Black rice (*Oryza sativa* L.) extract modulates ultraviolet-induced expression of matrix metalloproteinases and procollagen in a skin cell model

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Received December 29, 2016; Accepted February 13, 2018

DOI: 10.3892/ijmm.2018.3508

Abstract. Exposure of the skin to ultraviolet (UV) radiation causes extracellular matrix (ECM) collapse in the dermis, owing to an increase in matrix metalloproteinase (MMP) production in both the epidermis and dermis, and a decrease in type I collagen expression in the dermis. Recently, black rice (*Oryza sativa* L.) was reported to have a wide range of pharmacological effects in various settings. However, the effects of black rice extract (BRE) on UV-irradiated skin cells have not yet been characterized. BRE treatment did not affect cell morphology and viability of HaCaT and human dermal fibroblasts (HDF). We demonstrated that BRE downregulated basal and UV-induced MMP-1 expression in HaCaT cells. Furthermore, BRE significantly increased type I procollagen expression, and decreased MMP-1 and MMP-3 expression in UV-irradiated HDF. The underlying mechanisms of these results involve a decrease in p38 and c-Jun N-terminal kinase activity, and suppression of UV-induced activation of activator protein-1 (AP-1). BRE reduced UV-induced reactive oxygen species production in HaCaT cells in a dose-dependent manner. Indeed, mass spectrometry revealed that BRE contained antioxidative flavonoid components such as cyanidin-3-O-β-D-glycoside and taxifolin-7-O-glucoside. These findings suggest that BRE attenuates UV-induced ECM damage by modulating mitogen-activated protein kinase and AP-1 signaling, and could be used as an active ingredient for preventing photoaging of the skin.

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Key words: black rice, matrix metalloproteinases, type I procollagen, reactive oxygen species, activator protein-1, mitogen-activated protein kinase

Introduction

During an individual's lifetime, human skin is continuously exposed to harmful environmental factors, such as ultra violet (UV) light. Even though UV light stimulates the synthesis of vitamin D (1), which has beneficial effects in terms of regulating the immune system and calcium homeostasis, excessive UV exposure can cause oxidative damage (2,3), immune suppression (4), collagen degradation (5,6), photoaging, and skin cancer (7-9). UV irradiation induces the synthesis of matrix metalloproteinases (MMPs) in the skin (5,6,10). MMPs are a family of zinc-dependent endopeptidases that play a major role in extracellular matrix (ECM) degradation, including collagen fibers. More specifically, UV induces activation of mitogen-activated protein kinase (MAPK) components, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK (p38), which in turn regulate activator protein-1 (AP-1), leading to increased MMPs expression, and decreased collagen production (10,11).

Black rice is a grain from the species *Oryza sativa* L. var. *japonica*. It contains various polyphenolic compounds, such as anthocyanins (12), which have antioxidative activity. It has been reported that black rice extract (BRE) has many pharmacological effects, including antioxidant (13) and anti-inflammatory (14-16) activity. Furthermore, black rice anthocyanin extracts have been studied in various types of cancer, and have been shown to suppress cancer cell invasion (17), metastasis (18), and angiogenesis (19,20). Additionally, black rice bran inhibits tyrosinase activity in cell-free *in vitro* systems, suggesting its potential as a melanogenesis inhibitor (21). Germinated black rice also enhances hyaluronan production in HaCaT keratinocytes (22). However, it remains unknown whether BRE prevents UV-induced ECM alteration. Therefore, the aim of the present study was to

investigate the effects of BRE in UV-irradiated HaCaT cells, and human dermal fibroblasts (HDF), by analyzing the expression of MMPs and collagen, and generation of reactive oxygen species (ROS), as well as the underlying signaling pathways involved.

Materials and methods

Reagents and antibodies. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 2'7'-dichlorofluorescein diacetate (DCF-DA), *N*-Acetyl-L-cysteine (NAC) and phosphatase inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protease inhibitor cocktail tablets were purchased from Roche Applied Science (Penzberg, Germany). Anti-MMP-1 antibody (Oncogene Research Products, Boston, MA, USA) and monoclonal anti-procollagen type I N-terminal extension peptide (SP1.D8) antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) were used. Phospho- ERK1/2, JNK, p38 MAPK, and c-Jun, and the total ERK1/2, JNK, p38 MAPK, and c-Jun were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-c-Fos, β-actin and lamin B were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Preparation of BRE. BRE was prepared, and provided by the Korea Food Research Institute (Seongnam-si, Korea). Briefly, whole grains of black rice were extracted with 50% ethanol using a microwave system. The ethanol solvent was then evaporated using a freeze dryer (yield: 2.68% of dry wt). Stock solutions of BRE were prepared by dissolving the powder in DMSO, and stored at -20˚C. The final concentration of DMSO in the medium was kept below 0.1%.

