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Protective effect of *Oxya chinensis sinuosa* methanol extract on UVB-induced damage in human retinal pigment epithelial cells

Hyun Jung Lim, Sohyun Park, Joon Ha Lee, In-Woo Kim, HaeYong Kweon, and Minchul Seo*

Department of Agricultural Biology, National Institute of Agricultural Sciences, Rural Development Administration, Wanju-gun, Jeonbuk 55365, Korea

Abstract

The human eye, constantly exposed to solar radiation, can be damaged by UV radiation. In particular, ultraviolet B (UVB)-induced damage plays an important role in retinal degeneration and cell aging. In this study, we investigated the protective effects of the methanol extract of *Oxya chinensis sinuosa* (OCM), an edible insect known for its high protein content (64.2%), and various pharmacological effects, on human retinal pigment epithelial cells. ARPE-19 cells were treated with OCM and subsequently UVB irradiated. Our results showed that OCM effectively attenuates UVB-induced cell damage by reducing MAPK phosphorylation (JNK and p38 MAPK). Additionally, OCM increased the phosphorylation of Akt, and cell cycle regulators, including p21 and p27, in a dose-dependent manner. Moreover, OCM treatment increased ARPE-19 cell proliferation by activating the S6K1/S6 pathway. This study suggests that OCM prevents UVB-induced retinal cell damage by increasing cell proliferation via ROS reduction, suggesting its potential as a functional therapeutic superfood against retinal cell damage.

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Introduction

The eyes are crucial sensory organs that gather nearly of daily information. Functional impairment of the eye is a major factor affecting quality of life (Kim *et al*., 2020). Normally, the eye is exposed to solar radiation during the daytime, which is filtered by the Earth's ozone layer and the lens of the eye. However, increased UV exposure owing to environmental pollution induces reactive oxygen species (ROS) and cell damage in the eyes (Cao *et al*., 2012; Marie *et al*., 2018). Human retinal pigment epithelial cells form a single-cell layer between the photoreceptors and the vascular context. They control the amount of light reaching the retina and protect retinal photoreceptors (Strauss, 2005), affected by aging and oxidative stress (Kaunppinen *et al*., 2012). If damage such as oxidative stress occurs in the retinal epithelial cells, the breakdown of photoreceptor cells follows, and visual acuity is damaged (Nilsson *et al.,* 2003). Additionally, UV irradiation is involved in the pathogenesis of age-related macular degeneration (AMD) by causing oxidative stress and DNA mutations in retinal cells (Godar *et al*., 1993). Exposure to ultraviolet (UV) sunlight causes cell damage, malignant transformation, and skin cancer. The UV radiation in sunlight reaching the ground is composed of solar ultraviolet A (UVA; 315-400 nm), UVB (280-315 nm), and UVC (200-280 nm) radiations, which are completely inhibited by the ozone layer. UVB is more energetic than UVA and can cause mutations in target genes (Carr *et al*., 2012). Although UVB with wavelengths ranging from 280 to 315 nm has health benefits, excessive

***Corresponding author.**
Minchul Seo, Ph.D.
Department of Agricultural Biology, National Institute of Agricultural Sciences, Rural Development Administration, Wanju-gun, Jeonbuk 55365, Korea
Tel: +82-63-238-2991 / FAX: +82-63-238-3833
E-mail: nansmc@korea.kr

