in animal models of Parkinson's disease [15, 16]. Acteoside protects against amyloid- β -induced neurotoxicity and reduces lipopolysaccharide-stimulated inflammation, attributable to the translocation of Nrf2 from the cytoplasm to the nucleus and binding to antioxidant response elements. The current available therapies for wet AMD, such as pegaptanib, ranibizumab, bevacizumab, and aflibercept, are used to inhibit excessive angiogenesis [17, 18]. However, no effective treatments exist for dry AMD, owing to the multiple pathological complications of this disease.

In this study, we demonstrate the protective effects of CTE in RPE cells and brown Norway (BN) rats. In addition, we applied long-term low luminance BLL exposure to both *in vitro* and *in vivo* models to evaluate the protective effects of CTE. We found that CTE reduced the expression of apoptosis-related proteins after exposure to BLL. Moreover, CTE reduced the number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells in the retina of BN rats after long-term exposure to low luminance BLL. This study provides further insights into the novel anti-apoptotic effects of CTE and information regarding a new strategy for the treatment of retinal degeneration.

Materials and Methods

Chemicals and products

CTE was generously provided by Sinphar Pharmaceutical Company (Yilan, Taiwan). *In situ* cell death detection kits (Cat. No. 11-684-817-910) were purchased from Roche (Mannheim, Germany).

RPE cell culture

RPE cells (ARPE-19 and ATCC® CRL-2302™) were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ at 37°C. ARPE-19 cells at passage 19–30 were maintained on 10-cm cell culture dishes (Orange Scientific, Braine-l'Alleud, Belgium). Upon passaging with 0.05% trypsin-ethylenediamine tetraacetic acid (Gibco), the cells were replated at a 1:4 ratio. Before testing, ARPE-19 cells were seeded onto 48-well plates (Orange Scientific) for cell viability analyses at a density of 1.0 × 10⁵ cells/mL and onto 6-cm dishes (Orange Scientific) for western blotting analyses at a density of 2.0 × 10⁵ cells/mL for 24 h. After ARPE-19 cells reached 80% confluency, the following experiments were performed.

Drug treatment and cell viability assay

RPE cells at passage 19–30 were seeded onto 48-well plates and co-treated for 24 h with different concentrations of CTE, hydrogen peroxide (H₂O₂), tert-butyl hydroperoxide (t-BHP), and sodium azide (NaN₃). The cells were then cultured with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) at a final concentration of 1 mg/mL and incubated for 30 min. Dimethyl sulfoxide (200 µL) was then added to each well to dissolve the cells. Absorbance was measured at 570 nm using a Sunrise™ spectrophotometer enzyme-linked immunosorbent assay reader (MRX-TC; Dynex Technologies, Chantilly, VA). Values were corrected for background absorbance by subtracting the appropriate blanks. Data are from five independent assays.

In vitro BL exposure protocol

The light source and cell plates were placed 25 cm apart, as described in our previous study [19]. Luminance was measured with a light meter (LM-81LX; Lutron Electronic Enterprise, Taipei, Taiwan). BLL peaked at 460 nm (60 lux) for the indicated time (0–48 h), depending on the experimental design.

Western blotting

After 0–48-h co-treatment with CTE and BLL exposure, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in a radioimmunoprecipitation assay lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM NaF, and protease/phosphatase inhibitor cocktail). The cell lysate was obtained via centrifugation at 12,000 × g for 35 min, and the supernatant was collected to determine protein concentrations via the Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein samples were separated by 10–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane. After the membrane was blocked with 5% non-fat milk and incubated in Tris-buffered saline with Tween 20 (TBST) for 1 h, immunoblotting was performed with primary antibodies specific for cleaved caspase-3, phospho-JNK, JNK, phospho-p38, p38, phospho-extracellular signal-related kinase (ERK), ERK, Bcl-2, Bax, Fas-associated protein with death domain (FADD), caspase-8 (GeneTex, Irvine, CA), Fas-ligand (Abcam, Cambridge, UK), and β-actin (Sigma-Aldrich, St. Louis, MO). The blots were then incubated with horse radish peroxidase-conjugated secondary antibodies (1:6000 in TBST; Cayman Chemical, Ann Arbor, MI) for 1 h at room temperature. Using an enhanced chemiluminescence kit (Millipore, Billerica, MA), the intensity of the bands on each blot was analyzed with Image J software after normalizing to β-actin.

