ISSN (Print) 1225-9918
ISSN (Online) 2287-3406
Journal of Life Science 2019 Vol. 29. No. 12. 1321~1328 DOI : https://doi.org/10.5352/JLS.2019.29.12.1321

Inhibitory Activity of Blueberries on UVB-induced Oxidative Stress and Matrix Metalloproteinase Expression in Human Skin Fibroblasts

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Received November 5, 2019 / Revised December 9, 2019 / Accepted December 9, 2019

Intermediate-wavelength solar radiation, also known as ultraviolet B (UVB: 290-320 nm) radiation, may cause premature aging and oxidative damage-dependent skin cancer in humans. UVB-induced formation of reactive oxygen species (ROS)－often a consequence of excessive exposure to these rays－could activate matrix metalloproteinases (MMPs) such as MMP-1 and MMP-3. These enzymes break down type I collagen in human fibroblasts. In this study, we assessed the antioxidant and anti-aging effects of ethyl acetate extract of blueberry (EEB). An antioxidant test in blueberries evaluated ROS production using CCD-986sk cells and DPPH assay. In order to evaluate the anti-wrinkle efficacy of blueberries, the MMP-1 production and type 1 procollagen synthesis evaluated and the expression of MMP 1, 3 were tested through Western blot and RT- PCR. EEB exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and reduced the production of UVB-induced ROS. Also, EEB inhibited UVB-induced processes associated with photoaging and skin cancer, such as reduction in procollagen production and increase in MMP-1 production. More precisely, EEB (50 µg/ml) markedly suppressed mRNA and protein levels of MMP-1 and -3. The anti-aging effects are attributable to the antioxidant activity of EEB. These findings indicate that EEB has a protective effect against UVB-induced aging in human fibroblast cells by regulating the levels of type-1 procollagen, MMP-1, and MMP-3.

Key words : Blueberry, fibroblast, matrix metalloproteinase, oxidative stress, ultraviolet

Introduction

Solar ultraviolet (UV) radiation damages human skin and causes premature skin aging, which is characterized by thickening of the epidermis, rough texture, coarse wrinkles, sallowness with wrinkles, telangiectasia, and mottled pigmentation [8]. It is also believed to be a major causative factor of skin cancer [19]. UV radiation can be divided into three categories based on biological activity and the extent of skin penetration (UVA: 315-400 nm, UVB: 280-315 nm, UVC: 100-280 nm) according to its wavelength. Short- wavelength UV radiation (UVC, the most damaging form of solar radiation, is filtered by the atmosphere, while most of the intermediate- and long-wavelength solar radiation (UVA and UVB) can reach the Earth's surface and penetrate human skin. This can cause harmful physiological effects on organisms, including premature aging of human skin [2]. The amount of UVB radiation that penetrates the skin is minimal, but it is the most active component of sunlight. It is also known to cause genetic toxicity and more sunburn than UVA [26]. UVB can easily affect macromolecules in the epidermal layers, thus affecting cellular functions, including the generation of reactive oxygen species (ROS) such as singlet oxygen, superoxide, and H_2O_2, which can mediate DNA damage [17]. ROS can cause oxidative stress on skin cells and can cause inflammation, erythema and skin wrinkles [1]. Skin wrinkle generation by ROS is expressed by causing changes in collagen, elastin, proteoglycans and fibronectin [27]. Also, UVB induces upregulation of matrix metalloproteinases (MMPs) and corresponding dermal collagen fibril breakdown to yield products such as type 1 collagen (procollagen), fibrillin, and disrupted dermal connective tissue [12]. Type I collagen is mainly synthesized by the fibroblasts in the skin and is responsible for skin elasticity and strength [30]. MMPs constitute a large family of zinc- and calcium-dependent endopeptidases, and they play a crucial role in the physiological progression of inflammation, tumor

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invasion, and skin aging [32]. To date, 28 MMP genes have been identified. MMP-1, -2, -3, and -9 are the members of the collagenase family [18]. UVB is particularly known to induce MMP-1, -3, and -9 expression in human dermal fibroblast cells [7]. Of all the MMPs, MMP-1 and MMP-3 are the major contributors to UVB-induced photodamage [3]. Research has shown some natural products to be effective in inhibiting the expression of MMPs to delay photoaging.