exposure to UVB can cause mitochondrial damage in cells, inflammation, and eye diseases (Fitsiou *et al*., 2021), including ROS production in retinal pigment epithelial cells, resulting in retinal damage (Biesemeier *et al*., 2008). ROS levels increase as early as 30 min after UVB exposure, and the apoptosis of retinal cells reaches 30.76% within 24 h, causing continuous chronic to the cells (Cao *et al*., 2012). Notably, the mitogen-activated protein kinase (MAPK) pathway is involved in retinal cell death caused by various factors, including ultraviolet (Roduit and Schorderet, 2008). These findings suggest that UVB irradiation affects the retina. Since the oxidative stress caused by ROS can cause eye damage, the possibility of eye diseases increases in the elderly (Silván *et al*., 2016). Most studies have considered the effect of UVB on skin exposure; however, research on the effect of UVB on the retina is limited. Owing to environmental pollution, lifestyle changes, and the intake of processed food, the rate of eye aging is rapidly increasing. Since functional superfoods related to eye health are limited to ω-3 and lutein, there is a need for research on various superfoods related to eye health (Koskela *et al*., 2016; Liu *et al*., 2017). Especially, there is not much research on *Oxya chinensis sinuosa* and retinal health improvement. Edible insects may function as good food sources and have advantages, such as low greenhouse gas emissions, low water consumption, and small breeding spaces (Kim *et al*., 2022a). Edible insects contain unsaturated fatty acids including ω-3, proteins (Stull and Weir, 2023; Nowakowsk *et al*., 2022; Lee *et al*., 2021), and chitin and other fiber that could influence gut health (Stull *et al*., 2018). Dietary fiber is an energy sources for gut microbiota in the intestine by increasing diversity in fecal microbiota. ω-3 can exert reverting microbiota composition and induce anti-inflammatory actin (Costantini *et al*., 2017). Edible insects are an excellent source of protein and other micronutrients, they have a protective effect on the intestine (Stull and Weir, 2023). Notably, *Oxya chinensis sinuosa*, a high-protein insect with a protein content of 64.2%, has been used in herbal medicine, folk medicine, and food materials (Chung and Kim, 2014). Recent studies on grasshoppers have focused on inflammation control mechanisms (Yoon *et al*., 2014), protective effects against cell death in SH-SY5Y cells which is human neuroblastoma cells (Park *et al*., 2006), anticancer immune activity (Chung and Kim, 2014), and antioxidant and antibacterial activities (Kim *et al*., 2015).

In this study, we investigated the potential of *Oxya chinensis sinuosa* methanol (OCM) extracts as a superfood that could protect human retinal pigment epithelial cells from UVB damage. We also discuss the possible molecular mechanisms underlying the OCM-mediated protective effects on ARPE-19 cells against UVB.

Materials and Methods

Preparation of *Oxya chinensis sinuosa* methanol extracts

Dried *Oxya chinesis sinuosa* ground to a powder using a commercially available blender. *Oxya chinensis sinuosa* powder was mixed with 70% methanol (0.1g/mL), ultrasonicated (250 J, 10 s, 2 times), and incubated at room temperature for 30 min. After extraction, the supernatants were filtered (0.45 μm syringe filter) and dried using a centrifugal evaporator (CVE-3100, EYELA, Tokyo, Japan). The OCM extract was kept frozen at -80 °C until use and dissolved in 20% DMSO when used for the experiment.

Cell culture

Human retinal pigment epithelial cells were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin (Gibco, Life Technologies Limited, Paisley, UK) at 37 °C in a 5% CO_2 humidified incubator.

Induction of cell damage through UVB irradiation

ARPE-19 cells were seeded at a density of 3×10^5 cells/well in 6-well plates for 24 h, and the supernatant was removed and replaced with phenol red-free DMEM/F-12 supplemented with 10% FBS and 1% penicillin-streptomycin. Cell damage was induced using UVB irradiation (50 mJ/cm^2).

MTS assay

ARPE-19 cells were seeded at 5×10^3 cells/well in 96-well plates and incubated for 18 h. Cells were treated with OCM (0, 0.1, 0.5, 1, or 2 mg/mL) and irradiated with UVB (50 mJ/cm^2). After incubation for 24 h for recovery, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] solution (Promega, Madison, WI, USA) was added to the plate and incubated for 3 h.

Following incubation, the optical density was determined at 490 nm using a microplate reader.

Measurement of ROS

To confirm the efficacy of OCM in preventing intracellular ROS due to UVB, ROS levels were measured using an intracellular ROS assay kit (Cell Biolabs, Danvers, MA, USA). ARPE-19 cells were pretreated with OCM (0.5, 1, or 2 mg/mL) for 1 h, stimulated with UVB at 50 mJ/cm^2, and cultured alone or in combination with OCM for 24 h. After adding DCF-DA (100 μM) at 37 °C for 1 h. The culture media was completely removed and washed twice or thrice with 1X phosphate-buffered saline (PBS). Then, serum-free phenol red DMEM/F12 and 2X cell lysis buffer were mixed with the treated cells and incubated at room temperature for 5 min. Finally, the cell lysate was transferred to a black plate and measured at 485 nm excitation and 530 nm emission wavelengths using a fluorescence plate reader (Thermo Fisher Scientific, USA).

