Free-Radical Scavenger NSP-116 Protects the Corneal Epithelium against UV-A and Blue LED Light Exposure

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The corneal epithelium is continuously exposed to oxygen, light, and environmental substances. Excessive exposure to those stresses is thought to be a risk factor for eye diseases. Photokeratitis is damage to the corneal epithelium resulting in a painful eye condition caused by unprotected exposure to UV rays, usually from sunlight, and is often found in people who spend a long time outdoors. In modern life, human eyes are exposed to artificial light from light-emitting diode (LED) displays of computers and smartphones, and it has been shown that short-wavelength (blue) LED light can damage eyes, especially photoreceptors. However, the effect of blue LED light on the cornea is less understood. In addition, it is important to develop new treatments for preserving human eyesight and eye health from light stress. Here, we used human corneal epithelial cells-transformed (HCE-T) cells as an in-vitro model to investigate the protective effect of NSP-116, an imidazolyl aniline derivative, against the oxidative stress induced by light in the corneal epithelium. Treatment with 10 μM NSP-116 significantly increased the cell viability and reduced the death ratio following UV or blue LED light exposure. Furthermore, NSP-116 treatment decreased light-induced reactive oxygen species production and preserved the mitochondrial membrane potential. Immunoblotting data showed that NSP-116 suppressed the stress response pathway. Finally, NSP-116 treatment prevented corneal epithelial apoptosis induced by blue LED light in an in-vivo mouse model. In conclusion, NSP-116 has a protective effect against oxidative stress and corneal cell death from both UV and blue LED light exposure.

Key words blue light-emitting diode light; NSP-116; UV ray; human corneal epithelium cells-transformed; reactive oxygen species

INTRODUCTION

The corneal epithelium is located in the outermost layer of the ocular surface and is exposed to a variety of environmental factors, including light, atmospheric substances, and bacteria. The corneal epithelium is a multilayered squamous epithelium, and the apical membrane of most superficial cells expresses transmembrane mucins that form the glycocalyx to maintain tear fluid and protect the eye from bacterial infection.1 Inflammation of the ocular surface and damage to corneal epithelial cells destabilize the tear fluid, increasing the risk of dry eye symptoms and bacterial infections.2 Therefore, protection of corneal epithelial cells and prevention of inflammation are critical to maintaining eye health.

Light with a short wavelength, especially UV rays, has higher energy, and its excess exposure could damage a living body. UV is classified as UV-A (315–380 nm), UV-B (280–315 nm), and UV-C (200–280 nm) by the effects on living organisms.3 In nature, the source of UV is solar rays. The ozone layer absorbs most of the UV-C and UV-B. As a result, 99% of UV on the Earth is UV-A.3 Excessive exposure to UV light results in temporary corneal damage diagnosed as photokeratitis, sunburn on the cornea, which is accompanied by severe pain.4 Photokeratitis is frequently found in people who do outdoor activities, such as farmers and people on snow mountains for winter leisure, or people exposed to a UV light source in their workplaces, such as welders.5 In addition, epidemiological studies have reported that dry eye symptoms are more likely in areas with high sun exposure, suggesting that UV light may affect the ocular surface negatively.6,7 Based on these findings, it is important to understand the effects of UV light on corneal epithelial cells and to construct methods to protect the cornea from UV light.

Light in the 380 to 700 nm wavelength is visible to human beings. Visible light transmits through the corneal layer and reaches the retina. Then, photoreceptors exchange the light stimuli into a chemical signal.8 Excess exposure to short-wavelength blue light can also induce photoreceptor damage.9 In modern life, human eyes are often exposed to light from a display with light-emitting diode (LED) on electrical devices such as a smartphone. Besides, people who stare at visual display terminals (VDT) for a long time have a high risk of dry eye syndromes.10 It has been reported that blue LED light irradiation results in oxidative stress, ER stress, activation of autophagy, and cell death.11 However, the effect of blue LED light on the cornea epithelium is less well understood.

