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Morin Protects Normal Human Dermal Fibroblasts from Ultraviolet B-induced Apoptosis

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Ultraviolet B (UVB) irradiation causes skin diseases by inducing cellular oxidative stress, photoaging, and inflammation. This study aimed to investigate the protective effects of morin against UVB-induced oxidative stress in normal human dermal fibroblasts (NHDFs). Morin has been reported to be a potential therapeutic candidate for oxidative stress-mediated diseases, neurodegenerative diseases, and inflammation. Since morin has been identified as a potential antioxidant, we speculated that morin could alleviate UVB-induced apoptosis in NHDFs. Cell viability and intracellular reactive oxygen species (ROS) levels were measured using the MTT assay, H_2DCFDA, and the DHE staining method, respectively. Lipid peroxidation and protein carbonyl formation were tested using ELISA kits. DNA fragmentation and comet assay were used to assess DNA damage. Apoptotic bodies were analyzed using Hoechst 33342 staining and TUNEL assay. The expression of apoptosis-related proteins was examined using Western blot analysis. Morin showed a cyto-protective effect by scavenging UVB-induced ROS, increasing the expression of antioxidant-related proteins and inhibiting UVB-induced oxidative alterations such as lipid peroxidation, protein carbonylation, and DNA damage. Morin protects against UVB-induced cell apoptosis by inhibiting Bcl-2-associated X protein, caspase-9, and caspase-3 expression, while increasing the expression of the anti-apoptotic protein Bcl-2. These effects of morin were conferred through decreased phosphorylation of p38 and c-Jun N-terminal kinase 1/2. The results demonstrated that morin may be developed as a preventive/therapeutic drug to be used to prevent UVB-induced skin damage.

Key words : Apoptosis, fibroblasts, morin, reactive oxygen species, ultraviolet B

Introduction

Nearly 90~99% ultraviolet A (UVA) and 1~10% ultraviolet B (UVB) solar ultraviolet radiation can be observed on the earth's surface [1]. The skin, as the outermost organ, faces external stress under solar light; therefore, maintaining skin homeostasis is important to escape from solar light-induced skin cancer [4]. UVB is a well-known inducer of reactive oxygen species (ROS) that contributes to apoptosis, photo-aging, and inflammation [6, 17, 36]. UVB has also been reported to accelerate skin photo-aging by altering aging-related protein expression in dermal fibroblasts [9].

Flavonoids have attracted the interest of researchers owing to their oxygen radical scavenging and antioxidant properties [5]. Morin (2′,3,4′,5,7-pentahydroxyflavone) is a yellowish pigment belonging to the flavonoid family that exists in mills (*Prunus dulcis*), fig (*Chlorophora tinctoria*), and other Moraceae used as food and herbal medicine [31]. Furthermore, morin plays various roles in oxidative stress-induced diseases, including neurodegenerative diseases, myocardial ischemia-reperfusion injury, and airway inflammation [10, 18, 29].

Recently, many scientists have drawn attention to flavonoid-containing foods, which are recognized as important anti-cancer adjuncts [2]. In addition, recent studies have provided evidence that flavonoid products may reduce the risk of skin cancer [22]. In this study, we explored the therapeutic potential of morin in UVB-induced skin fibroblast damage by assessing its antioxidant properties. We also attempted to

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elucidate the cyto-protective mechanism of morin in UVB-induced apoptosis.

Materials and Methods

Cell culture and UVB irradiation

Normal human dermal fibroblasts (NHDF) (Lonza, Walkersville, MD, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotic-antimycotic solution at 37°C under 5% CO_2. The UVB source was a CL-1000M UV crosslinker (UVP, Upland, CA, USA), which was used to apply UVB radiation with an energy spectrum of 280~320 nm (peak intensity, 304 nm).

Cell viability

Cells were treated with morin (Sigma-Aldrich Co., St. Louis, MO, USA) at 5, 10, 25, 50, and 100 μM or exposed to UVB (0, 25, 50, 100, 150, and 200 mJ/cm^2) and incubated at 37°C for 24 hr. At this time, 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco Inc., Cleveland, OH, USA) was added to each well to obtain the formazan crystals, which were dissolved in dimethyl sulfoxide (DMSO). Finally, the absorbance was measured at 540 nm using a scanning multi-well spectrophotometer [20].

UV/visible light absorption

To check the light absorption spectra of morin, it was diluted in DMSO at a ratio of 1:1,000 (v/v). The solution was observed under UV light (range, 200~400 nm) using a Biochrom Libra S22 UV/visible light spectrophotometer (Biochrom Ltd., Cambridge, UK).