Extraction and quantification of cell protein

For protein extraction, ARPE-19 cells were seeded at 3 × 10^5 cells/well in 6-well plates and incubated for 24 h. The cells were pretreated with different concentrations (0, 0.1, 0.5, 1, and 2 mg/mL) of OCM for 24 h and then irradiated with UVB (50 mJ/cm^2). The cells were washed twice with PBS and lysed using M-PER™ Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA, USA) containing Halt™ Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific). The protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific).

Western blot analysis

Proteins were separated using Bolt™ 4-12% Bis-Tris Plus gel (Invitrogen, Carlsbad, CA, USA) and transferred to a PVDF membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with TBST containing 5% blocking buffer for 1 h at room temperature and then blotted with antibodies against β-actin, ATK, phospho-AKT, p38, phospho-p38, JNK, phospho-JNK, ERK, phosphor-ERK, p21, p27, mTOR, phospho-mTOR, S6, phospho-S6, S6K1 and phospho-S6K1 (all antibodies were purchased from Cell Signaling Technology, USA) overnight at 4 °C. After incubation, the membrane was washed several times with TBST washing buffer and incubated with a horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. Finally, band expression was detected using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate on an Alliance Q9 gel documentation system (UVITEC, Cambridge, UK).

Statistical analysis

Data are presented as mean ± standard deviation (SD) of at least three independent experiments. Differences between the two groups were evaluated using Student's t-test. SPSS version 18.0 K (SPSS Inc., Chicago, IL) was used for the statistical analysis, and p values <0.05 were considered statistically significant.

Results and Discussion

Effects of OCM on cell viability in ARPE-19 cells

To evaluate the cytotoxicity of OCM extract on human retinal pigment epithelial cells (ARPE-19), cell viability was determined using the MTS assay. The OCM extract was not cytotoxic to ARPE-19 cells at concentrations up to 2 mg/mL (Fig. 1A).

Effects of OCM on UVB-induced cell death in ARPE-19 cells

Our results showed that UVB irradiation (50 mJ/cm^2) induced cell death in ARPE-19 cells, as demonstrated by the 60% cell viability (Fig. 1B). In another study, low-dose UVB irradiation (50 mJ/cm^2) induced a significant degree of apoptosis in ARPE-19 cells (Cao *et al*., 2012). Notably, *Oxya chinensis sinuosa* was shown to exert anti-wrinkle effects on skin damaged by UVB and protect skin cells by reducing the expression of MMPs (Im

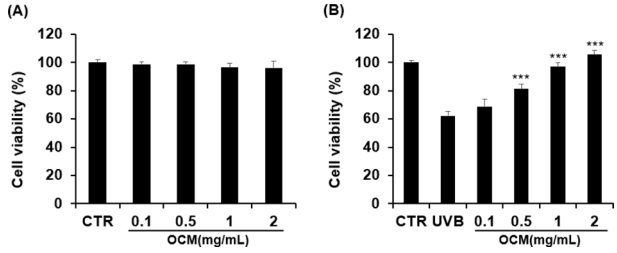

Fig. 1. Cell viability assay. (A) Cell viability of ARPE-19 retinal epithelial cells treated with OCM, as determined by MTS assay. (B) Cell viability of UVB-radiated ARPE-19 cells, as determined by MTS assay. Data are the mean ± SD. Significance: ***P < 0.001 in comparison with the UVB-treated group. Each measurement was conducted in triplicate. CTR, control; OCM, *Oxya chinensis sinuosa* methanol extracts; UVB, ultraviolet B.

et al., 2019). We examined whether the *Oxya chinensis sinuosa* (OCM) extract helps recover ARPE-19 cells damaged by UVB. Our findings suggested that OCM treatment decreased the persistent cytotoxicity of UVB-induced cell damage in ARPE-19 cells (Fig. 1B). When treated with 2 mg/mL OCM, the cell viability of the UVB-treated group was comparable to that of the control group.