Excessive light exposure has been shown to cause oxidative stress by leading to the formation of free radicals and other reactive oxygen species (ROS), including superoxide anions.12,13 Thus, free radical scavenging may be useful to protect against light-induced oxidative tissue damage. NSP-116, 4-(4-acetyl-piperezin-1-yl)-2-(1H-imidazol-1-yl) aniline, is a novel imidazolyl aniline derivative (Fig. 1A). NSP-116 has a free radical scavenging activity against superoxide anions and hydroxyl radicals.15 It has been shown that NSP-116 has a protective effect against photooxidative damage on photoreceptors15 and...
ischemia by stroke in the brain. Therefore, NSP-116 might preserve corneal structure under oxidative stress induced by excessive light exposure as well.

Here, we examined the effect of NSP-116 on photo-oxidative damage in the cornea. UV-A and blue LED light irradiation induced cell death and mitochondrial damage in human corneal epithelial cells-transformed (HCE-T) cells. This damage was suppressed in the presence of NSP-116. Furthermore, NSP-116 reduced cell death in mouse cornea exposed to blue LED light. In summary, NSP-116 is a candidate for novel treatment of light induced corneal damage.

MATERIALS AND METHODS

Cell Culture Immortalized human corneal epithelial cell lines (HCE-T) were obtained and maintained as previously reported. HCE-T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (FUJIFILM-Wako, Osaka, Japan) with 0.5% dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan), 5µg/mL insulin, 2.75µg/mL transferrin, 3.35µg/mL selenium (ITS-G supplement, FUJIFILM-Wako), 10ng/mL recombinant human epidermal growth factor (R&D Systems, Minneapolis, MN, U.S.A.), 5% fetal bovine serum (FBS; VALEANT, Costa Mesa, CA, U.S.A.). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. Cells were passaged by trypsinization every 2 or 3 d.

UV-A or Blue LED Light Exposure to HCE-T Cells in Vitro and NSP-116 Treatment HCE-T cells were seeded into 96 well plates (Corning, Corning, NY, U.S.A.) at 5000 cells per well and incubated for 24h. The medium was replaced with fresh medium containing 1% FBS. After 30min, the cells were treated with 1, 3, or 10µM NSP-116 or vehicle (PBS with 1% DMSO) and incubated for 1h. The cells were then exposed to UV-A or blue LED light. UV-A light exposure was performed according to a previous report. HCE-T cells pretreated with NSP-116 were exposed to 5J/cm² UV-A light (365nm UV-A light source, CL-1000L UV Crosslinkers; Ultraviolet Products Ltd., Cambridge, U.K.) for 0.5h. After UV-A irradiation, the cells were incubated for 24h.

Blue LED light exposure was performed by a modified protocol of previous reports. Briefly, HCE-T cells were exposed to blue LED light (464nm, M-Trust Co., Ltd., Hyogo, Japan) at 1000lx for 24h. Blue LED light exposure was performed in a CO₂ incubator under a humidified atmosphere of 5% CO₂ at 37°C.

NSP-116 was obtained from Nippon Soda Co., Ltd. (Tokyo,
Cell Death and Viability Assays Cell death and cell viability assays were performed as reported previously.19 In short, the cell nuclei were stained at 24 h after UV-A light exposure (0.5 h) or after the end of blue LED light exposure (24 h). HCE-T cells were incubated with 16.2 μM Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and 1.5 μM propidium iodide (PI; Thermo Fisher Scientific) for 30 min. Images were then collected using BIORÉVO BZ-X710 (Keyence, Osaka, Japan), and the cell death rate was calculated from the number of PI-positive cells as a percentage of the number of Hoechst 33342-positive cells.

Cell viability was measured by using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). At 24 h after UV-A light exposure (0.5 h) or after the end of blue LED light exposure (24 h), CCK-8 reagent was added to each well at 10 μL and incubated for 2 h. At 0 and 2 h, the absorbance was measured using a Varioskan Flash 2.4 microplate reader (Thermo Fisher Scientific) at 450 nm. The relative cell viability was calculated from the absorbance change between 0 h (blank) and 2 h, and shown as the percentage of the control.

ROS Production Assay Intracellular ROS production was determined using CM-H2DCFDA (Thermo Fisher Scientific). At 24 h after UV-A light exposure (0.5 h) or after the end of blue LED light exposure (24 h), the cells were treated with CM-H2DCFDA at a final concentration of 10 μM for 1 h. Fluorescent signals were measured using a Varioskan Flash 2.4 microplate reader at excitation/emission: 495/527 nm. The ROS production rate was corrected by the number of live cells calculated by Hoechst 33342/P1 staining.