Also, research is ongoing to develop pharmaceuticals with antioxidant properties that can counteract the detrimental effects of excess ROS production. Dietary intake of fruit and vegetables has been implicated as a natural way of achieving the same result [6]. Berries are known to be beneficial to health. Studies on blueberry extracts have revealed an abundance of antioxidants, such as flavonoids (anthocyanins, flavonols, and proanthocyanins) and phenol [21]. Most of these studies are focused on the antioxidant, anti-diabetic, and anti-cancer properties of blueberry extracts; the anti-aging properties are rarely investigated. In this study, we clarified the roles of MMPs and type I procollagen in the potential anti-aging effects exerted by blueberries on UVB-irradiated human CCD-986sk fibroblasts.

Materials and Methods

Plant materials and extraction

Highbush 'Duke' blueberries (*Vaccinium corymbosum L.*) were provided by Cosmofarm Inc. (Hammonton, NJ, USA) in June 2012. The blueberries were immediately harvested, rinsed with deionized water, freeze-dried, and then pulverized into a fine powder using a grinder. Further, 50 g of blueberry powder was soaked in 500 ml of 70% EtOH for 24 hr at room temperature. The mixture was then filtered three times through Whatman filter paper (no. 2). The extraction process was repeated three times. The solvent was evaporated from the total extract to obtain 17.4 g of concentrated extract, which was partitioned with n-hexane (1,500 ml), EtOAc (1,500 ml), and n-BuOH (1,500 ml) to yield dried n-hexane-(0.23 g), EtOAc-(1.42 g), n-BuOH-(1.38 g), and H_2O-soluble (16.0 g) residues. Various concentrations of the EtOAc extracts (EEB) were used in further experiments.

Cell culture

CCD-986sk human skin fibroblast cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All media required for cell growth were purchased from Gibco BRL (Franklin Lakes, NJ, USA). Cells were grown in 10% fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin (100 U/ml) at 37℃ in a 5% CO_2 incubator. The cells were stained with Trypan blue to eliminate dead cells and then counted using a hemocytometer. Aliquots (100 µl) of the cell suspension were plated at $5×10^4$ cells/well in 96-well plates and exposed to UVB at 312 nm (20 mJ/cm^2) for 1 min (Bio-Sun lamps, Vilber Lourmat, Marine, France). A volume of 20 µl EEB (1-100 µg/ml) was added to cells, whereas an equal volume of water was added to the control samples. The cells were grown for 24 hr and used for further experiments.

Cell viability

The reagent 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl-tetrazoliumbromide (MTT) was purchased from Sigma-Aldrich (St.Louis, MO, USA). The MTT cell proliferation assay was performed by modifying a previouslyl published protocol [22]. A total of 20 µl of MTT solution (5 mg/ml) was added to 180L of cell suspension and incubated for 4 hr. The MTT solution was then removed, and 150 µl of dimethylsulfoxide (DMSO) was added and incubated for 30 min. Cell toxicity was determined by measuring the absorbance of each well at 540 nm using a microplate reader (Spark® Cyto, Tecan, Salzburg, Austria).

Measurement of ROS production

We measured ROS production using the ROS-Glo™ H_2O_2 Assay system (Promega, Madison, WI, USA) [11]. In brief, cells ($2×10^5$/well in a 96-well plate) were seeded for 24 hr in a CO_2 atmosphere at 37℃. After incubation, the cells were treated with or without UVB (20 mJ/cm^2). Subsequently, EEB at a concentration of 10, 25, or 50 µg/ml was added to the wells. The supernatants of EEB-treated cell media were then obtained, and ROS production was estimated using the ROS-GloTM H_2O_2 Assay. The levels of activated ROS were measured using a luminescence spectrometer (Perkin Elmer, MA, USA).

Measurement of MMP-1 production

The production of MMP-1 was determined by an enzyme-linked immunosorbent assay (ELISA) kit [20]. Aliquots of supernatants of EEB-treated cell media were transferred to immunowell plates and incubated at 37℃ for 2 hr. The wells were blocked with casein and subsequently incubated

with an anti-MMP-1 antibody at 37℃ for 1 hr. The plates were then washed with a wash buffer, incubated with a secondary antibody linked to peroxidase at 37℃ for 1 hr, thoroughly re-washed with wash buffer, and subsequently re-incubated with tetramethylbenzidine (TMB) until color development. The absorbance was measured with a microplate reader at 450 nm.