Effects of OCM on MAPK signaling pathway

MAPKs consist of the extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun NH2-terminal kinase (JNK), which are affected by intracellular or extracellular factors, and the signaling transduction is activated through protein phosphorylation (Cao *et al*., 2012). Upon activation of these pathways, transcription factors translocate into the nucleus (Kim *et al*., 2022b). MAPKs are activated by ultraviolet irradiation, oxidative stress, and pro-inflammatory cytokines, including IL-1β and TNF-α. Moreover, they are involved in the expression of various genes related to inflammation, cell proliferation, and apoptosis, increasing cell death and toxicity (Silván *et al*., 2016). In particular, UV-activated signal transduction pathways are mediated through MAPKs, including ERK, JNK, and p38 kinase

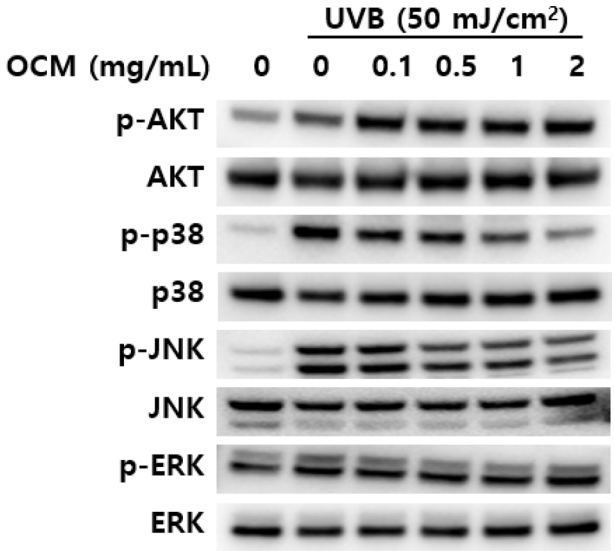

Fig. 2. Effect of OCM on MAPKs and AKT pathway in UVB-radiated ARPE-19 cells. Western blotting for MAPKs and phosphor-Akt in ARPE-19 cells pretreated with OCM (0, 0.1, 0.5, 1, and 2 mg/mL) for 1 h, irradiated with UVB (50 mJ/cm^2), and incubated for 24 h. AKT, protein kinase B; p38 MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinases; ERK1/2, extracellular regulated kinase 1/2; OCM, *Oxya chinensis sinuosa* methanol extracts; UVB, ultraviolet B.

(Bode and Dong, 2003). Western blot analysis showed that the phosphorylation of MAPK was increased by UVB irradiation in ARPE-19 cells (Fig. 2). In other studies, phosphorylation was shown to increase when mice were UVB-irradiated (Im *et al*., 2019). Interestingly, MAPK phosphorylation was markedly decreased by OCM. Also, it inhibited the expression of inflammatory cytokines, such as IL-6, IL-1β, and TNF-α, by potentially regulating MAPK signaling. Our research demonstrated that OCM suppressed the intracellular levels of phosphorylated p38 and JNK; however, phosphorylated ERK increased slightly in UVB-induced ARPE-19 cells. ERK activation is crucial for cell survival, growth, and differentiation, whereas the activation of JNK and p38 is strongly associated with apoptosis (Bode and Dong, 2003; Li *et al*., 2002; Zwang and Yarden, 2006). Based on our study, we believe that treatment with OCM helped the proliferation of ARPE-19 cells damaged by UVB irradiation via the activation of ERK signaling and attenuation of p38 and JNK signaling. Therefore, OCM regulates cell survival and apoptosis by modulating MAPKs.

The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) pathways are related to cell viability. These pathways activate various intracellular signaling pathways, including those involved in cell proliferation, growth, and angiogenesis (Carnero, 2010). In particular, Akt is involved in survival-related proliferation. Moreover, Akt blocks apoptosis by downregulating pro-apoptotic proteins, such as Bcl-2 and caspase-9, via the PI3K/Akt pathway (Osaki *et al*., 2004). Our results demonstrate the effect of the OCM extract on Akt signaling in UVB-damaged ARPE-19. Our study demonstrates that OCM treatment increases the expression of phosphorylated Akt in a dose-dependent manner (Fig. 2). Our results suggest that OCM inhibits intracellular apoptosis and activates pathways related to cell growth and proliferation, thus promoting the recovery of UVB-damaged ARPE-19 cells.