Intracellular H_2O_2 detection

The 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, OR, USA) method was used to detect intracellular H_2O_2 level in NHDF cells. For H_2O_2 fluorescence detection, cells were plated on 96 well plate, incubated for 16 hr, and then exposed with UVB (0, 25, 50, 100, 150, and 200 mJ/cm^2) alone or co-cultured with morin (0,5, 10, 25, 50, and 100 μM) followed by exposure to UVB (100 mJ/cm^2). After incubation for 30 min, cells were treated with 25 μM H_2DCFDA (Molecular Probes, Eugene, OR, USA) dye and incubated for another 30 min. DCF fluorescence was measured with a PerkinElmer LS-5B spectrofluorometer (Waltham, MA, USA). For image analysis, cells were seeded onto a chamber glass slide at 0.6×10^5 cells/well and cultured for 16 hr. The cells were incubated for 1 hr with morin (25 μM) and exposed with UVB (100 mJ/cm^2). H_2DCFDA (100 μM) was added to each well, and the stained cells were assessed under a FV1200 laser scanning confocal microscopy (Olympus, Tokyo, Japan) [24].

Measurement of intracellular superoxide anion radical level

To detect superoxide anion radical level in the cells, we estimated the oxidation of dihydroethidium (DHE). The cells were treated with morin and/or UVB and incubated with DHE (10 μM) for 30 min. After incubation, fluorescence intensity was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The intensity of emission in the red range was assessed based on histograms generated using CellQuest Pro 5.1 software (Becton-Dickinson, Bedford, MA, USA) and ModFit LT 3.2 software (Verity Software House, Topsham, ME, USA).

Western blot analysis

Cells treated with morin and/or UVB were harvested. Then, the cells were washed once with ice-cold phosphate-buffered saline and lysed using lysis buffer. The supernatant containing proteins was collected, and protein concentrations were determined. Next, the supernatant was divided into aliquots, which were subjected to electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel. The separated proteins were transferred onto nitrocellulose membranes and incubated with the primary and secondary antibodies. Protein bands were obtained with an X-ray film using the enhanced chemiluminescence Western blotting detection kit (Amersham, Buckinghamshire, UK). The following antibodies were used: anti-actin (sc-8432), anti-Bcl-2 (sc-7382), Bcl-2-associated X protein (Bax; sc-7480), anti-catalase (sc-271803), anti-CuZn superoxide dismutase (CuZnSOD) (sc-17767), anti-glutamatecysteine ligase catalytic subunit (GCLC) (sc-390811) (Santa Cruz Biotechnology, Dallas, TX, USA), anti-caspase-3 (#9662), anti- caspase-9 (#9504), anti-heme oxygenase 1 (HO-1) (#70081), anti-phosphorylated c-Jun N-terminal kinase (JNK)1/2 (#9251), and anti-phosphorylated p38 (#4511) (Cell Signaling Technology, Beverly, MA, USA).

Lipid peroxidation assay

Lipid peroxidation was detected by measuring the level of 8-isoprostane, which is a stable end product of lipid peroxidation, in the NHDF medium. The assay was performed according to the instructions of the commercial enzyme im-

munoassay (Cayman Chemical, Ann Arbor, MI, USA) [23].

Protein carbonyl formation

NHDF were pretreated with morin for 1 hr. The cells were then exposed to UVB radiation and incubated for 24 hr. The extent of protein carbonyl formation was examined using the Oxiselect^{TM} Protein Carbonyl ELISA Kit (Cell Biolabs, San Diego, CA, USA) [35].

DNA fragmentation

Cells were treated with morin and/or UVB was applied to this experiment. Cellular DNA fragmentation was detected by analyzing cytoplasmic histone-associated DNA fragments using a DNA fragmentation assay kit (Roche Diagnostics, Portland, OR, USA).

Single-cell gel electrophoresis (comet assay)

DNA damage was assessed using the comet assay [28]. The cell suspension was embedded in 1% low-melting agarose and then applied to the surface of the slide with 1% normal melting agarose. After solidification, the slides were immersed in a solution to lyse the cell membrane. Lysed cells were subjected to electrophoresis. After washing 3 times with neutralizing buffer, the slides were stained with ethidium bromide for observation of broken DNA strands using a fluorescence microscope and image analyzer (Komet 5.5, Kinetic Imaging Ltd, Wirral, UK). The tail length and percentage of total fluorescence in the comet tails were recorded for 50 cells per slide.

Nuclear staining with Hoechst 33342

The cells were treated with the DNA-specific fluorescent dye Hoechst 33342 (Biomol GmbH, Hamburg, Germany) to detect apoptotic bodies. After 10 min of incubation, images of the nuclear condensation of NHDF were observed under an Olympus 100 W Mercury Power Supply (Tokyo, Japan) [20].

Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed using an *in situ* cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions [16]. Briefly, flow cytometry analysis for the TUNEL assay was performed by seeding the cells on a culture dish at a density of 2×10^5 cells/mL. Sixteen hours after plating, cells were treated with morin and/or UVB. Twenty-four hours later, cells were fixed with 4% paraformaldehyde for 1 hr at 15~25℃ and permeabilized for 2 min in 0.1% sodium citrate solution containing 0.1% Triton X-100. After washing in phosphate-buffered saline, the sections were incubated with the TUNEL reaction mixture for 1 hr at 37°C. The cells were analyzed using a flow cytometer. The intensity of emission in the green range was assessed based on histograms generated by CellQuest Pro 5.1 software (BD) and ModFit LT 3.2 software (Verity Software House).