Type-I procollagen synthesis assay

Human dermal fibroblasts (5×10^4 cells) were seeded into 24-well plates, and the medium was decanted 24 hr later. The cells were then washed twice with phosphate-buffered saline, and serum-free medium was added to the wells. After 24 hr, EEB (10-25 μg/ml) was added, and the cells were cultivated for 48 hr. The culture medium was collected after stimulation (treatment with UVB at 312 nm (20 mJ/cm^2) and used for the assessment of collagen synthesis. The collagen content was determined using the Procollagen type I C- peptide assay kit (Takara Bio Inc., Otsu Shiga, Japan).

RNA isolation, cDNA synthesis, and Real-time quantitative polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. CDNA synthesis from total RNA was done using GoSctipt™ Reverse Transcription System (Promega). PCR reactions were carried out using a B7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Specific primer pairs were as follows: MMP-1 forward 5′-AGC GTG TGA CAG TAA GCT AA -3′, MMP-1 reverse 5′- GTT TTC CTC AGA AAG AGC AGC AT - 3′, MMP-3 forward 5′- TTG TTC TTT GAT GCA GTC AGC -3′, MMP-3 reverse 5′- GAT TTG CGC CAA AAG TGC - 3′, beta-actin forward 5′- ATT GTT GCC ATC AAT GAC CC-3′, beta-actin reverse 5′- AGT AGA GGC AGG GAT GAT-3′.

Western blot analysis for MMP-1 and MMP-3

Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CCD-986sk cells were grown and harvested by centrifugation at 12,000 rpm for 20 min at 4℃, after adding 10 ml radio-immune precipitation assay (RIPA) buffer with protease inhibitor cocktails. The supernatant was saved, and protein concentration was measured using the Bradford assay [29] Western blot analysis was performed according to a standard procedure using specific antibodies. The signals were detected using Super Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). The protein signals were analyzed using a LAS 4000 image analyzer (Fujifilm Life Science, Tokyo, Japan). The sample protein signals were then divided by the internal control signals using Image J software.

Statistical analysis

Each experiment was performed in triplicate. The data are presented as a mean ± standard error of the mean (SEM). Statistical significance was considered at $p<0.05$ or $p<0.01$.

Results

DPPH radical-scavenging activity of EEB

Present study aimed to reveal the biological functions EEB. DPPH radical-scavenging activity of EEB (5-1,000 μg/ml) was evaluated first. As shown in Fig. 1, the DPPH radical-scavenging activity of EEB significantly increased in a dose-dependent manner.

Measurement of EEB cytotoxicity using MTT cell proliferation assay

To determine the cytotoxicity of EEB, MTT cell proliferation assay was performed. EEB (10, 25, 50, 75, and 100 μg/ml) was added to CCD-986sk human skin fibroblast cells. After this addition, 70% of the cells remained viable at an EEB concentration of 100 μg/ml. At EEB concentrations <75 μg/ml, the cell viability was at least 90%, as shown in Fig. 2.

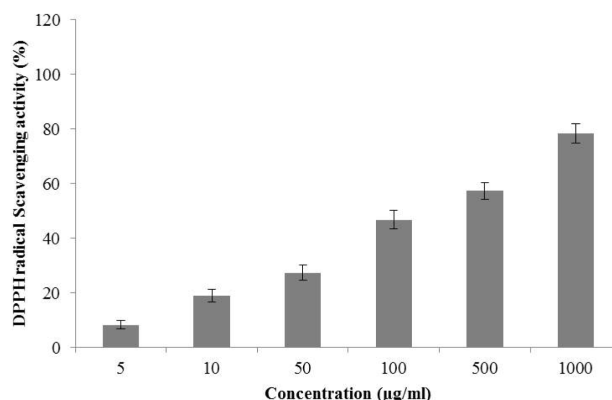

Fig. 1. DPPH radical-scavenging activity of ethyl acetate extract of blueberries (EEB). All experiments were performed in triplicate. The shown values represent mean ± SEM of three different assays.