Immunofluorescence

RPE cells were fixed with 4% paraformaldehyde in PBS for 15 min. Triton X-100 in PBS (0.2%) was then added to the cells for 15 min, followed by 5% fetal bovine serum in PBS for 30 min; the cells were then incubated with an anti-cleaved caspase-3 antibody at a 1:250 ratio for 24 h (Cell Signaling Technology, Danvers, MA). A goat anti-rabbit IgG secondary antibody (Dylight 594) was used at a ratio of 1:200 (Vector Laboratories, Burlingame, CA). Cell nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI; AAT Bioquest, Sunnyvale, CA). Changes in fluorescence were observed with a laser CS SP5 confocal spectral microscope imaging system (Leica, Teban Gardens, Singapore).

TUNEL staining

DNA fragments, which can be detected by a TUNEL assay, are a significant hallmark of apoptosis. We used an *In Situ* Cell Death Detection Kit (Cat. No. 11-684-817-910; Roche) to measure apoptotic programmed RPE cell death as per the manufacturer's protocol. Briefly, we fixed RPE cells with 4% paraformaldehyde and labeled the DNA strand breaks in an enzymatic reaction to detect TUNEL-positive cells, which presented with a green-fluorescent stain. The stained cells were then scanned and analyzed with a Tissue FAXS-plus Imaging System (TissueGnostics, Vienna, Austria). Ten thousand events were counted in the gated RPE region, and we analyzed the images for the number of TUNEL-positive cells with TissueQuest/HistoQuest software (TissueGnostics).

Animal experiments

Male BN rats (300–350 g body weight) were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and kept for 2–3 months. The rats were maintained in a 12-h/12-h light/dark cycle at $26 \pm 1^\circ\text{C}$, 35–47% relative humidity ratio, and *ad libitum* access to water and food. Untreated rats were maintained in the dark to serve as controls. The study protocols were approved by the Institutional Animal Care Use Committee of Taipei Medical University (approval number: LAC-2016-0442). All experimental procedures involving the use of animals complied with the Association for Research in Vision and Ophthalmology statements for the use of animals in ophthalmic and vision experimental research.

BLL-induced rat model of retinal degeneration

We used a periodic low-luminance long-term BLL exposure-induced model of retinal degeneration, as described in our previous study [19]. Briefly, BN rats were divided into three groups: a control group, which was kept under dark conditions; a BLL-exposed group, which was exposed to periodic BLL in the dark without pupil dilation (460 nm, 150 lux) for 3 h per day for 60 days; and a CTE-treated plus BLL-exposed group, which was pre-treated with CTE (100 mg/kg body weight) for 14 days and then co-treated with periodic BLL exposure for 3 h per day for 60 days. All rats were returned to the animal colonies and the normal light/dark cycle (250 lux, 12 h/12 h) was resumed at the end of the experimental period.

Statistical analysis

Significant differences were calculated using one-way analysis of variance followed by Tukey's post hoc test and multiple comparisons test. Statistical significance was determined by *p*-values of <0.05 .

Results

CTE protects RPE cells from oxidative stress damage

To evaluate the cytotoxicity of CTE via the MTT assay, several concentrations of CTE (0–500 $\mu\text{g/mL}$) were applied to RPE cells. CTE treatment had no obvious toxic effects on RPE cell viability at any of the concentrations tested (Fig. 1A). Therefore, 50 and 100 $\mu\text{g/mL}$ CTE were used in the subsequent experiments. To investigate the anti-apoptotic effects of CTE, different oxidative inducers (H_2O_2 , t-BHP, and NaN_3) were co-administered with CTE to RPE cells for 24 h. H_2O_2 , NaN_3 , and t-BHP decreased cell viability and damaged RPE cells in a dose-dependent manner (slashed bars, Fig. 1B, 1C, and 1D); cell viability was significantly lower in cells treated with 0.03 mM H_2O_2 , 0.3 mM NaN_3 , and 0.3 mM t-BHP than in the control group.

Fig. 1. Cistanche tubulosa extract (CTE) protects retinal pigment epithelial (RPE) cells against H₂O₂, tert-butyl hydroperoxide (t-BHP), and NaN₃-induced cytotoxicity. (A) RPE cells were treated with the indicated concentrations of CTE (0–500 μg/mL) for 24 h, and cell viability was measured via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (B, C, D) RPE cells were treated with the indicated concentrations of CTE (50 or 100 μg/mL), followed by H₂O₂ (0.03–1 mM), t-BHP (0.003–1 mM), and NaN₃ (0.3–10 mM) treatment for 24 h. Significant differences were calculated using one-way analysis of variance followed by Tukey's multiple comparison test. *p<0.05 compared with the control group; #p<0.05 compared with the H₂O₂-, t-BHP-, and NaN₃-exposed groups. The experiments were repeated three times, and similar results were obtained.