Statistical analysis

All measurements were performed in triplicate, and all values are presented as mean ± standard error. Statistically significant differences were analyzed using analysis of variance (ANOVA) with Tukey's test. A p-value <0.05 was considered to be statistically significant.

Results

Cyto-protective effect of morin on UVB-exposed NHDF cells

The cell viability was greater than 95% for all morin concentrations used. Therefore, morin was not cytotoxic to NHDF cells (Fig. 1A). In contrast, the cells exposed to UVB had low cell viability at doses over 100 mJ/cm^2, suggesting intracellular cytotoxicity in NHDF cells (Fig. 1B). However, morin protected the cells from UVB (100 mJ/cm^2)-induced cell death in a dose-dependent manner, with significantly high cell viability at doses over 25 μM (Fig. 1C). To further investigate the cyto-protective effects of morin, we used 25 μM morin as the optimal concentration for subsequent experiments. Morin absorbed UVB, showing a maximum peak at 271 nm (Fig. 1D). These results indicated that morin protects cells against UVB-induced cytotoxicity.

Antioxidant effect of morin on intracellular ROS induced by UVB in NHDF cells

Cells were exposed to different doses of UVB to assess intracellular H_2O_2 generation, and the results showed that UVB induced intracellular H_2O_2 at a dose of 100 mJ/cm^2 (Fig. 2A). Thus, we selected UVB at 100 mJ/cm^2 as the optimal dose for subsequent experiments. Next, the ROS scavenging ability of morin was detected, and the results proved that morin (25, 50, and 100 μM) significantly reduced UVB-induced H_2O_2 level (Fig. 2B). Furthermore, under a confocal microscope, an increase in green fluorescence (intracellular H_2O_2) induced by UVB was observed, and morin-treated cells