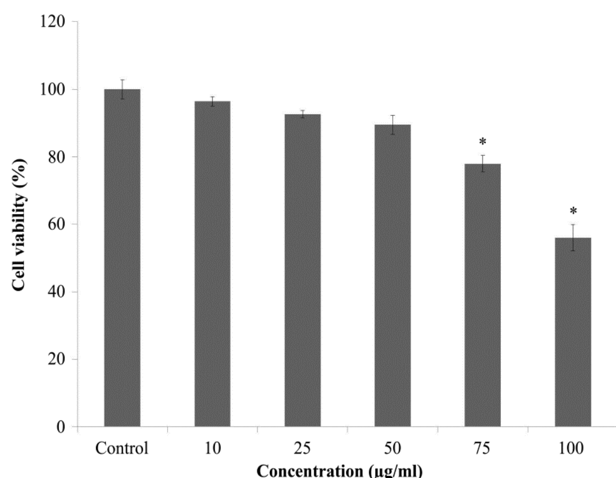

Fig. 2. The viability of CCD-986sk cells after treatment with ethyl acetate extract of blueberries (EEB). MTT assay was performed to measure viabilities of cells. All experiments were performed in triplicate. The shown values represent mean ± SEM of three different assays. *indicates $p < 0.05$, ** indicates $p<0.01$.

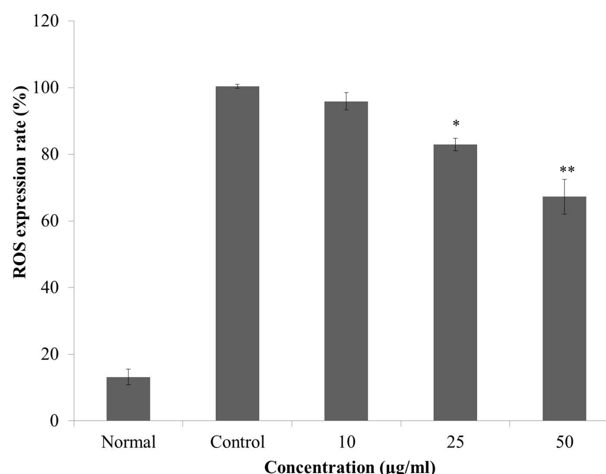

Fig. 3. Effects of ethyl acetate extract of blueberries (EEB) on the generation of reactive oxygen species (ROS) induced by UVB irradiation of human skin fibroblasts. CCD-986sk cells were grown and exposed to UVB for 1 min and were then treated with indicated concentrations of EEB. Normal; non-UVB-irradiated cells, Control; UVB-irradiated cells without EEB treatment. Shown values represent mean ± SEM of three different assays. *indicates $p<0.05$, ** indicates $p<0.01$.

Effect of EEB on ROS generation

ROS are harmful molecules produced as by products of normal cellular functions or exposure to external triggers, and their continuous production may damage the cells [5]. At a molecular level, excess ROS can overwhelm cellular antioxidants, thereby potentiating phenotypic alterations of the human skin [13]. Previous in vitro studies have shown increased ROS production in UVB-irradiated cells compared to unirradiated controls [4]. Our study showed a marked decrease in ROS production over a range of EEB concentrations (Fig. 3). We also found that EEB provided maximum protective effects at concentrations of 25 and 50 μg/ml. The results confirmed the potential of EEB to inhibit the generation of intracellular ROS.

Effect of EEB on UVB-induced MMP-1 production and type-I procollagen synthesis

Matrix metalloproteinases (MMPs) are well known as factors involved in inflammation, tumor invasion, and skin aging. Also, the activities of MMP are much higher under various stimuli, including UVB [25]. Thus, we sought to determine the stimulation of UVB in human skin fibroblast cells and the protective effect of EEB. Cells were exposed to UVB (20 mJ/cm^2), and the secreted MMP-1 was measured by using an ELISA kit. Fig. 4A shows that UVB irradiation led to the activation of MMP-1 in CCD-986sk cells, but this effect did not continue after completion of the treatment. Taken together, the evidence suggests that EEB promotes collagen synthesis. Compared with sham-treated and UVB-irradiated controls, EEB-treated cells showed increased production of type-1 procollagen at EEB concentrations of 10, 25, and 50 μg/ml (Fig. 4B). Type-1 procollagen amounts corresponding to these EEB concentrations were 15.8%, 38.3%, and 62%, respectively (Fig. 4B). The results indicated that the expression of MMP-1 was reduced, while the production of procollagen was increased after treating UVB-irradiated cells with EEB. Also, the inhibition of MMP-1 expression and the production of procollagen in UVB-irradiated cells were related to the antioxidant effect of EEB.