Effects of OCM on molecular marker of cell cycle regulators

Cell cycle regulation is modulated by cyclins, cyclin-dependent kinases (CDKs), and their inhibitors (Sheer and McCormick, 2002). Cyclin-dependent kinase inhibitors such as p21 and p27 are important regulators of the cell cycle (Hao *et al*., 2016). P21 is a p53-inducible protein that facilitates DNA repair, differentiation, and cell cycle arrest (Klopfleisch and Gruber, 2009). Both the loss of p21 expression and over-

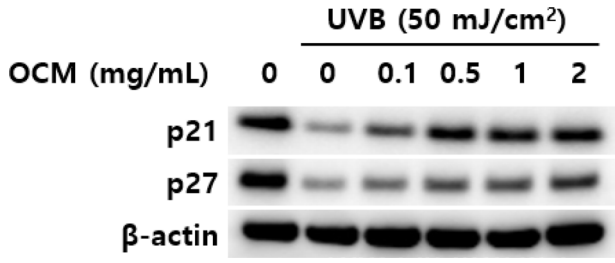

Fig. 3. Effect of OCM on cell cycle regulatory proteins in UVB-radiated ARPE-19 cells. Western blotting for cell-cycle-related proteins in ARPE-19 cells pretreated with OCM (0, 0.1, 0.5, 1, and 2 mg/mL) for 1 h, stimulated by UVB (50 mJ/cm^2), and incubated for 24 h. OCM, *Oxya chinensis sinuosa* methanol extracts; UVB, ultraviolet B.

activation are associated with cancer progression (Abukhdeir and Park, 2008). For example, p21 and p27 are downregulated in breast cancer (Hseu *et al*., 2008). Similar to p21, p27 promotes cell cycle progression by interacting with cyclins and CDKs. Mutations in these proteins can lead to uncontrolled cell proliferation (Kiyokawa *et al*., 1996). Loss of the p27 protein has been demonstrated in 60% of human carcinomas (Viglietto *et al*., 2002). p21 depletion significantly enhances UVB-induced apoptosis, and its rescue reduces apoptosis in keratinocytes. Increased p21 expression enhances Akt activation, which protects the cells from apoptosis (Lei *et al*., 2010). Notably, Akt regulates the phosphorylation of p21 and p27 and contributes to cell proliferation via cell cycle progression. Hao reported that when ARPE-19 cells were treated with resveratrol, the expression of p21 and p27 markedly increased, resulting in cell proliferation (Hao *et al*., 2016).

Among various natural products, *Oxya chinensis sinuosa* has recently been registered as a food in the Korean Food Standards Codex of the Ministry of Food and Drug Safety (MFDS) (Kim *et al*., 2016). A recent study reported that *Oxya chinensis sinuosa* protects against UVB damage, but the specific molecular mechanism is unknown. To determine whether OCM treatment induces ARPE-19 cell proliferation under UVB irradiation, the effect of OCM on the expression of cell cycle regulatory molecules, including p21 and p27, was examined using western blotting. As shown in Fig. 3, OCM treatment (0.1, 0.5, 1, and 2 mg/mL) for 24 h protected the UVB-damaged cells and increased the expression of p21 and p27. Our results suggest that OCM increases the expression of p21 and p27 and the phosphorylation of Akt, which protects cells from apoptosis upon UVB irradiation, resulting in the proliferation of ARPE-19 cells.

Effects of OCM on mTOR/S6K1/S6 pathway

The mammalian target of rapamycin (mTOR) is a phosphoinositide 3-kinase (PI3K)-related kinase family that plays a central role in cell growth and survival via mTOR complex1 and mTOR complex2 (Laplante and Sabatini, 2009; Wang *et al*., 2022). Some reports have suggested that overactivation of mTOR is involved in tumorigenesis (DeBerardinis *et al*., 2008; Fruman and Rommel, 2014; Strimpakos *et al*., 2009). Furthermore, the PI3K/Akt/mTOR pathway is important for cellular nutrient and energy metabolism (Fernandes and Demetriades, 2021).