Cell line and culture conditions. Two types of cells were used in this study: HaCaT cells, which is a spontaneously transformed human keratinocyte cell line and primary HDF, isolated from the foreskin of young volunteers (aged 10-19 years). The present study was approved by the Institutional Review Board (IRB no. 1101-116-353) at Seoul National University Hospital and conducted according to the Declaration of Helsinki. All subjects provided written informed consent. HaCaT (immortalized human keratinocytes) and HDF between fifth to fifteenth passages were used for all experiments. The HaCaT cells and HDF were maintained in Dulbecco's modified Eagle's medium (DMEM; Welgene, Daegu, Korea) containing 10% fetal bovine serum (FBS; Welgene) and penicillin/streptomycin (400 U/ml, 50 g/l) at 37˚C in a humidified condition with 5% CO_2.

UV irradiation and BRE treatment. The cells were starved of media for 24 h, and washed twice and replaced with PBS prior to UV irradiation. Philips TL 20W/12 RS fluorescent sun lamps with an emission spectrum between 275 and 380 nm (peak, 310-315 nm) were used as the UV source, as previously described (23). To block UVC (<290 nm) wavelengths, Kodacel filter (TA401/407; Kodak, Rochester, NY, USA) was placed 2 cm in front of UV lamp. Waldmann UV meter (model 585100, Villingen-Schwenningen, Germany) was used to measure UV irradiance. Immediately after UV irradiation (HaCaT cell; 50 mJ/cm^2, HDF; 100 mJ/cm^2), PBS was replaced with serum-free media with or without BRE for indicated time periods.

MTT assay. The cell viability was measured by MTT assay. The HaCaT ($2x10^4$ cells/well) and HDF ($5x10^4$ cells/well) cells were seeded into 96-well plates and were maintain until 80% confluency, and then cells were UV-irradiated or non-irradiated. The cells were post-treated with various concentrations of BRE for 48 h. After incubation, MTT solution (0.5 mg/ml in PBS) was added into culture plates and further incubated for 4 h. The formazan crystals were dissolved in DMSO after culture media was removed and were quantified at 570 nm using ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA).

Nuclear protein extraction. HaCaT cells were scraped from culture dishes, suspended in ice-cold cytoplasmic extraction buffer (20 mM Tris, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5% Nonidet P-40, protease inhibitor cocktail) and incubated on ice for 10 min. Subsequently, nuclear pellets were collected by centrifugation at 6,000 rpm for 5 min at 4˚C and were re-suspended with extraction buffer (cytoplasmic extraction buffer with 400 mM NaCl, 5% glycerol) and incubated on ice for 30 min with shaking. The pellets were centrifuged at 12,000 rpm for 10 min at 4˚C to obtain the nuclear fraction.

Western blot analysis. To determine the amount of MMPs and procollagen secreted into the culture medium, equal amounts of culture medium were separated using polyacrylamide gel electrophoresis, and then the separated proteins were electro-transferred onto a PVDF membrane (Amersham, Buckinghamshire, UK). The membrane was blocked with blocking buffer, and then incubated with appropriate primary antibodies. After incubation, the membranes were washed, then incubated with horseradish peroxidase-conjugated secondary antibody. Enhanced chemiluminescence detection system (GE Healthcare) was used to visualize the protein bands. To analyze MAPK signaling, HaCaT cells were lysed with RIPA lysis buffer (Millipore Corp., Billerica, MA, USA) containing protease and phosphatase inhibitors. Furthermore, to analyze AP-1 activation, the nuclear proteins were extracted from the cells as described above.

Reverse transcription and real-time quantitative PCR (RT-qPCR). RNAiso Plus (Takara Bio Inc., Shiga, Japan) was used to isolate the total RNA from the HaCaT cells and HDF (24). Isolated total RNA (1 μg) was used as templates for cDNA synthesis using a First Strand cDNA Synthesis kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions, which was then used for real-time PCR. The PCR cycling conditions were 50˚C for 2 min and 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Quantitation of PCR reactions was performed by 7500 Real-time PCR System (Applied Biosystems Life Technologies, Foster City, CA, USA) with the SYBR Premix Ex Taq II kit (Takara Bio Inc.) using suitable primers as follows: hMMP-1 forward, 5'-AAGCGTGTGACAGTAAGCTA-3' and reverse, 5'-AACCGGACTTCATCTCTG-3', hMMP-3 forward, 5'-CTCACAGACCTGACTCGGTT 3' and reverse,