Mitochondrial Membrane Potential Assay The mitochondrial membrane potential was detected using a JC-1 Mitochondrial Detection Kit (Dojindo Laboratories). At 24 h after UV-A light exposure (0.5 h) or after the end of blue LED light exposure (24 h), the medium was changed to fresh medium containing 2 μM JC-1 dye and incubated for 30 min. Subsequently, the medium was removed, and imaging buffer was added. Fluorescent signals were measured using a Varioskan Flash 2.4 microplate reader. Red fluorescence (ex/em: 540/605 nm) shows JC-1 aggregation, which indicates the normal condition mitochondrion. Green fluorescence (ex/em: 480/510 nm) shows JC-1 monomer, which indicates the damaged mitochondria. The mitochondrial membrane potential was calculated as the ratio of red/green fluorescence intensity. Representative images were collected using BIORÉVO BZ-X710.

Western Blotting Analysis HCE-T cells were seeded on a 24 well-plate (seeding density: 25000 cells/well) and then incubated for 24 h under a humidified atmosphere of 5% CO2 at 37°C. After treatment with 10 μM NSP-116 or vehicle (PBS with 1% DMSO), the cells were incubated for 1 h. Subsequently, the cells were exposed to 5 J/cm2 UV-A light or 1000 lx blue LED light. At 24 h after UV-A light exposure (0.5 h) or after the end of blue LED light exposure (24 h), the cells were washed by PBS, and lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1% NP-40) containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). The cell lysates were centrifuged at 12000 × g for 20 min at 4°C. Protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, U.S.A.) with bovine serum albumin as a standard. An equal volume of protein sample and sample buffer solution (FUJIFILM-Wako) was mixed and boiled for 5 min. The protein sample was subjected to electrophoresis on a 5% to 20% gradient SDS-polyacrylamide gel (SuperSep Ace; FUJIFILM-Wako), and the separated proteins were subsequently transferred to a polyvinylidenedifluoride membrane (Immobilon-P; Merck Millipore Corp., Billerica, MA, U.S.A.). For immunoblotting, the following primary antibodies were used: rabbit anti-phospho-p38 mitogen-activated protein kinase (MAPK) antibody (Cat# 9211), rabbit anti-p38 MAPK antibody (Cat# 9212), rabbit anti-phospho-nuclear factor-kappaB (NF-κB) (Ser536) antibody (Cat# 3033), rabbit anti-NF-κB antibody (Cat# 8242) (Cell Signaling Technology, Beverly, MA, U.S.A.), and mouse anti-β-actin antibody (Sigma-Aldrich, Cat# A2228). All primary antibodies were used at 1:1000 dilution. A horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Pierce Biotechnology, Rockford, IL, U.S.A.) and an HRP-conjugated goat anti-mouse antibody were used as secondary antibodies (1:2000 dilution). Immunoreactive bands were visualized using an Immunostar-LD (FUJIFILM-Wako). The signal detection and analysis were performed using Amersham Imager 680 Analysis Software (GE Healthcare, Chicago, IL, U.S.A.).

Animals All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. Seven-week-old male ddY mice were purchased from Japan SLC Ltd. (Hamamatsu, Japan). The mice were housed under a 12-h light–dark cycle (lighting: 8:00 a.m.–8:00 p.m.) at 24 ± 2°C and 55 ± 15% humidity, were fed a solid diet (CE-2; CLEA, Tokyo, Japan), and had free access to water.

Blue LED Light Irradiation in Vivo ddY mice were irradiated with 30000 lx blue LED light (456 nm, Cree; Durham, NC, U.S.A.) for 1 h twice daily (8:30 and 16:30) for 10 d. Mice were kept in a mirrored cage without anesthesia and exposed to blue LED light from the light source located 45 cm above. They were also treated with 5 μL of eye drops containing 10 μM NSP-116 (4% polyethylene glycol, 0.1% Tween-80, and 0.01% DMSO in PBS) or vehicle both before and after exposure to blue LED light.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Staining Ten days after the start of blue LED light irradiation, mouse eyes were excised and fixed in 0.1 M phosphate buffer (PB) containing 4% paraformaldehyde (pH 7.4) for 4 h at 4°C. Then, the eyes were placed successively in 5, 10, 15, 20, and 25% sucrose-containing 0.1 M PB (pH 7.4). Eyes were then embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan). By using a cryostat (Leica, Wetzlar, Hesse, Germany), 15 μm thick corneal sections were prepared at −20°C and placed on MAS-coated cover glasses (Matsunami Glass, Osaka, Japan).