Normally, retinal cells are sensitive to ROS due to their exposure to light, and ROS is related to PI3K/Akt/mTOR pathway (Wang *et al*., 2022). PI3K/Akt signaling regulates the mTOR pathway, which regulates cell growth. Furthermore, the activity of ribosomal p70S6 kinase (S6K1) is regulated by the mTOR pathway (Brown *et al*., 1995; Burnett *et al*., 1998; Oshiro *et al*., 2007). Especially, S6K1 is activated by mTORC1. The phosphorylation of S6 is used as a marker of the degree of mTORC1 activation. Thus, Akt, mTOR, and S6K1 signaling are related to cell proliferation and differentiation.

Western blotting was performed to observe the expression patterns of the mTOR, S6K1, and S6, associated with cell growth and proliferation, following OCM treatment. Our results showed that UVB-induced mTOR phosphorylation was not significantly

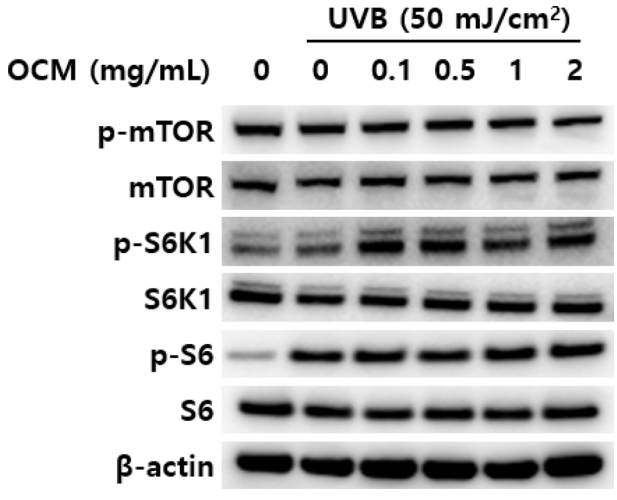

Fig. 4. Effect of OCM on mTOR signaling proteins in UVB-radiated ARPE-19 cells. Western blotting for mTOR signaling proteins in ARPE-19 cells pretreated with OCM (0, 0.1, 0.5, 1, and 2 mg/mL) for 1 h, stimulated by UVB (50 mJ/cm^2), and incubated for 24 h. OCM, *Oxya chinensis sinuosa* methanol extracts; UVB, ultraviolet B; mTOR, mammalian target of rapamycin; S6K1, ribosomal protein S6 kinase beta-1; S6, ribosomal protein s6.

altered by OCM treatment and demonstrated an increasing tendency of phosphorylated S6K1, which in turn affects phosphorylated-S6 (Fig. 4). However, phosphorylated-mTOR, S6, and S6K1 by UV irradiation showed a tendency to decrease upon treatment of HaCaT keratinocytes with deep water (Lee *et al*., 2019).

Generally, the phosphorylation of S6K1 and S6 is controlled by mTOR (Lee *et al*., 2019). Decreased S6 phosphorylation inhibits proliferation and migration of human retinal capillary endothelial cells (Wang *et al*., 2022). Photocarcinogenesis in skin cells is characterized by excessive cell proliferation and reduced apoptosis. As cells become cancerous due to UVB exposure, the phosphorylation of mTOR and S6K1 increases, leading to cell proliferation. This phenomenon can be inhibited using natural products and drugs. Considering the results of our study, ARPE-19 cells were damaged by UVB, and OCM treatment activated Akt phosphorylation and increased S6K1 and S6 phosphorylation, promoting cell proliferation and protecting the cells from damage.