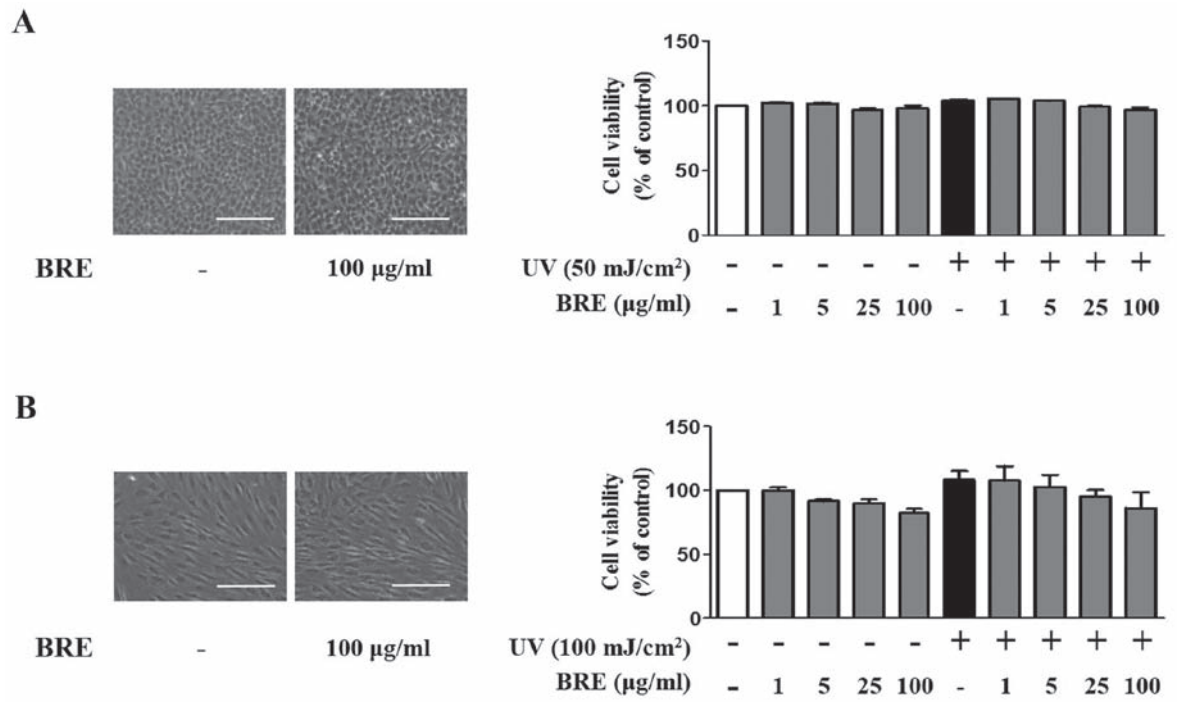

Figure 1. Effects of BRE and UV irradiation on the cell viability in HaCaT cells and HDF. Cell morphology and viability were examined in (A) HaCaT cells and (B) HDF treated with the indicated concentration of BRE without or with UV irradiation (50 mJ/cm^2 and 100 mJ/cm^2 for HaCaT cells and HDF, respectively). The optical densities were measured and the percentage of viability was calculated. Values are mean ± SEM of four independent experiments. Scale bar indicates 200 μm. BRE, black rice extract; UV, ultraviolet; HDF, human dermal fibroblasts.

5'-CACGCCTGAAGGAAGAGATG-3', h type I procollagen forward, 5'-CTCGAGGTGGACACCACCCT-3' and reverse, 5'-CAGCTGGATGGCCACATCGG-3' h36B4 forward, 5'-TCGACAATGGCAGCATCTAC-3' and reverse, 5'-TGATGCAACAGTTGGGTAGC-3'. Relative mRNA expression was analyzed using the $2^{-\Delta\Delta Ct}$ methods, and 36B4 was used as an internal control. Data are expressed as the fold number of gene expression.

Measurement of ROS production. To quantify intracellular oxidative stress, the cells were pretreated with NAC (2 μM) or BRE for 1 h, and then washed with PBS. After washing, the cells were labeled with 20 mM DCF-DA at 37˚C for 1 h. To induce ROS generation, the cells were exposed to UV irradiation at a dose of 50 mJ/cm^2. The cells were then washed twice with PBS, and the relative ROS level was determined using a Victor3 multilabel plate reader (PerkinElmer, Inc., Waltham, MA, USA), with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. At the same time, fluorescence images were captured under a fluorescence microscope (DMIL; Leica Microsystems GmbH, Wetzlar, Germany) with a 20x0.75 NA objective lens.

Ultra performance liquid chromatography-quadrupole-time of flight (UPLC-Q-TOF) mass spectrometry (MS). To identify black rice metabolites, metabolites from 50% ethanolic extract of black rice were analyzed by an ultra performance liquid chromatography-quadrupole-time of flight (UPLC-Q-TOF) MS (Waters Corp., Milford, MA, USA). The extract was injected into an Acquity UPLC BEH C18 column (2.1x100 mm, 1.7 μm; Waters Corp.) at a column temperature of 40˚C. Mobile phase consisted of water with 0.1% formic acid (FA) and acetonitrile with 0.1% FA at a flow rate of 0.35 ml/min for 9 min. The eluents were ionized by electrospray ionization (ESI) with positive or negative mode and analyzed using a Q-TOF MS. The scan range of TOF MS data was from 50 to 1,500 m/z with a scan time of 0.2 sec. The capillary voltage was set at 3 or 2.5 kV for positive mode and negative mode, respectively, while the sample cone voltage was 40 V. The desolvation flow rate was 900 l/h at a temperature of 400˚C and source temperature set to 100˚C. Leucine-enkephalin ([M+H] = m/z 556.2771) was used as a reference for lock mass at a frequency of 10 sec. The MS/MS spectra were obtained using collision energy ramps from 20 to 45 eV. Metabolites were identified by Unifi software with various LC/MS databases.