However, co-treatment with 50 and 100 μg/mL CTE attenuated the H₂O₂-, NaN₃-, and t-BHP-induced RPE cell damage and reversed the decrease in cell viability (black bars, Fig. 1B, 1C, and 1D). The above data suggest that CTE rescues RPE cells from oxidative stress damage.

CTE inhibits BLL-induced RPE cell death

In our previous study, we found that BLL is cytotoxic to RPE cells, which was attributable to changes in the expression of Bcl-2 and Bax. In the present study, we further examined the protective effects of CTE on BLL-induced RPE cell damage. We placed two blue electric LED plates in a cell culture incubator. The distance between the light source and cell plates was 25 cm, and BLL intensity was 60 lux, as measured by a light meter (Fig. 2A). An MTT assay

Fig. 2. Cistanche tubulosa extract (CTE) protects retinal pigment epithelial (RPE) cells against blue light-induced cell cytotoxicity. (A) To investigate the effect of blue light-emitting diode light (BLL)-induced apoptosis in RPE cells, we placed a BLL apparatus in a cell culture incubator. The distance between the light source and cell plates was 25 cm, and the intensity of BLL was 60 lux, as measured by a light meter. (B) RPE cells were exposed for 0, 6, 12, 24, and 48 h to BLL (460 nm, 60 lux), and cell viability (%) was measured by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (C) RPE cells were treated with the indicated concentrations of CTE (50 or 100 µg/mL), followed by BLL exposure for 6–48 h, and cell viability was measured. Significant differences were calculated using one-way analysis of variance followed by Tukey's multiple comparison test. Values are expressed as the means $\pm$ standard deviation. * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the BLL groups. The experiments were repeated three times, and similar results were obtained.

demonstrated that BLL exposure for different periods of time (6–48 h) caused RPE cell death in a time-dependent manner (Fig. 2B). The BLL-induced reductions in cell viability after 24 and 48 h were significantly attenuated when the cells were co-treated with CTE (100 µg/mL) (Fig. 2C). These data suggest that CTE protects RPE cells from BLL-induced phototoxicity.

CTE inhibits BLL-induced apoptosis in RPE cells by regulating the Bcl-2/Bax and Fas/FasL pathways

On the basis of the potential protective effects of CTE in RPE cells, we further investigated the possible involvement of CTE in this process. Two apoptotic signaling pathways were examined, i.e., the Bax/Bcl-2 and Fas/Fas ligand (FasL) pathways, and related proteins (e.g., pro-caspase-8 and pro-caspase-3; Fig. 3A and 3B). BLL exposure (6–48 h) was found to upregulate the protein expression of Bax/Bcl-2, FasL, and FADD in a time-dependent manner (Fig. 3A). Moreover, BLL downregulated the protein expression of procaspase-3 and procaspase-8, resulting in more cleavage forms of caspase-3 and caspase-8 after BLL exposure, which is consistent with our previous study [19]. To evaluate the protective effects of CTE further, different concentrations of CTE were applied to BLL-exposed RPE cells (Fig. 3B). After 48-h BLL exposure, CTE (50 µg/mL) decreased the Bax/Bcl-2 ratio. Further, CTE (100 µg/mL) decreased the protein expression of FasL and FADD, but significantly increased the levels of procaspase-8 and procaspase-3 (Fig. 3C and 3D). Studies have shown that caspase-3 activation is a marker of apoptosis in RPE cells [20]. To confirm the protective effects of CTE further, we performed immunofluorescent staining of cleaved caspase-3. BLL exposure for 48 h was found to induce caspase-3 cleavage and translocation into the nucleus, resulting in RPE cell apoptosis (yellow arrows, Fig. 4A). However, when BLL-exposed cells were co-treated with CTE (100 µg/mL), the BLL-induced expression of cleaved caspase-3 was inhibited. To understand the anti-apoptotic effects of CTE better, we used TUNEL staining to detect DNA fragments in RPE cells. After 48-h BLL exposure, TUNEL-positive cells increased from $1.78 \pm 0.36\%$ to $23.82 \pm 0.33\%$; this effect was attenuated by co-treatment with CTE ($23.82 \pm 0.33\%$ to $2.22 \pm 0.11\%$; Fig. 4B, 4C, and 4D). These results indicate that CTE treatment strongly protects RPE cells from BLL-induced apoptosis by mediating the Bax/Bcl-2 and FasL/FADD pathways.