To detect apoptotic cells, TUNEL staining was performed using an In Situ Cell Death Detection kit (Roche Biochemicals, Mannheim, Germany) according to the manufacturer’s
protocol. Frozen sections were thawed from −80°C at the time of staining, left at −20°C for 1 h, left at 4°C for 1 h, and then dried at room temperature for 2 h. The sections were then surrounded using a Super PAP pen (Daido Sangyo, Saitama, Japan) to prevent the reaction solution from running off. The sections were washed three times with PBS and incubated in 0.1% sodium citrate solution containing 0.1% Triton X-100 for 1 h. The sections were then washed three times in PBS and incubated at 37°C for 1 h in a TUNEL reaction mixture prepared according to the manufacturer’s protocol. For nuclear staining, the sections were incubated in Hoechst 33342 (dilution: 1:1000) solution for 30 min. After nuclear staining, the sections were sealed in Fluoromount (Diagnostic BioSystems, Pleasanton, CA, U.S.A.). Three sections of each eye were captured under a fluorescence microscope (Keyence), and the number of TUNEL-positive cells was counted. The length of the corneal arc was measured using ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.), and the number of TUNEL-positive cells per 1000 µm of corneal arc was calculated. The mean of the measurements on the three images was used as the number of TUNEL-positive cells per eye.

**Statistics** All data are presented as means ± standard error of the mean (S.E.M.). Statistical analyses were performed with Student’s t-test or Dunnett’s test or Tukey’s test using the Statistical Package for the Social Sciences 15.0J for Windows software (SPSS Japan Inc., Tokyo, Japan). A value of \( p < 0.05 \) was considered to indicate statistical significance.

**RESULTS**

**Effects of NSP-116 on Photo-Oxidative Damage in Human Corneal Epithelial Cells** To examine the effect of NSP-116 (Fig. 1A) on UV-A induced cell damage, human corneal epithelial cells-transformed (HCE-T) cells were treated with 1, 3, or 10 µM NSP-116 and exposed to UV-A for 24 h (Fig. 1B). HCE-T cell death rate was increased to 27.1 ± 2.0% by UV-A exposure, and 10 µM NSP-116 significantly reduced the cell death rate to 17.3 ± 1.5% (Figs. 1C, D). In addition, pretreatment with 10 µM NSP-116 inhibited the reduction in cell viability of HCE-T induced by UV-A irradiation (Fig. 1E).

Next, we investigated the effect of NSP-116 on blue-LED light-induced human corneal epithelial cell damage (Fig. 2A). Even though the cell death rate was less, blue LED light also induced cell death rate to 7.8 ± 1.7%, and NSP-116 at 10 µM suppressed the increase of cell death rate to 2.6 ± 0.6% (Figs. 2B, C). Additionally, pretreatment with 10 µM NSP-116 inhib-
...ited the reduction in cell viability of HCE-T induced by blue LED light exposure (Fig. 2D).

Effect of NSP-116 on ROS Production Induced by UV-A or Blue LED Light in HCE-T Cells It has been reported that one of the main causes of cell damage induced by light exposure is intracellular ROS accumulation.\textsuperscript{18,19} Therefore, we investigated the effect of NSP-116 on ROS production by UV-A or blue LED light using CM-H\textsubscript{2}DCFDA, an intracellular ROS indicator. UV-A irradiation increased the intracellular ROS levels. Blue LED light exposure also increased ROS levels.

![Graph](https://example.com/graph1.png)

**Fig. 3.** NSP-116 Suppresses UV-A and Blue LED Light-Induced ROS Production

(A) Intracellular ROS levels determined by CM-H\textsubscript{2}DCFDA probe 24h after 5J/cm\textsuperscript{2} UV-A irradiation for 0.5h. (B) Intracellular ROS levels determined by CM-H\textsubscript{2}DCFDA probe in HCE-T cells exposed to 1000lx blue LED light for 24h. Data are expressed as means ± S.E.M. (n = 6). *p < 0.05, **p < 0.01, Student’s t-test vs. Control, *p < 0.05, **p < 0.01, Dunnett’s test vs. Vehicle.