Statistical analysis. The results are presented as means ± SEM, and statistical analyses were performed using a Student's t-test or one-way ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of BRE on cell viability in HaCaT and HDF. To examine the effect of BRE on skin cells, we first tested the cytotoxicity of BRE using an MTT assay. BRE (5-100 μg/ml) did not influence the viability of HaCaT cells, with or without UV irradiation (Fig. 1A). A slight, but not significant, reduction in the viability of HDF was observed after treatment with 5-100 μg/ml of BRE (Fig. 1B). Under the same experimental conditions, 100 μg/ml of BRE did not change cell morphology. These results indicate that BRE is not significantly cytotoxic to HaCaT cells or HDF. Therefore, 5-100 μg/ml of BRE was used for the subsequent experiments.

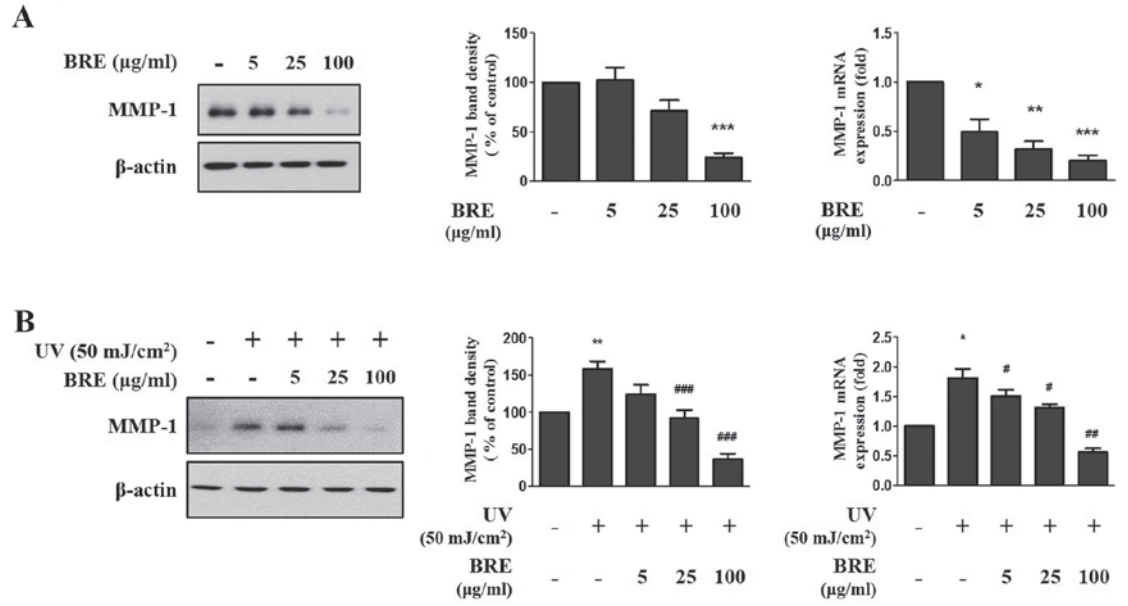

Figure 2. The inhibition effect of BRE on MMP-1 expression in HaCaT cells. HaCaT cells were post-treated with the indicated concentration of BRE for 48 h, (A) without or (B) with UV irradiation (50 mJ/cm^2). The MMP-1 protein level in the conditioned medium was investigated by western blot analysis and relative band density was analyzed using ImageJ software. Actin was used as endogenous control. The MMP-1 mRNA level was analyzed by RT-qPCR, respectively. 36B4 mRNA was used to normalize each mRNA expression level. The bar graphs show the mean ± SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the non-irradiated control cells. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 compared with the UV-irradiated control cells. BRE, black rice extract; MMP, matrix metalloproteinase; UV, ultraviolet; RT-qPCR, reverse transcription-quantitative PCR.