CTE inhibits BLL-induced phosphorylation of stress response proteins in RPE cells

Previous studies have demonstrated that MAPK pathways, such as ERK, JNK, and p38 MAP, are activated in RPE cells and in the retina after exposure to ultraviolet light or BL [21, 22]. Hence, to investigate the regulatory effects of CTE on the MAPK stress response pathway after BLL exposure, 100 µg/mL CTE was applied to RPE cells under different BLL exposure times (Fig. 5A). Western blot analysis indicated that phosphorylated ERK, JNK, and p38 were activated upon BLL exposure, but significantly inhibited upon co-treatment with CTE in RPE cells (Fig. 5B). The above results indicate that CTE inhibits BLL-induced RPE cell damage, which might be associated with inhibition of MAPK in RPE cells.

CTE rescues BLL-induced retinal damage after long-term periodic exposure in a rat model

To evaluate the protective effects of CTE, we exposed BN rats to long-term BLL exposure for 60 days and evaluated the effects of CTE. As shown in the schematic presented in Fig. 6A, BN rats were divided into three groups: control group, BLL-exposed group, and CTE plus BLL group (Fig. 6A). In the CTE plus BLL group, BN rats were continuously supplemented with CTE (100 mg/kg body weight) by oral gavage once daily for 14 days before BLL exposure. On day 0, BN rats were co-treated with CTE (100 mg/kg body weight) and exposed to BLL for 3 h per day for 60 days. After 60 days of BLL exposure, the animals were sacrificed for western blot analysis and immunohistochemistry staining. In the CTE plus BLL group, the expression of procaspase-3 protein in rat eye homogenates was higher after CTE treatment than in the BLL group (Fig. 6B and 6C). These data illustrate that CTE prevents long-term low luminance BLL-induced retinal phototoxicity and apoptosis by inhibiting the activation of cleaved caspase-3. Immunohistochemistry staining provided more evidence regarding the protective effects of CTE on the structure and physiological pathways of the retina (Fig. 7). Further, after BLL exposure we found that the inner neuron layer was diminished and observed morphological deformations at the outer neuron layer (ONL), including the inner segment/outer segment in both the central and peripheral retina, which is consistent with our previous study (Fig. 7B). Importantly, CTE prevented RPE cell deformation, neuronal

Fig. 3. Cistanche tubulosa extract (CTE) attenuates blue light-induced caspase-3 activation via the Bcl-2/Bax and Fas/FasL signaling pathways in retinal pigment epithelial (RPE) cells. (A) Apoptosis-related proteins (Bax, Bcl-2, Fas ligand [FasL], Fas-associated protein with death domain [FADD], procaspase-3 [pro-cas-3], and procaspase-8 [pro-cas-8]) were evaluated via western blot analysis after exposure to blue light for 0, 6, 12, 24, and 48 h. (B) RPE cells were treated with various concentrations of CTE (10, 50, and 100 μg/mL), followed by blue light exposure for 48 h. (C, D) Quantification of protein levels relative to the β-actin control was performed by densitometry and expressed as means ± standard error (n = 3). *p<0.05 compared with exposure to the blue light group; #p<0.05 compared with the blue light-treated groups.

Fig. 4. Cistanche tubulosa extract (CTE) inhibits blue light-induced cell apoptosis in retinal pigment epithelial (RPE) cells. (A) The cells were co-treated with CTE (100 $\mu\text{g/mL}$) and/or blue light exposure for 48 h, and caspase-3 activation was measured via immunofluorescence microscopy. Representative images demonstrate cleaved caspase-3 (cleaved Cas3, red) and 4'-6-diamidino-2-phenylindole (DAPI, blue) dual staining of the cells. (B) To investigate the end-point of apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was performed; the yellow arrows represent TUNEL-positive cells. (C) The number of cells exhibiting TUNEL fluorescence was counted and expressed as a percentage of DAPI-positive cells. TUNEL data were also quantified by TissueQuest/HistoQuest software (TissueGnostics). (D) Quantified TUNEL data are shown. Significant differences were calculated using the Mann-Whitney U-test. Values are expressed as the means $\pm$ standard deviation ($n = 4$). * $p < 0.05$ compared with the control group; # $p < 0.05$ compared with the blue light-treated groups. Scale bars = 20 μm .