![Graph](https://example.com/graph2.png)

**Fig. 4.** NSP-116 Suppresses UV-A and Blue LED Light-Induced Mitochondrial Dysfunction

(A) Representative images of JC-1 staining, an indicator of mitochondrial membrane potential, in HCE-T cells at 24h after exposure to UV-A light for 0.5h. JC-1 aggregate and monomer stand for normal and damaged mitochondria, respectively. (B) The rate of JC-1 aggregation and JC-1 monomerization at 24h after UV-A irradiation. (C) Representative images of JC-1 staining in HCE-T cells exposed to blue LED light for 24h. (D) The rate of JC-1 aggregation and JC-1 monomerization in HCE-T cells exposed to blue LED light for 24h. Data are expressed as means ± S.E.M. (n = 6). ***p < 0.01, Tukey’s test vs. Control, **p < 0.01, Tukey’s test vs. Vehicle. Scale bar = 200µm.
production. NSP-116 at 10 µM suppressed the ROS production induced by UV-A and blue LED light. In addition, NSP-116 treatment without light exposure decreased the intracellular ROS levels (Figs. 3A, B).

Mitochondria are a major source of intracellular ROS production. 20) To assess the mitochondrial membrane potential, JC-1 staining was performed. The exposure to either UV-A or blue LED light increased the damage to mitochondria indicated by green fluorescence JC-1 monomer (Figs. 4A, B). The ratio of damaged mitochondria was significantly reduced in the presence of NSP-116 (Figs. 4C, D).

Effect of NSP-116 on the Stress Response Signaling Pathways by UV-A or Blue LED Light in HCE-T Cells

To investigate the activation of stress response proteins, we performed an immunoblotting analysis. The p38 MAPK pathway is activated by ROS such as superoxide anions from UV-induced stimulation, which induce apoptosis and inflammation. 21) NF-κB is known to be activated by UV light, X-rays, and free radicals and to promote inflammation and apoptosis. 22) The phosphorylation of p38 MAPK was promoted by UV-A or blue LED light irradiation (Figs. 5A, C, E, G). NSP-116 10 µM inhibited the phosphorylation of p38 MAPK (Figs. 5A, C, E, G). In addition, phosphorylated NF-κB was increased by UV-A or blue LED, and NSP-116 treatment

Fig. 5. NSP-116 Suppresses UV-A and Blue LED Light-Induced Stress Response Pathway Activation

(A, B) The expression level of each protein measured by Western blotting of HCE-T cell lysates of 24h after 5J/cm² UV-A irradiation for 0.5h. The representative images of p38 MAPK (A) and NF-κB (B). Quantitative analysis of phospho-p38 MAPK. (C) Quantitative analysis of phospho-NF-κB. (D) The expression level of each protein measured by Western blotting of HCE-T cell lysates of 1000lx blue LED light exposure for 24h. The representative images of p38 MAPK (E) and NF-κB (F). (G) Quantitative analysis of phospho-p38 MAPK. (H) Quantitative analysis of phospho-NF-κB. Data are expressed as the means ± S.E.M. (n = 4-5) **p < 0.01 vs. Control, ##p < 0.01 vs. Vehicle, Tukey’s test.
inhibited the phosphorylation of NF-κB (Figs. 5B, D, F, H). Interestingly, UV-A and blue LED light irradiation decreased the total NF-κB expression levels (Figs. 5B, F, Supplementary Figs. 1A, B).

**Evaluation of the Effect of NSP-116 in an in Vivo Blue LED Mouse Model** To evaluate the effect of NSP-116 in vivo, we performed blue LED light irradiation on ddY mice and NSP-116 treatment by eye drops and assessed the damage to the corneal epithelium by TUNEL staining. Blue LED light irradiation increased TUNEL-positive apoptotic cells in the corneal epithelium layer. In particular, the upper side of the cornea was more likely to be exposed to the light (Fig. 6B). The number of TUNEL-positive cells in the corneal epithelium layer was increased significantly to 3.3 ± 0.6 cells per 1000 μm corneal arc by blue LED light irradiation, and 10 μM NSP-116 eye drops significantly inhibited the increase of TUNEL-positive cells to 2.5 ± 0.8 cells per 1000 μm corneal arc in the whole cornea (Fig. 6C). Additionally, TUNEL-positive cells on the upper side of the cornea were increased by blue LED light irradiation, and NSP-116 suppressed them significantly (Vehicle group; 4.1 ± 0.9 cells/1000 μm, NSP-116 treatment group; 2.2 ± 0.5 cells/1000 μm) (Fig. 6D). In the lower side of the cornea where cells were exposed to light weakly, TUNEL-positive cells were significantly increased by