EEB decreased MMP expression at mRNA and protein level

This study also investigated the inhibitory effect of EEB on MMP-1 and MMP-3 mRNA expression in UVB-irradiated cells. To verify whether MMP-1 and MMP-3 mediated anti-aging effects of EEB, we quantified MMP-1 and MMP-3 mRNA levels by RT-PCR before and after 48 hr EEB treatment of UVB-irradiated cells by RT-PCR (Fig. 5). The results showed an increase in both mRNA levels immediately after treatment followed by a decrease over time. In the case of MMP-1, the mRNA level decreased by a factor of 2 following EEB treatment. Western blotting results also revealed that

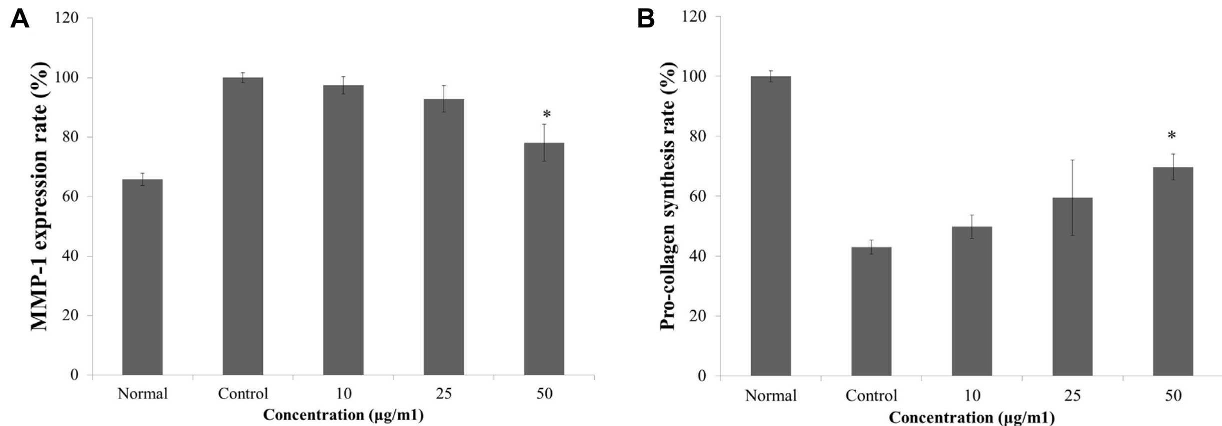

Fig. 4. (A) Matrix metalloproteinase (MMP)-1 inhibition and (B) type-1 procollagen synthesis in CCD-986sk cells after treatment with ethyl acetate extract of blueberries (EEB). Fibroblast cells were exposed to UVB for 1 min and treated with EEB (10, 25, or 50 μg/ml). MMP-1 inhibition and Type-1 procollagen synthesis were measured with an assay kit. Normal; non-UVB-irradiated cells, Control; UVB-irradiated cells without EEB treatment. Data represent mean ± SEM of three different assays. * indicates $p<0.05$.