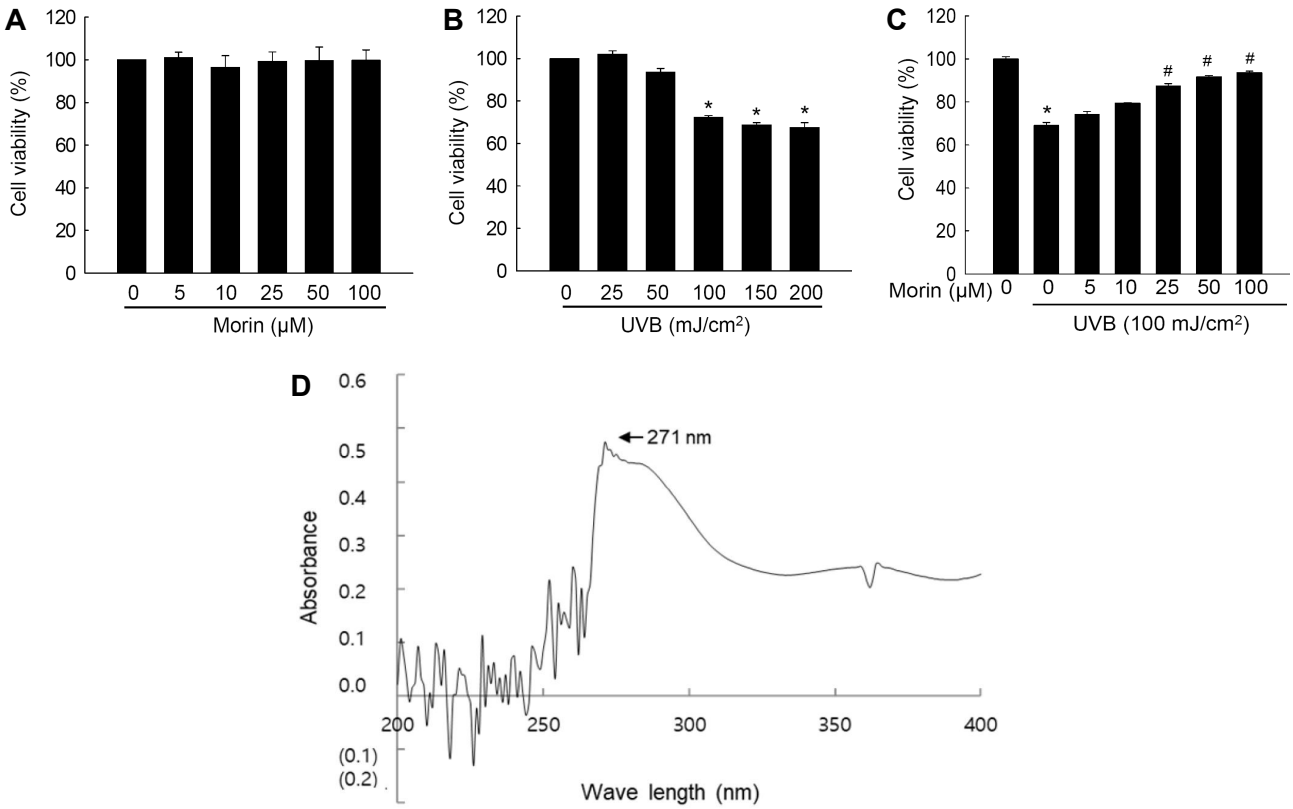

Fig. 1. Cyto-protective effect of morin on UVB-exposed NHDF cells. (A) The viability of morin-treated cells at various concentrations (5, 10, 25, 50, and 100 μM) in NHDF cells was detected by MTT. (B) The viability of UVB-exposed cells (25, 50, 100, 150, and 200 mJ/cm^2) in NHDF cells was detected by MTT. *significantly different from control cells (p<0.05). (C) The protective effects of morin (5, 10, 25, 50, and 100 μM) in NHDF cells treated with UVB (100 mJ/cm^2) were assessed by MTT. *significantly different from control cells (p<0.05); $^\#$significantly different from UVB-exposed cells (p<0.05). (D) The light/UV absorption of morin was detected by a UV/visible light spectrophotometer. Arrow indicates the absorbance peak at 271 nm.

presented a low level of H_2O_2 (Fig. 2C). In addition, DHE staining revealed that UVB-induced superoxide anion radical generation was blocked by morin (Fig. 2D). To investigate whether the cyto-protective effect of morin against oxidative stress was associated with antioxidant enzymes, antioxidant enzyme-related proteins were measured by Western blot analysis in morin-and/or UVB-exposed cells. In the presence of morin, the protein expression levels of catalase, CuZnSOD, GCLC, and HO-1 were increased within 24 hr (Fig. 2E). Although the protein expression levels of catalase, CuZnSOD, GCLC, and HO-1 in UVB-exposed cells were lower than those in control cells, morin treatment restored these protein expression in UVB-exposed cells. These results further indicated that morin exerted antioxidant effects in NHDF cells.

Protective effect of morin on macromolecules damaged by UVB

UVB radiation-induced excessive intracellular ROS can damage cellular macromolecules, such as proteins, mitochondria, nuclear DNA, and lipids [19]. In addition, ROS-induced 8-isoprostane secretion can be detected in skin cells and used as a specific indicator of lipid peroxidation [27]. The results showed that 8-isoprostane level was significantly increased in UVB-exposed cells, and were decreased by morin pretreatment (Fig. 3A). Furthermore, in UVB-exposed cells, protein carbonyl content was significantly high compared to control cells; however, morin significantly inhibited UVB-induced protein carbonyl formation (Fig. 3B). UVB radiation also causes single- and double-strand breaks and DNA damage [8]. It was also confirmed that DNA fragmentation, which was induced by UVB exposure, was suppressed in the presence of morin (Fig. 3C). Damaged cellular DNA was visualized by electrophoresis, which produced an elongated nuclear tail. Compared with the control group, the cells had a considerably long nuclear tail after UVB irradiation; however, in morin-pretreated cells, the nuclear tail was shortened