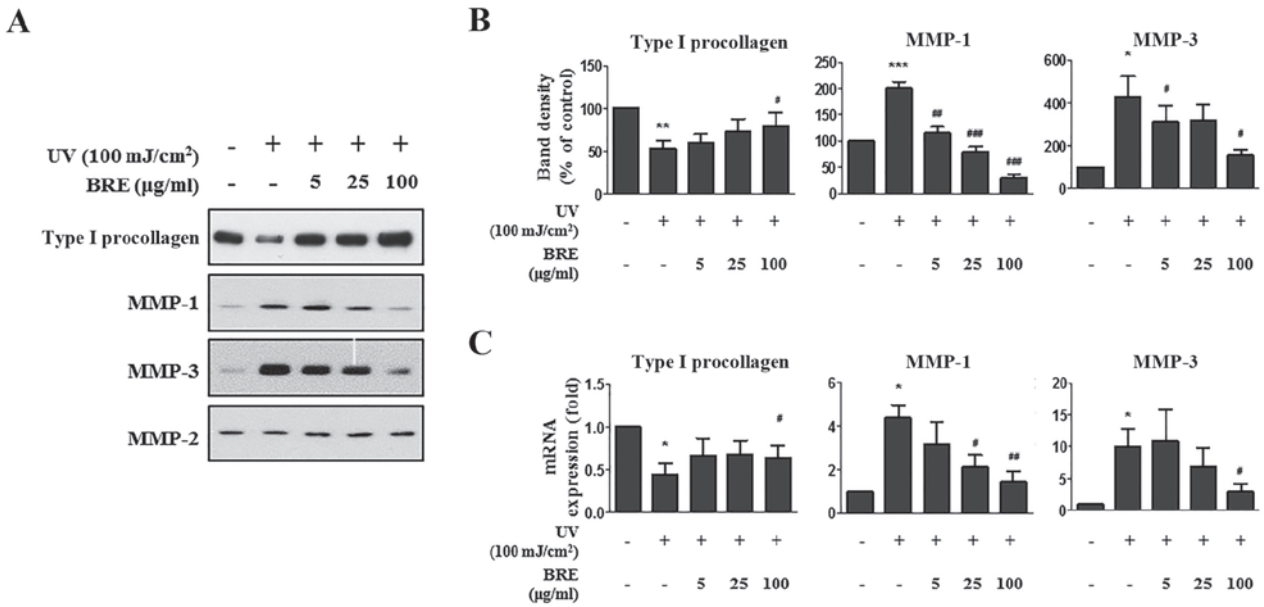

Figure 3. The effect of BRE on MMPs and procollagen expression in HDF. HDF were exposed to 100 mJ/cm^2 of UV irradiation and post-treated with BRE. The MMPs and procollagen expression levels were then examined. (A) The western blot shows the MMP-1, MMP-2, MMP-3, and procollagen protein levels in the conditioned medium from the UV-irradiated HDF treated with BRE for 48 h. (B) Relative band density was analyzed using ImageJ software. MMP-2 was used as endogenous control. (C) The MMP-1, MMP-3, and procollagen mRNA levels in the UV-exposed HDF, following treatment with the indicated concentration of BRE for 48 h, were assessed using RT-qPCR. 36B4 mRNA was used to normalize each mRNA expression level. The graphs show the mean ± SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 compared with the non-irradiated control cells. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 compared with the UV-irradiated control cells. BRE, black rice extract; MMP, matrix metalloproteinase; HDF, human dermal fibroblasts; UV, ultraviolet; RT-qPCR, reverse transcription-quantitative PCR.

BRE inhibits MMP-1 expression in HaCaT cells. To determine the effect of BRE on MMP-1 production in HaCaT cells, the cells were post-treated with BRE for 48 h, with or without UV irradiation. The basal level of MMP-1 expression was decreased to 24% in 100 μg/ml BRE treated cells compared with non-treated cells by western blot analysis. Additionally, MMP-1 mRNA level also showed similar result. BRE reduced basal MMP-1 expression in a dose-dependent manner (0.5±0.2, 0.3±0.1, and 0.2±0.1-fold, respectively) (Fig. 2A). Furthermore, BRE ameliorated UV-induced MMP-1 expres-