Effects of OCM on ROS production in UVB-radiated ARPE-19 cells

A ROS assay was performed to determine ROS generation in UVB-radiated ARPE-19 cells treated with OCM. We confirmed that the production of intracellular ROS rapidly increased after UVB irradiation. When ARPE-19 cells were irradiated with UVB for 24 h, the concentration of intracellular ROS decreased in the presence of various concentrations (0.5, 1, and 2 mg/mL) of OCM. The relative ROS content of the cells irradiated with UVB for 24 h was 300%, which was three times that of the control group. When these UVB-damaged ARPE-19 cells were treated with 0.5, 1, and 2 mg/mL OCM, the ROS content was 220%, 196%, and 160%, respectively (Fig. 5). OCM inhibits ROS levels in ARPE-19 cells after UVB irradiation. This is consistent with the report that *Oxya chinensis sinuosa* displays excellent antioxidant properties (Kim *et al*., 2015).

Several studies have reported that the ingredients of fruits and green tea exert protective effects on retinal cells damaged by UVB (Cao *et al*., 2012; Hsieh *et al*., 2018; Xu *et al*., 2010). Excessive ROS production can inhibit cell proliferation and cause pathological symptoms of retinal degenerative diseases such as AMD. The upregulation of ROS in the mitochondria can cause apoptosis (Kadenbach *et al*., 2009). In addition, UVB irradiation damages cells by inducing DNA damage (Sparrow *et al*., 2000). Polyphenols, abundant in fruits and vegetables, have high antioxidant activity, inhibit DNA fragmentation, and restore damaged cells from low-concentration radiation. Polyphenols repair damaged DNA and increase the expression of the DNA repair enzyme poly ADP-ribose polymerase (PARP) (Xu *et al*., 2010). Polyphenols have cell-enhancing effects as they increase the activity of DNA repair and antioxidant enzymes.

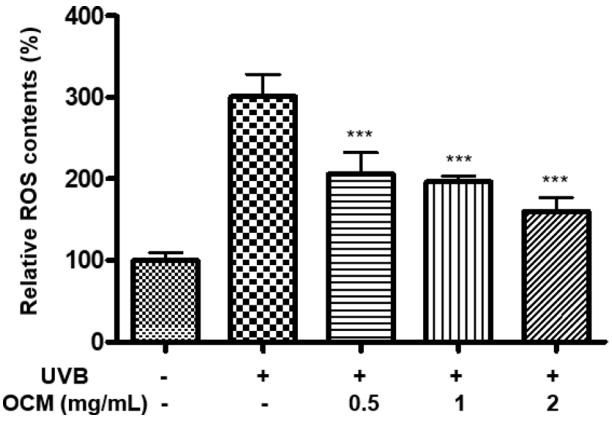

Fig. 5. Effect of OCM on ROS production in UVB-radiated ARPE-19 cells. ROS levels in ARPE-19 cells pretreated with various concentrations of OCM (0.5, 1, and 2 mg/mL), irradiated with UVB (50 mJ/cm^2), and incubated for 24 h. Data are the mean ± SD. Significance: ***$P < 0.001$ in comparison with the UVB-treated group. Each measurement was conducted in triplicate. OCM, *Oxya chinensis sinuosa* methanol extracts; UVB, ultraviolet B; ROS, reactive oxygen species.

Oxya chinensis sinuosa, an edible insect used in our study, has high DPPH radical scavenging activity and contains polyphenols with high antioxidant capacity (Kim *et al*., 2015). Polyphenols can prevent oxidation, act as antioxidants *in vivo*, maintain health, and prevent disease (Whang *et al*., 2001). The polyphenols contained in *Oxya chinensis sinuosa* are thought to reduce ROS in retinal cells damaged by UVB. UVB increases ROS production and simultaneously increases the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH), which neutralize ROS before cell damage (Birben *et al*., 2012). In addition, *Oxya chinensis sinuosa* activates antioxidant enzymes, such as SOD and CAT, in mice (Im *et al*., 2019). In this study, we conclude that OCM enhanced antioxidative activity and attenuated ROS production in UVB-induced ARPE-19 cells. Therefore, *Oxya chinensis sinuosa* helps repair damaged cells, inhibits apoptosis, and increases the cell survival rate, thereby protecting retinal cells from UV damage. In conclusion, OCM could be used as a

preventive, therapeutic, and functional superfood against UVB-induced eye damage.

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