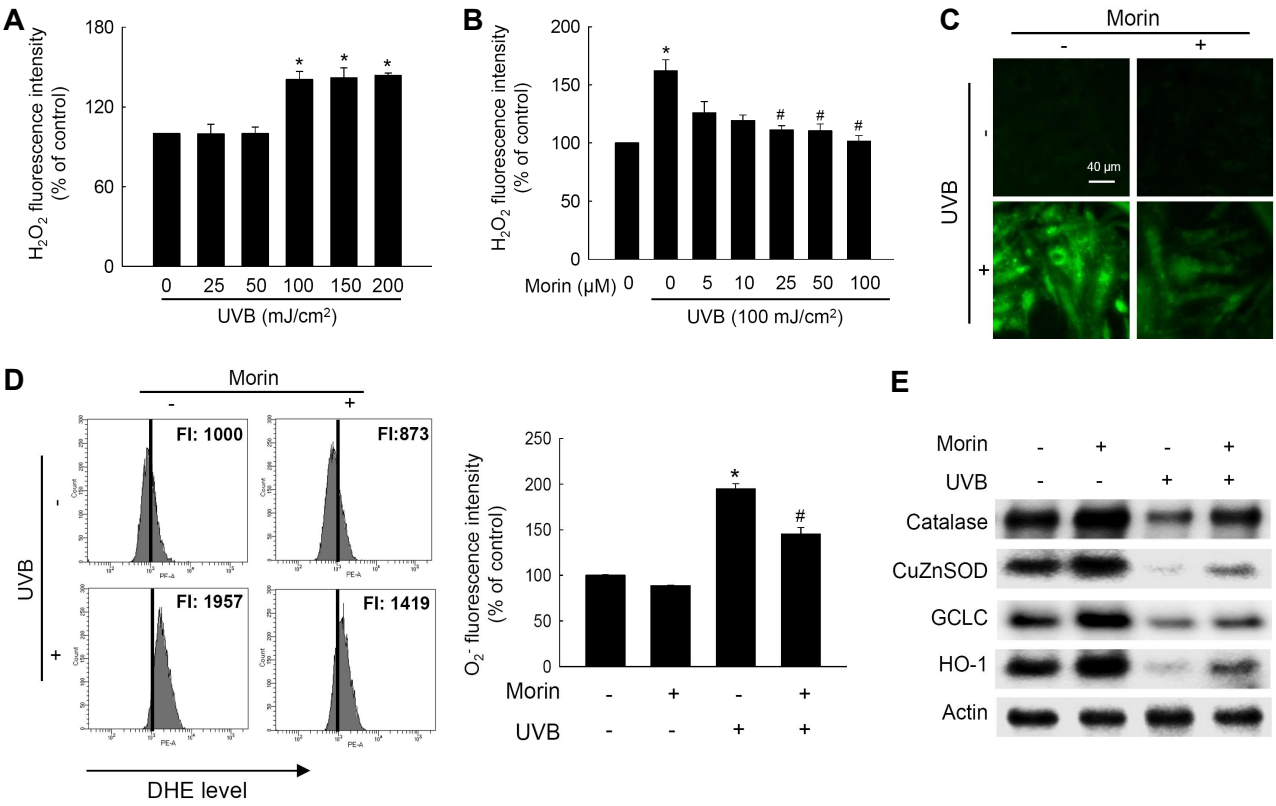

Fig. 2. Antioxidant effect of morin on ROS induced by UVB. (A, B) The intracellular H_2O_2 generation was examined by spectrofluorometry after H_2DCFDA staining (A) in UVB (25, 50, 100, 150, 200 mJ/cm^2)-exposed cells and (B) in UVB (100 mJ/cm^2)-exposed cells after morin treatment at 5, 15, 25, and 50 μM. *significantly different from control (p<0.05); $^\#$significantly different from UVB-exposed cells (p<0.05). (C) H_2O_2 level was detected by a confocal microscope after H_2DCFDA staining. (D) Superoxide anion radical was detected by flow cytometry after DHE staining. *significantly different from control cells (p<0.05); $^\#$significantly different from UVB-exposed cells (p<0.05). (E) The expression levels of catalase, CuZnSOD, GCLC, and HO-1 were monitored by Western blot analysis.

(Fig. 3D). Therefore, the results presented in Fig. 3 suggest that morin shields lipids, proteins, and DNA from UVB-induced oxidative damage.

Inhibitory effect of morin on UVB-induced apoptosis

To confirm whether morin inhibits UVB-induced apoptosis, we determined the amount of apoptotic bodies in UVB- or morin-pretreated cells. In UVB-exposed cells, apoptotic body formation was significantly upregulated. In contrast, in morin-pretreated cells, the number of apoptotic bodies was decreased (Fig. 4A). UVB-exposed cellular nuclei were stained with TUNEL reagent and analyzed by flow cytometer. The number of TUNEL-positive cells, a characteristic of apoptosis, was significantly higher in UVB-exposed cells (TUNEL-positive index: 2.08) than in control cells (TUNEL-positive index: 1) (Fig. 4B). In contrast, TUNEL-positive cells were significantly reduced in UVB-exposed cells pretreated with morin (TUNEL-positive index: 1.27) compared to UVB-exposed cells (TUNEL-positive index: 2.08) (Fig. 4B). Two typical proteins, Bcl-2 and Bax, are involved in the suppression and promotion of apoptosis, respectively [30]. In the morin-treated group, Bcl-2 was upregulated and Bax was downregulated (Fig. 4C). It is well known that the caspase cascade is activated during apoptosis caused by UVB exposure [36]. In the UVB-stimulated NHDF cells, the active forms, cleaved caspase-9 and cleaved caspase-3 were upregulated, whereas these were downregulated in the morin-pretreated cell group (Fig. 4C). It was demonstrated that phosphorylation of p38 and JNK 1/2 is activated by UVB-induced apoptosis in dermal fibroblasts [21]. As shown in Fig. 4D, our data also revealed that UVB induced phosphorylation of p38, and JNK 1/2. However, morin significantly inhibited the phosphorylation of p38 and JNK following UVB exposure. These results indicate that morin protects cells against apoptosis induced by UVB via inhibition of p38 and JNK signaling.