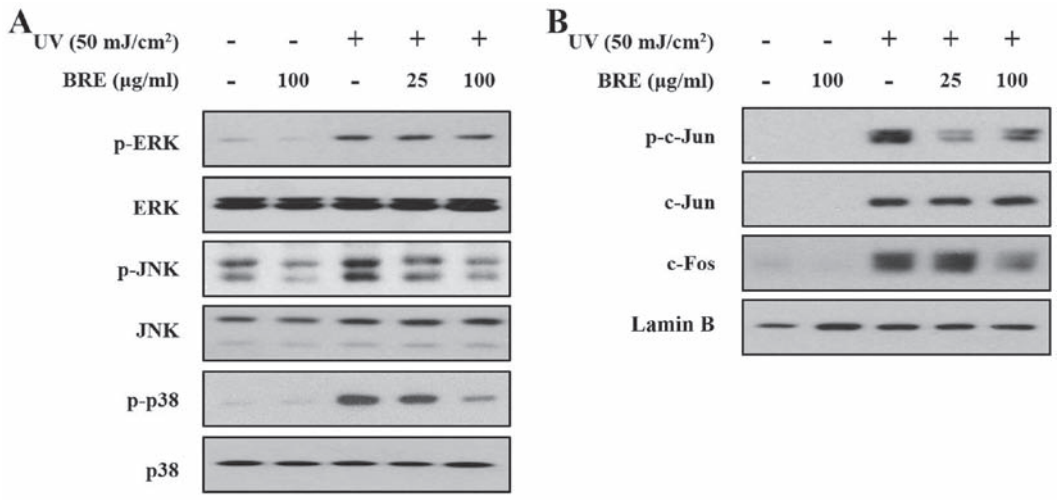

Figure 4. The effect of BRE on UV-induced MAPK signaling, and AP-1 (c-Jun/c-Fos) activity in HaCaT cells. The effects of BRE on UV-induced MAPK signaling, and AP-1 activity were examined. The HaCaT cells were post-treated with the indicated concentration of BRE for 15 min, with or without UV irradiation (50 mJ/cm^2). (A) The phosphorylation status of ERK, JNK, and p38 was then assessed using western blot analysis. (B) UV-irradiated or non-irradiated HaCaT cells were also post-treated with BRE for 4 h, and the nuclear levels of phospho-c-Jun, c-Jun and c-Fos were analyzed. The blots shown are representative images of three independent experiments. BRE, black rice extract; UV, ultraviolet; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

sion (158% of control) in a concentration-dependent manner (25, and 100 μg/ml BRE showed 92 and 36% of control, respectively). The MMP-1 mRNA level was 1.8±0.3-fold higher in the UV-exposed cells than in the control cells. However, when the cells were treated with 5, 25, and 100 μg/ml BRE, the expression of MMP-1 was markedly suppressed (1.5±0.2, 1.3±0.1, and 0.5±0.1-fold, respectively) (Fig. 2B).

BRE suppresses UV-induced MMP-1 and MMP-3 expression, and decreased procollagen expression in HDF. To investigate the effect of BRE in UV-irradiated HDF, the cells were post-treated with BRE for 48 h after UV irradiation (100 mJ/cm^2). The amount of procollagen and MMPs (MMP-1, MMP-2, and MMP-3) secreted into the culture medium was analyzed, and the mRNA levels were analyzed using total RNA. The results showed that MMP-1 and MMP-3 secretion dramatically increased in the UV-irradiated HDF compared to that in the control cells (200 and 430% of the control, respectively), but treatment with 5, 25, or 100 μg/ml of BRE inhibited UV-induced MMP-1 (115, 80 and 31% of the control, respectively) and MMP-3 production (315, 320 and 155% of the control, respectively). Furthermore, UV-induced suppression of procollagen production (46% of control) was recovered in 100 μg/ml of BRE treated cells (80% of control) (Fig. 3A and 3B). The MMP-2 level was unaffected, and served as a loading control. The expression level of MMP-1 in the UV irradiated cells was 4.4±1.0-fold higher than that in the non-irradiated cells. However, treatment with 25 and 100 μg/ml BRE significantly reduced the MMP-1 expression level to just 2.1±1.0 and 1.5±0.8-fold higher than the control value, respectively. The MMP-3 expression level was also significantly upregulated by UV irradiation (10.0±4.9-fold). However, the cells treated with 100 μg/ml of BRE showed significantly inhibited MMP-3 expression (2.9±2.0-fold). Next, we investigated the change in procollagen production following UV and BRE treatment. While type I procollagen production was suppressed (0.4±0.3-fold) in the HDF following UV exposure, treatment with 100 μg/ml BRE increased the type I procollagen protein and mRNA levels (0.6±0.3-fold) in the HDF (Fig. 3C).

Effect of BRE on UV-induced MAPK signaling and AP-1 activity. To elucidate whether MAPK signaling pathways are involved in the attenuation of UV-induced MMP-1 expression in HaCaT cells, the cells were treated with the indicated concentrations of BRE for 4 h before UV irradiation (50 mJ/cm^2). As shown in Fig. 4A, UV irradiation significantly increased the phosphorylation of ERK, JNK, and p38 within 15 min. Post-treatment with BRE diminished the UV-induced phosphorylation of JNK and p38 in a dose-dependent manner, but ERK phosphorylation was unaffected.

AP-1 is a transcription factor composed of c-Jun and c-Fos subunits (10,25). Next, we analyzed c-Jun phosphorylation and c-Fos expression in nuclear extracts from the UV-irradiated HaCaT cells. The cells were irradiated with UV, and then incubated for 4 h with the indicated concentrations of BRE. Although UV exposure increased c-Jun and c-Fos expression, and c-Jun phosphorylation, BRE treatment suppressed c-Jun phosphorylation, and c-Fos expression in the nucleus (Fig. 4B).