Fig. 5. Cistanche tubulosa extract (CTE) inhibits blue light-induced c-Jun N-terminal kinase (JNK) and p38 phosphorylation in retinal pigment epithelial (RPE) cells. (A) The effects of CTE (100 µg/mL) on the protein expression of phosphorylated extracellular signal-related kinase (ERK), JNK, and p38 after blue light exposure for 0–360 min are shown. (B) Proteins detected in (A) were quantified relative to β-actin. Proteins were quantified by densitometry, and the results are expressed as means ± standard error (n = 3). Significant differences were calculated using one-way analysis of variance followed by Tukey's multiple comparison test. *p<0.05 compared with exposure at 0 h; #p<0.05 compared with blue light exposure at the same time.

cell damage, and overall thinning of the retina in the CTE plus BLL group (Fig. 7C). Further, we did not observe TUNEL-positive cells in the central and peripheral retina of normal rats (Fig. 8Ab and 8Bb). Conversely, after BLL exposure for 60 days, TUNEL-positive cells were detected frequently in the ONL of the central and peripheral retina (Fig. 8Ae and 8Be). DAPI staining of the ONL of the central retina was markedly decreased in the BLL group (Fig. 8Ad). However, the presence of TUNEL-positive cells was attenuated and DAPI staining of the central and peripheral retina ONL was increased in the CTE plus BLL group (Fig. 8Ai and 8Bi).

Fig. 6. Cistanche tubulosa extract (CTE) inhibits blue light-emitting diode light (BLL)-induced caspase-3 activation in retinal homogenates. (A) A schematic of the experimental design is shown. Brown Norway rats were divided into three groups: control group, in which the rats were maintained in the dark; BLL-exposed group, in which the rats were exposed to periodic BLL for 3 h per day for 60 days; and a CTE plus BLL group, in which the rats were co-treated with CTE and BLL. # Represents the concentration of CTE (100 mg/kg body weight). (B) Pro-caspase 3 (Pro-Cas-3) protein levels in retinal homogenates were evaluated by western blot analysis. (C) Quantification of protein levels relative to the β -actin control was performed by densitometry and expressed as means $\pm$ standard error ($n = 4$). Significant differences were calculated using the Mann-Whitney U-test. * $p < 0.05$ compared with control group; # $p < 0.05$ compared with BLL group.

Fig. 7. Cistanche tubulosa extract (CTE) protects the central and peripheral retina against blue light-emitting diode light (BLL) exposure. (A, B, C) Representative areas for the central and peripheral retina are indicated by red and blue rectangular frames, respectively. The images in each rectangular frame are shown at a higher magnification. (A) The complete structure of the retina is shown for the control group. (B) Results from the BLL group, showing reductions in retinal thickness of both the central and peripheral retina, are presented. (C) Oral administration of 100 mg/kg body weight CTE alleviated BLL-induced decrements in retinal thickness. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigment epithelial cells ($n = 4$ for each group). Scale bars = 10 μm .

Fig. 8. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in a Cistanche tubulosa extract (CTE)-treated and blue light-emitting diode light (BLL)-exposed brown Norway rat model. (A, B) Central and peripheral retina tissue sections were subjected to TUNEL (green) and 4'-6-diamidino-2-phenylindole (DAPI, blue) counterstaining of retinal sections; representative TUNEL fluorescent images are shown. The yellow arrows indicate TUNEL-positive cells. DAPI staining of nuclei was performed as a background stain. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; IPL, inner plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer (n = 4 for each group). Scale bars = 20 μ m.

Discussion

Retinal degeneration is a genetic and multifactorial ocular disease that causes blindness. AMD is the most common cause of blindness among the elderly in developed countries and is representative of multifactorial retinopathy [23]. Improvements in technology, such as those observed for digital devices, have resulted in an increase in the amount of time people spend using their handsets, which has led to the development of smartphone dependency and even addiction. The increasing distribution rate of these “all-in-one” digital devices worldwide has dramatically affected our mental and physical health since 2010. A clinical case study reported that retinal degeneration occurred when long-term smartphone use was simulated [24]. Exposure to excessive BL increases oxygen consumption and ROS generation,

causing the accumulation of large amounts of toxic retinoid adducts and retinal damage [25]. Clinical trials have indicated that daily supplementation with an antioxidant cocktail slows down the progression of retinal cell atrophy [26]. As a result, the discovery of therapeutic natural compounds for use as anti-oxidant supplements can be extremely beneficial for the prevention of oxidative stress-induced retinal degeneration.