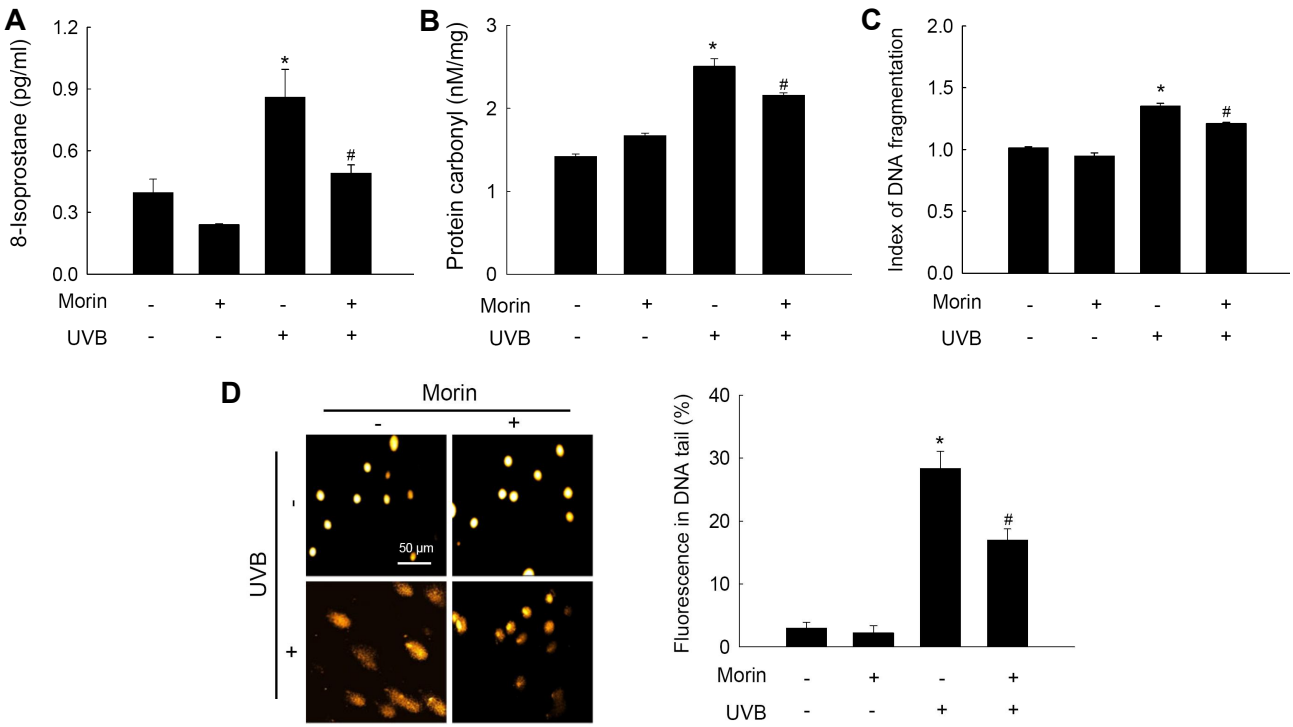

Fig. 3. Protective effect of morin on macromolecules damaged by UVB. (A) Lipid peroxidation was detected by measuring level of 8-isoprostane in each group. *significantly different from control cells (p<0.05); #significantly different from UVB-exposed cells (p<0.05). (B) Carbonyl formation was assessed by the protein carbonyl ELISA kit. *significantly different from control cells (p<0.05); #significantly different from UVB-exposed cells (p<0.05). (C) DNA fragmentation was analyzed with the DNA fragmentation ELISA kit. *significantly different from control cells (p<0.05); #significantly different from UVB-exposed cells (p<0.05). (D) Comet assay assessed DNA damage in each group. The fluorescence of the DNA tail was provided in each group. *significantly different from control cells (p<0.05); #significantly different from UVB-exposed cells (p<0.05).

Discussion

UVB-induced cellular disorders can cause a variety of harmful reactions, including erythema, immunosuppression, pigmentation, and premature aging [12]. In the skin fibroblast model, UVB-damaged cells induced senescence via the induction of DNA damage and expression of matrix metalloproteinase-1 [14]. In addition, UVB could induce apoptosis in mouse embryonic fibroblasts, which is regulated by the p62 signaling pathway [7]. To identify potential protective compounds, we conducted experiments to determine the protective effect of morin on UVB-induced apoptosis in NHDF. This study demonstrated that morin protected NHDF against UVB-induced oxidative stress and demonstrated that morin can play a key role in alleviating ROS levels. Morin showed efficient light absorption at 271 nm, within the UVB range (280~320 nm). As a result, it is reasonable to presume that the cyto-protective effects of morin can be applied to inhibit UVB-induced cell damage.