BRE reduces UV-induced intracellular ROS generation. To determine whether BRE can reduce UV-induced ROS production, HaCaT cells were pre-treated with BRE or NAC for 1 h before UV irradiation (50 mJ/cm^2). The intracellular ROS levels measured using DCF-DA assay were 82.0±7.6% and 73.8±8.0% of the control value, following treatment with 25 and 100 μg/ml BRE, respectively. Furthermore, the UV-irradiated cells displayed dramatically increased ROS generation (234.0±9.6%) when compared with the control, whereas treatment with 25 and 100 μg/ml BRE significantly attenuated the intracellular ROS level to 183.0±10.2% and 169.2±5.9%, respectively (Fig. 5A). Under the same experimental conditions, a decrease in the ROS level following

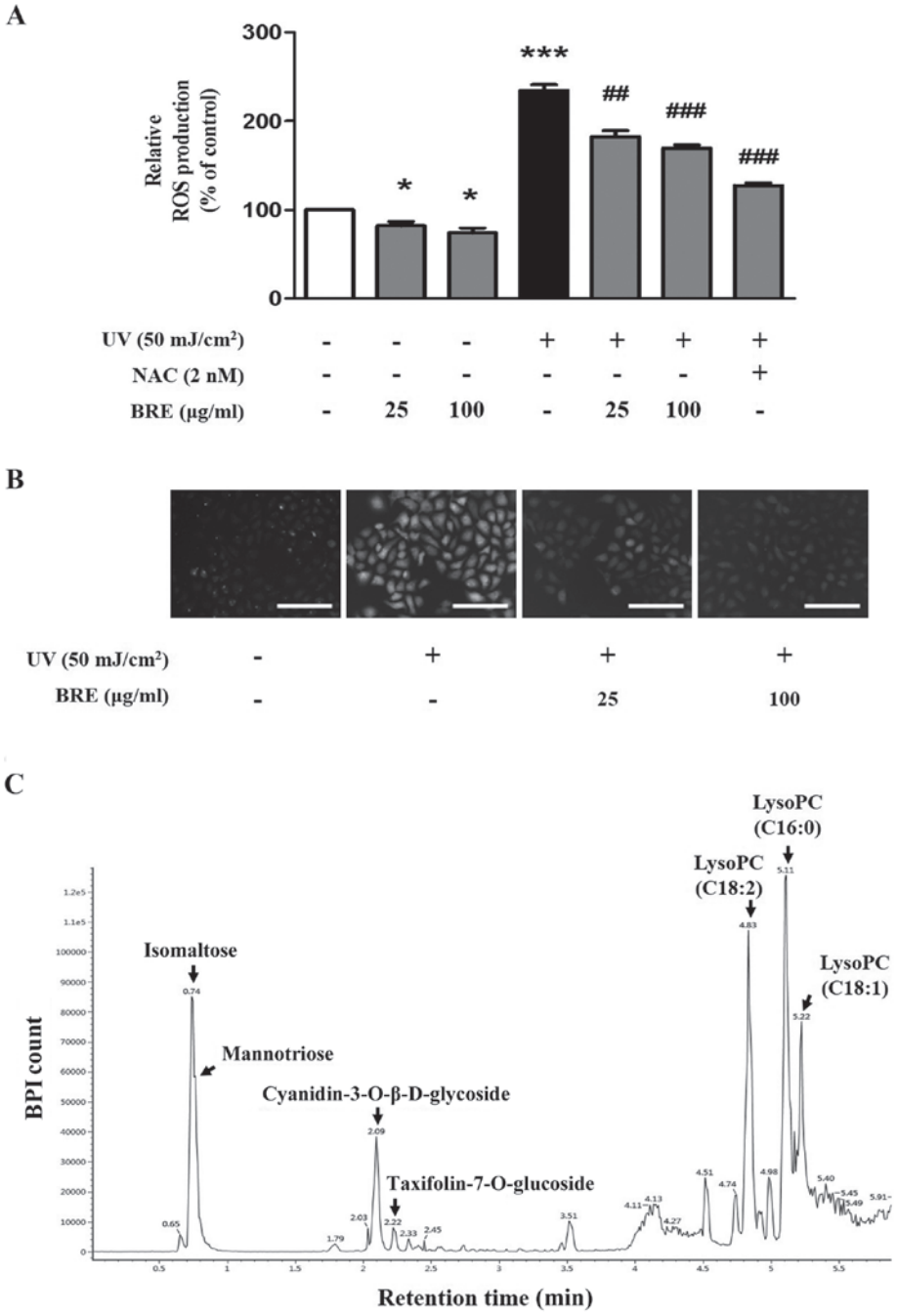

Figure 5. BRE reduced intracellular oxidative stress in HaCaT cells. The cells were pre-treated with BRE or NAC for 1 h before UV exposure, and the relative ROS levels were analyzed using DCF-DA. (A) The difference in DCF fluorescence generated from DCF-DA by intracellular ROS was measured using a multi-label plate reader, and (B) florescence microscopy. (C) BRE was analyzed using UPLC-Q-TOF MS profiles. The graph shows the mean ± SEM of at least three experiments. *P<0.05, ***P<0.001 compared with the non-irradiated control cells. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 compared with the UV-irradiated control cells. Scale bar indicates 200 μm. BRE, black rice extract; NAC, *N*-acetylcysteine; UV, ultraviolet; ROS, reactive oxygen species, DCF-DA, 2'7'-dichlorofluorescein diacetate.