![Fig. 6](image)

(A) Image of blue LED irradiation and the corneal section. (B) Typical images of TUNEL stained corneal sections at 10d after blue LED irradiation. Scale bar = 500 μm (left). Scale bar = 200 μm (right). Quantitative analysis of the number of TUNEL-positive cells per 1,000 μm corneal arc is shown in (C) whole cornea, (D) upper side of cornea, and (E) lower side of cornea. Data are expressed as means ± S.E.M. (n = 9–11). *p < 0.05, **p < 0.01, Tukey’s test vs. Control, *p < 0.05, Tukey’s test vs. Vehicle.
blue LED light irradiation, but there is no effect on apoptosis by NSP-116 eye drop treatment (Fig. 6E).

DISCUSSION

In the present study, we evaluated the effect of NSP-116 against photo-oxidative damage in the corneal epithelium. In HCE-T, both UV-A or blue LED light induced cell death (Figs. 1, 2) and increased intracellular ROS levels (Fig. 3). In addition, UV-A and blue LED light decreased mitochondrial membrane potentials (Fig. 4) and activated stress response signaling pathways (Fig. 5). In vivo, blue LED light irradiation promoted corneal epithelium apoptosis (Fig. 6). NSP-116 had a protective effect against this photo-oxidative corneal epithelium damage (Fig. 7), and it can be a novel candidate for treating or preventing corneal diseases related to oxidative damage.

NSP-116 is a compound with a strong radical scavenging activity against hydroxyl radicals and superoxide anions and is a potential prophylactic and therapeutic agent for inflammatory and oxidative stress-related diseases. UV-A generates hydroxyl radicals, superoxide anions, hydrogen peroxide, and singlet oxygen. Blue light has been reported to generate hydroxyl radicals, superoxide anions, and singlet oxygen in mitochondria as well. Hydroxyl radicals cause lipid peroxidation, protein denaturation, and DNA damage and induce apoptosis in cells. Previously, some free radical scavengers have shown a protective effect against photo-oxidative damage. For example, Edaravone, a free radical scavenger used clinically for brain protection, has been shown to have a protective effect against photodamage of the retina. In addition, crocetin, which is known to have an antioxidant effect via free radical scavenging, has been shown to reduce UV-induced oxidative stress in the skin and to have a protective effect against light-induced retinal damage, suggesting that free radical scavenging may be effective against photo-oxidative tissue damage. These results suggest that NSP-116 prevented the oxidative stress in corneal epithelial cells and inhibited apoptosis by scavenging the hydroxyl radicals and superoxide anions that were excessively generated by UV-A and blue LED light exposure.

In the present study, we showed that UV-A and blue LED light irradiation of corneal epithelial cells enhanced and activated p38 MAPK phosphorylation (Figs. 5A, C, E, G). p38 MAPK activation induces the transcription factor activator protein-1 (AP-1), which promotes inflammation and apoptosis. In addition, activation of p38 MAPK cleaves Bid, a member of the B-cell lymphoma 2 (BCL-2) protein family, and abolishes the mitochondrial membrane potential, which releases cytochrome c into the cytoplasm and activates caspases, resulting in apoptosis and cell death. In corneal epithelial cells, it is known that p38 MAPK is activated during either UV-B irradiation or high osmotic cell damage. However, blue LED light irradiation of cone cells has been shown to activate p38 MAPK via ROS. The present study indicated that activation of the p38 MAPK pathway also contributed to corneal epithelial cell death (Figs. 1, 2, 6) and mitochondrial damage (Fig. 4) caused by UV-A and blue LED light irradiation. Because NSP-116 has a radical scavenging ability for superoxide anions, it was suggested that NSP-116 suppresses the activation of p38 MAPK by scavenging the superoxide anions generated by UV-A and blue LED light irradiation. However, the direct effect of NSP-116 on p38 MAPK activity is not clear, and further studies are needed to better understand the mechanism of action of NSP-116.