A review pointed out that excessive ROS can damage various macromolecules, such as DNA, lipids, and proteins [25]. Our data show that morin attenuates UVB-induced lipid peroxidation, protein carbonylation, and DNA damage in NHDF cells. Moreover, morin has antioxidant and cyto-protective effects in V79-4 cells and skin dermal fibroblasts upon oxidative stress exposure [15, 32]. Our data showed that morin increased the expression of antioxidant-related proteins, such as catalase, CuZnSOD, GCLC, and HO-1. In addition, the combination of ROS and DNA damage induced by UVB contributes to skin cell apoptosis [33]. In addition, Bcl-2 is the target of a UV-inducible apoptosis switch [11]. Our data also showed a reduction in the formation of apoptotic bodies and TUNEL-positive cells in morin- and UVB-co-treated cells compared to UVB-treated cells. In addition, pretreatment with morin markedly upregulated the expression of the anti-apoptotic protein Bcl-2 and downregulated the expression of pro-apoptotic proteins (Bax, active caspase-9, and active caspase-3) induced by UVB. Furthermore, MAPK signaling

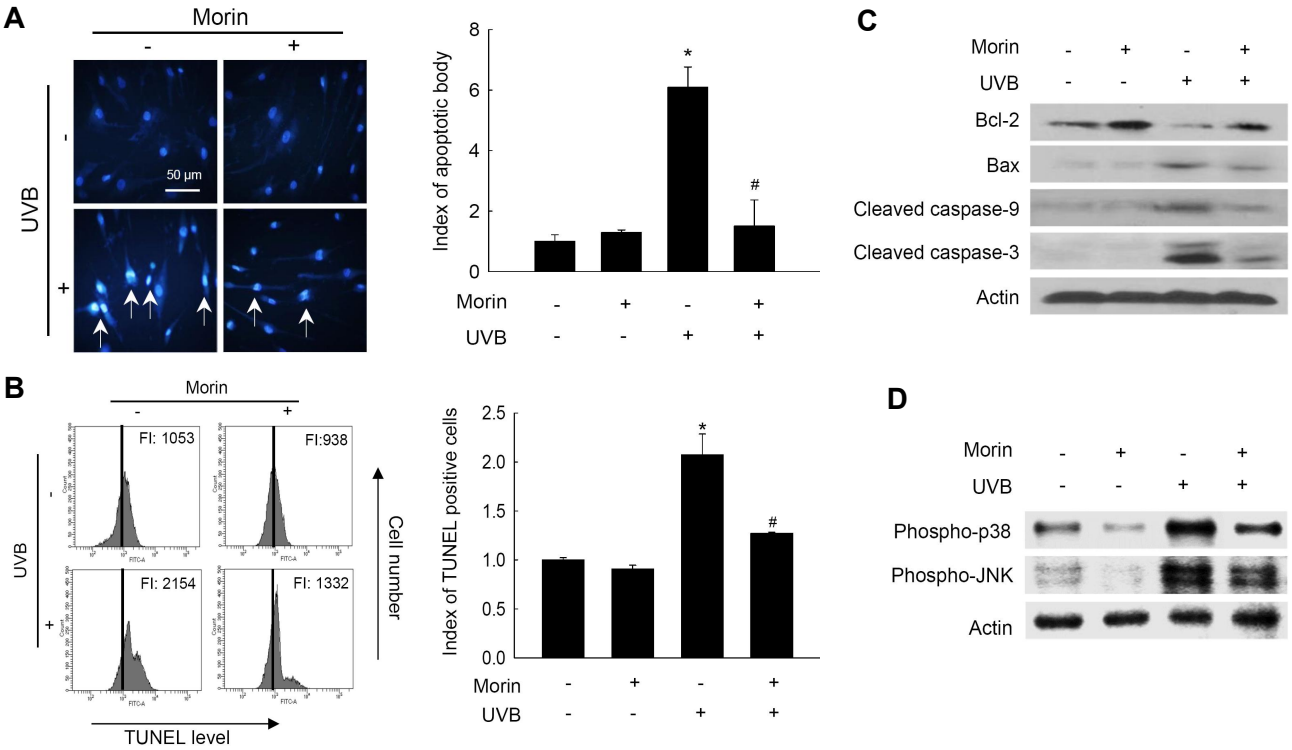

Fig. 4. Inhibitory effect of morin on UVB-induced apoptosis. (A) Apoptotic body formation (arrows) was assessed by fluorescence microscopy after Hoechst 33342 staining. *significantly different from control cells (p<0.05); #significantly different from UVB-exposed cells (p<0.05). (B) TUNEL-positive cells were detected by flow cytometry after the TUNEL reaction. *significantly different from control cells (p<0.05); #significantly different from UVB-exposed cells (p<0.05). (C, D) The expression levels of (C) Bcl-2, Bax, cleaved caspase-9, and cleaved caspase-3 and (D) phospho-p38, and phospho-JNK1/2 were monitored by Western blot analysis.