BRE treatment was also observed using fluorescence microscopy (Fig. 5B). Analysis of the components in BRE using the optimized UPLC-Q-TOF MS method showed that polysaccharides such as isomaltose and mannotriose were detected at 0.74 min and lysophosphatidylcholine (LysoPC) components at 4.83, 5.11 and 5.22 min. The flavonoid components, cyanidin-3-O-β-D-glycoside (C3G), known as an anthocyanin and taxifolin-7-O-glucoside (T7G), were detected at 2.09 and 2.22 min, respectively (Fig. 5C). These results indicated that the photoprotective effect of BRE in skin cells may be attributed to antioxidant effects possibly by flavonoids.

Discussion

UV-induced DNA damage and ROS initiate various changes in expression of genes, which are involved in inflammatory responses that lead to pre-mature skin aging, known as 'photoaging' (26,27). Therefore, the application of antioxidants and/or anti-inflammatory compounds is thought to enhance

resistance to photoaging. Previous investigations have suggested that BRE has antioxidative effects in HepG2 cells and C57BL/6 mice, through the induction of superoxide dismutase and catalase activity (13). Furthermore, anthocyanins from black rice bran have been shown to have ROS-scavenging activity *in vitro* (28). BRE also relieves skin inflammation in a chemical-induced inflammatory mouse model (15). However, the precise effects and underlying mechanisms of BRE in UV-induced responses in skin cells are still unknown.

In the present study, we demonstrated that BRE has potent photoprotective effects against UV-irradiated changes in skin cells, possibly via ROS/MAPK signaling pathways. UV irradiation induces MMP expression, which leads to the breakdown of collagen, a dominant component of connective tissue (29,30). We demonstrated that BRE inhibits UV-induced MMP-1 expression in HaCaT cells, and MMP-1 and MMP-3 expression in HDF.

In the skin, collagen is necessary to maintain resiliency and strength, and decreased collagen expression is observed in photoaged skin (31,32). In the present study, UV-exposed HDF showed a reduced level of type I procollagen, which was recovered by BRE treatment. Our findings indicate that BRE could prevent or rescue UV-induced ECM alterations via a dual pathway involving inhibition of MMP expression and increase of procollagen expression.

Oxidative stress induced by UV irradiation is one of the pivotal triggers of MMP-1 and MMP-3 upregulation by both the AP-1 dependent and independent (p38 activity) pathways in HDF (33). Thus, reducing ROS levels could be an effective strategy to prevent photo-damage (26,27,34). In the present study, BRE significantly reduced intracellular ROS generation in HaCaT cells. In addition, BRE inhibited UV-induced AP-1 activity (c-Jun/c-Fos), and MAPK (JNK/p38) signaling, possibly owing to its antioxidative effects.

Botanical phenolic acids and flavonoids exert antioxidant effects and these are commonly used in the prevention of photoaging (35,36). Black rice contains abundant flavonoids and polyphenols, including cyanidin-3-O-glycoside chloride (C3G), peonidin-3-O-glucoside chloride, and ferulic acid (13,37). C3G is a typical anthocyanin, which shows antioxidative effects in various conditions (38-40). In addition, taxifolin has been reported to be detected in black rice bran (41) and has antioxidant properties (42,43). UPLC-Q-TOF MS result showed that BRE here contains considerable amounts of C3G and T7G. Therefore, the antioxidative and protective effect of BRE on UV-irradiated skin cells are thought to be attributed to flavonoids, such as C3G or T7G in BRE.

One of the most important protective mechanisms of flavonoids is their antioxidative ability through the activation of Nrf2-dependent genes. Under normal physiological conditions, inactive Nrf2 is located in the cytoplasm with its inhibitor Keap1. Under high ROS conditions, Nrf2 is released from Keap1 and moved into the nucleus, regulating the expression of downstream target genes via antioxidant response element (ARE) binding sites in the promoter (44). Flavonoids are potent inducers of the ARE/Nrf2/Keap1 signaling pathway, and the anthocyanin is the most prominent among the flavonoids (45,46). Thus, the antioxidant activity through Nrf2 activation by anthocyanin contained in BRE is also a possible mechanism.

In conclusion, BRE attenuates markers of photoaging including reduction of collagen and increases of MMPs in skin cells. The underlying mechanism involved in these beneficial effects could be the inhibition of ROS generation and AP-1 activation *in vitro*. Further study employing proteomic analysis by mass spectroscopy is warranted to determine other factors in addition to MMP-1 and collagen. Additionally, if stability, safety, and efficacy are demonstrated in human clinical studies, BRE could be used as a cosmetic ingredient for preventing photoaging of the skin.

Acknowledgements

Not applicable.

Funding

This research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea (grant nos. HI12C1723 and HI14C1277).

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MH, JSB and DHL designed the study and performed the experiments. HSS provided the black rice extract and analyzed UPLC-Q-TOP. JJB performed the cell viability assay and was involved in drafting the article. JHC had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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