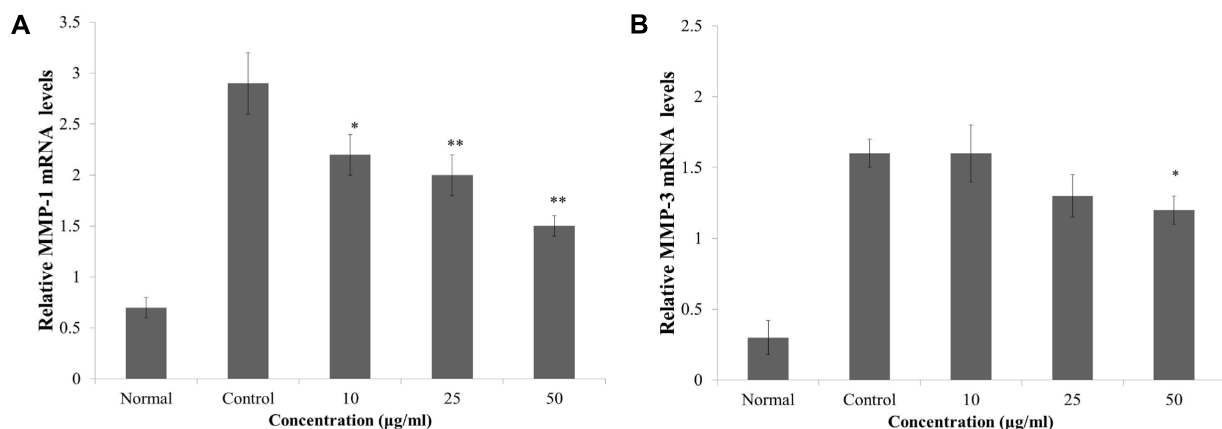

Fig. 5. The expressions of matrix metalloproteinase (MMP)-1 (A) and -3 (B) mRNA were evaluated using realtime PCR. CCD-986sk cells were grown and exposed to UVB for 1 min, after which the cells were treated with 10, 25, or 50 μg/ml of EEB. The levels of mRNA were detected by RT-PCR. β-Actin mRNA was used as an internal control. Normal; non-UVB-irradiated cells, Control; UVB-irradiated cells without EEB treatment. Shown values represent mean ± SEM of three different assays. * indicates $p<0.05$, ** indicates $p<0.01$.

25 μg/ml and 50 μg/ml of EEB reduced MMP-1 and MMP-3 protein levels by 15.7% and 38.3%, respectively (Fig. 6A). Similarly, treatment with 25 and 50 μg/ml of EEB reduced the MMP-3 protein level by 22% and 33.6%, respectively in vitro.

Discussion

Oxidative stress has a potential of damaging cellular components and organelles, which can result in skin photoaging [24]. Among various fruits and vegetables, berries are easily accessible and also beneficial to health. Blueberry extracts are known to contain abundant antioxidant components [16]. Antioxidant, antidiabetic, and anti-cancer effects are classic effects of blueberry; however, our study mainly focused on the anti-aging effect of blueberry extract on the human skin. UVB, which affects the skin and causes aging, is known to increase levels of MMPs. MMPs are known to play a crucial role in inflammation, tumor invasion, and skin aging [23]. Also, MMP secretion from keratinocytes and fibroblasts causes wrinkles, as a symptom of skin aging [28]. UV rays, oxidative stress, and cytokines are well known factors that contribute to increase in MMP activity [31].Their active form a mainly act as epidermal descomposers; MMP-1 degrades