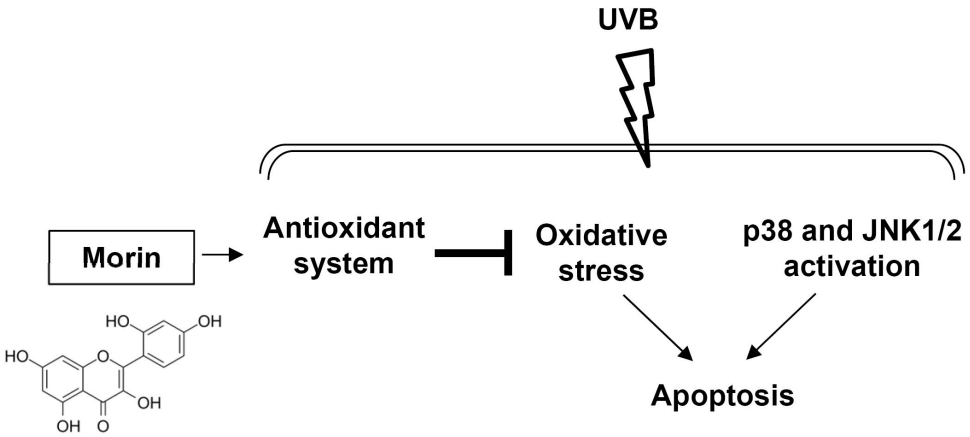

Fig. 5. Protective effect of morin via inhibition of oxidative stress against UVB-induced apoptosis. UVB damaged NHDF cells by inducing excessive ROS, which contributed to lipid peroxidation, protein carbonylation, and DNA damage. However, morin inhibited UVB-induced ROS by activating the antioxidant system, and prevented the oxidation of intracellular macromolecules. In addition, morin inhibited p38 MAPK and JNK1/2 of apoptotic signal pathway activated by UVB. Thus morin can protect cells from UVB-triggered apoptosis.

plays an important role in controlling cell proliferation, survival, and death. The p38 and JNK are the main MAPK subfamily in mammalian cells, and oxidative stress caused by excessive ROS can initiate apoptotic signaling through phosphorylation of p38 and JNK [13, 26, 34]. This study revealed that UVB-induced p38 and JNK activation was reduced in

morin-pretreated cells, demonstrating the cyto-protective effect of morin by enhancing the antioxidant activity in UVB-induced cells.

In summary, the cyto-protective activity of morin against UVB radiation is related to the reduction in ROS by activating the antioxidant system and reducing oxidative damage to cellular components, which leads to cellular apoptosis (Fig. 5). Therefore, morin can be used as a therapeutic agent to protect human skin from the harmful effects of UVB irradiation. The above findings can provide a foundation for future studies to examine the bioavailability and photoprotective function of morin in vitro to determine the underlying mechanism. Therefore, the above findings provide evidence that morin could be viewed as a potential therapeutic candidate for photoprotection of the skin.

Acknowledgment

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 자외선 B로 유도된 아포토시스로부터 모린의 정상 인간 피부 섬유아세포 보호효과

박정언[1†] · 진오현[1†] · 박미경[2] · 강경아[2] · 페르난도 핀카 디바게 사미라 마두샨[1] · 헤라스 무디야세라게 우다리 라크미니 헤라스[1] · 현진원[1,2*]
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자외선 B 조사는 세포의 산화 스트레스, 광노화, 염증을 유발하여 피부 질환을 유발한다. 본 연구의 목적은 인간 피부 섬유아세포에서 자외선 B 조사로 유도된 산화적 스트레스에 대한 모린의 보호 효과를 연구하는 것이다. 모린은 산화적 스트레스로 매개된 질환, 신경 퇴행성 질환, 염증의 잠재적인 치료 후보로 보고되었다. 모린이 항산화제로 보고되고 있기에, 본 연구에서는 모린이 피부 섬유아세포에서 산화적 스트레스 억제를 통한 UVB 유도 아포토시스를 완화할 수 있다고 추측했다. 세포생존율과 세포 내 활성 산소종 레벨은 각각 MTT 분석법, H_2DCFDA 및 DHE 형광 염색 방법을 사용하여 측정하였다. 단백질 카르보닐 형성과 지질 과산화는 ELISA 키트를 사용하여 측정하였다. DNA 분절법, comet assay는 산화적 DNA 손상을 평가하는데 사용되었다. Apoptosis 현상은 TUNEL 분석 및 Hoechst 33342 염색법을 사용하여 분석하였다. 아포토시스 관련 단백질의 발현은 Western blot 분석을 사용하였다. 모린은 자외선 B로 유도된 활성 산소종을 제거하고, 항산화 관련 단백질을 증가시켜 지질 과산화, 단백질 카르보닐화 및 DNA 손상을 억제하여 세포를 보호하였다. 모린은 항아포토시스 단백질 Bcl-2의 발현 증가 및 Bax, caspase-9와 caspase-3 발현을 억제함으로써 자외선 B로 유도된 세포 사멸로부터 보호하였다. 이러한 효과는 또한 p38 및 JNK 1/2의 인산화 감소에 의해 매개되었다. 따라서 모린이 자외선 B로 유도된 피부 손상에 대한 예방/치료 약물로 개발될 수 있음을 나타낸다.