Few data exist regarding the role of CTE in oxidative stress-induced retinopathy. In this study, we examined the pharmacological effects of CTE on low-intensity long-term BLL exposure-induced retinal degeneration, as well as cell damage and apoptosis resulting from exposure to various oxidants such as H_2O_2 , t-BHP, and NaN_3 . We found that treating RPE cells that have been exposed to different oxidants with CTE (50 and 100 $\mu g/mL$) significantly increased viability (Fig. 1B to 1D), suggesting that CTE is a strong anti-oxidant.

Current studies have shown that among the phenylethanoid glycosides, echinacoside, acteoside, and isoacteoside are the most active compounds in CTE, which is used in traditional Chinese medicine and has been reported to have neuroprotective, antibacterial, anti-oxidative, anti-apoptotic, and anti-allergic effects [27, 28]. Echinacoside is potentially a powerful protective compound; studies have shown that echinacoside significantly reduces 6-hydroxydopamine-induced ROS production and attenuates mitochondria-related apoptosis by inhibiting interleukin (IL)-1 β and IL-6 in PC-12 cells [29]. In addition to echinacoside, acteoside is a member of the dihexose family and is another principal constituent found in the stems of *C. tubulosa*. Studies have shown that acteoside inhibits MPP^+ -induced neuronal death and protects SH-SY5Y neuronal cells against β -amyloid [30]. Many therapeutic properties are associated with acteoside, including anti-allergic, anti-neurotoxic, anti-inflammatory, anti-apoptosis, and anti-proliferative properties [31–33]. Isoacteoside, an isoform of acteoside, inhibits the IL-1 β -induced expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in human umbilical vein endothelial cells, providing evidence of ROS reduction [34].

In this study, a significant loss in cell viability, as well as increases in the Bax/Bcl-2 ratio and FasL and FADD protein levels after BLL exposure were observed. However, co-treatment with CTE significantly attenuated the BLL-induced activation of the intrinsic Bax/Bcl-2 and extrinsic Fas/FasL signaling pathways (Fig. 3). Though BL treatment yielded numerous TUNEL-positive RPE cells, CTE administration significantly reduced the number of apoptotic cells and provided protective effects (Fig. 4). Kuang et al. further confirmed that CTE reduces Bax protein expression and upregulates Bcl-2 protein expression in H_2O_2 -injured PC-12 cells [35]. Moreover, CTE possesses neuroprotective effects against tumor necrosis factor α -induced increases in caspase-3 activity in SH-SY5Y cells, demonstrating the anti-apoptotic effects of CTE [27]. BLL-induced phototoxicity has been reported to correlate with increased oxidative stress in RPE cells via nuclear factor- κ B, p38 MAP, and ERK inactivation [36]. We found that CTE decreased the phosphorylation of JNK and p38 during BLL exposure, providing evidence for CTE-mediated cellular protective effects against BLL.

Previously, we found that low-intensity long-term BLL exposure induces retinopathy in a rat model [19]; as a continuation of that study, we orally administered CTE to BN rats. BL-induced retinal degeneration and damage has been studied in other models [37, 38]. Excessive light exposure reduces ONL thickness, owing to the stress response [39, 40]. In a Sprague-Dawley rat model, free radical production increased and ONL thickness decreased after exposure to 750 lux BLL, demonstrating the phototoxicity of BLL [41]. In this study, we found that the oral administration of CTE prevented the effects of long-term periodic BLL exposure in both the peripheral and central retina of BN rats (Fig. 8). These results suggest that CTE protects the retina from periodic low-luminance long-term BLL exposure-induced retinal degeneration. In addition, a phase III clinical trial at the University of Wisconsin investigated the effects and safety of CTE therapy on the duration and severity of illness (ClinicalTrials.gov identifier: NCT00065715, University of Wisconsin-Madison Department of Family Medicine Madison, Wisconsin, United States, 2014).

Conclusion

The information above suggests that CTE is a powerful protective compound against oxidative stress. Further, to the best of our knowledge, this is the first study to investigate the effects of CTE in retinal cells. This study provides further information regarding the effects and potential use of CTE in patients with retinopathy.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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