We showed that the transcription factor NF-κB, which induces inflammation and apoptosis, is activated by UV-A and blue LED light irradiation of corneal epithelial cells (Figs. 5B, D, F, H). In the corneal epithelium of mice with UV-B-induced photokeratitis, increased ROS induces nuclear translocation of NF-κB, which promotes inflammation and apoptosis. Administration of astaxanthin, which has a high radical scavenging capacity, has been shown to inhibit nuclear translocation of NF-κB, inflammation, and apoptosis induced by UV-B. In addition, NF-κB and TUNEL-stained apoptotic cells co-localize with NF-κB in light-induced retinal photoreceptor damage in mice, suggesting that NF-κB activation is involved in oxidative light-induced apoptosis. In this study, the increased expression of phosphorylated NF-κB, which was increased by blue LED light irradiation, was suppressed by treatment with the radical scavenger NSP-116 (Figs. 5B, D, F, H). These results suggest that exposure of corneal epithelial cells to blue LED light caused corneal epithelial cell damage via the NF-κB pathway and induced apoptosis through an increase in intracellular ROS.

In addition to increased phosphorylation of NF-κB, we also

![Fig. 7. Schematic Diagram of UV-A- and Blue LED-Induced Corneal Epithelial Cell Damage and Mechanism of Protection by NSP-116](Image)

In the corneal epithelium, UV-A and blue LED light increased intracellular ROS (Fig. 3). ROS decreased the mitochondrial membrane potential (Fig. 4), activated stress response pathways (Fig. 5), and finally caused cell death, especially apoptosis (Figs. 1, 2, 6). The free radical scavenger NSP-116 showed a protective effect against this cell damage induced by UV-A and blue LED light via ROS scavenging.
found that its total protein amount was decreased by both UV and blue LED light irradiation, (Figs. 5B, F, Supplementary Figs. 1A, B). On the other hand, total p38 MAPK and extracellular signal-regulated kinase (ERK)1/2 were not significantly changed by light irradiation, which consistent with a previous study on corneal epithelium, suggesting that UV- and blue LED light-induced total protein level change would be specific in certain signaling proteins including NF-xB. There is a possibility that light irradiation may affect not only the intracellular distribution of NF-xB but also its turn-over. Further investigation is needed to reveal how UV- and blue LED light-induced stress impairs NF-xB expression.

In the present study, we showed that topical treatment with eye drops containing the radical scavenger NSP-116 ameliorated the apoptosis of the mouse corneal epithelium induced by blue LED light (Fig. 6). Excessive light exposure has been reported to cause oxidative stress-mediated inflammation, apoptosis, and dry-eye like symptoms in the mouse cornea, and blue light may contribute significantly. In vitro, exposure of corneal epithelial cells to blue light may also cause oxidative stress and mitochondrial damage, which is consistent with the damage that occurs in vivo. It has been reported that bilberry and its constituents have a protective effect against blue light-induced corneal epithelial cell damage in vitro, and in the present study, the radical scavenger NSP-116 showed a protective effect (Figs. 1–4), suggesting that the antioxidant approach is useful against corneal epithelial photo-oxidative damage. Previous clinical reports have shown that prolonged use of a VDT and increased exposure to sunlight can increase the risk of dry eye symptoms. Oxidative stress and inflammation are thought to play a role in the development of dry eyes, and it is known that lipid peroxides, which are produced by oxidative stress and proinflammatory proteins such as matrix metalloproteinase (MMP)-9, are increased in the tear fluid of dry eye patients. Antioxidants such as omega-3 fatty acids and bilberry have been reported to be useful in the prevention and treatment of dry eyes. In conclusion, this study indicates that the blue LED light-induced mouse corneal damage model may be a useful tool in the search for methods to prevent and treat corneal oxidative damage such as dry eye symptoms.

In conclusion, our results indicate that NSP-116 protects corneal epithelial cells from photooxidative cytotoxicity by scavenging the reactive oxygen species generated by excessive light exposure, suggesting that NSP-116 may be a novel corneal protection agent.

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Supplementary Materials The online version of this article contains supplementary materials.

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