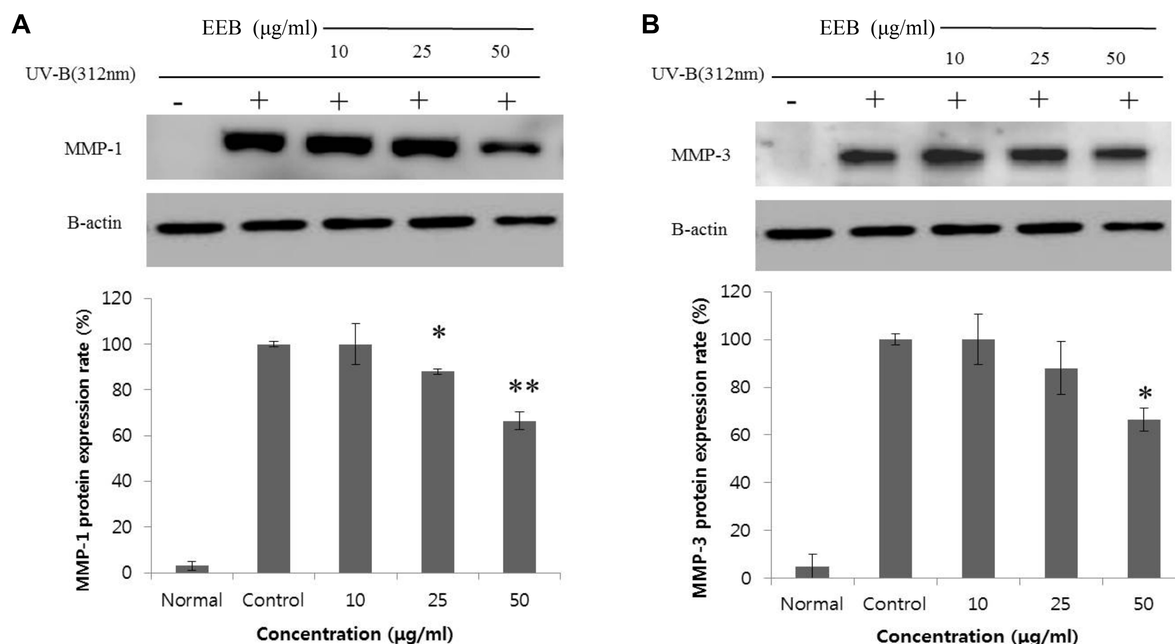

Fig. 6. Protein levels of matrix metalloproteinase (MMP)-1 and -3 were investigated after treatment of CCD-986sk fibroblasts with ethyl acetate extract of blueberries (EEB) through western blotting analysis. After 24 hr incubation of CCD-986sk cells, they were exposed to UVB radiation for 1 min and were treated with 10, 25, or 50μg/ml of EEB. Antigen-specific antibodies as primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were used. Chemiluminescence was detected using enhanced chemiluminescence (ECL) solution. All experiments were performed in triplicate. Normal; non-UVB-irradiated cells, Control; UVB-irradiated cells without EEB treatment. The values represent mean ± SEM of three different assays. * indicates $p<0.05$, ** indicates $p<0.01$.

type-1 collagen, which is synthesized in the fibroblasts in the skin to maintain skin elasticity and solidity, whereas MMP-3 degrades the basement membrane of the skin [10]. In this manner, UVB induces skin aging. Several studies have reported the antioxidant and anti-aging effects of crude fruit extracts. For example, the anthocyanins in bilberries have been reported to scavenge UVA-stimulated production of ROS [14], and strawberry extracts have been shown to possess strong anti-inflammatory [9] and antioxidant activities [33]. Previous studies have shown that some natural foods and their derived products have an inhibitory effect on MMP expression due to reduced photodamage [15] In the light of this observation, this study demonstrated the antioxidant properties of EEB measured by decreased ROS production and a DPPH free radical-scavenging assay. We showed that EEB treatment decreases mRNA and protein levels of MMP-1 and MMP-3 in UVB-irradiated human skin fibroblast cells in vitro. These findings indicate that EEB has anti-aging properties. As a result, EEB may be considered as a functional food and an essential excipient in cosmetic products. Further studies on the antioxidant effects of EEB are, however, necessary to understand the molecular mechanisms underlying its various pharmacological effects. In summary, we have counteracted in vitro the known effect of UV irradiation on the MMP-mediated destruction of a major component of the dermis, i.e., collagen. EEB diminished UVB-irradiation-induced photoaging by inhibiting MMP-1 and MMP-3 and elevating type I procollagen production through the scavenging of ROS. The data obtained indicated that EEB protected against photoaging induced by UVB irradiation in human fibroblast cells.

Acknowledgement

This work was supported by the Technology development Program (S2687105) funded by the Ministry of SMEs and Startups (MSS, Korea).

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초록 : 인간피부 섬유아세포에서 UVB 유도된 산화 스트레스와 기질금속단백질가수분해효소 발현에 블루베리의 저해능

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자외선B(UVB: 290-320 nm) 방사선으로도 알려진 중간 파장 태양광선은 인간에게 조기 노화 및 산화손상에 의존하는 피부암을 유발할 수 있다. UVB가 유발하는 반응성 산소종(ROS)의 형성은 종종 이러한 광선에 과도하게 노출되는 결과로 MMP-1과 MMP-3와 같은 매트릭스 금속단백질(MMP)을 활성화할 수 있다. 이 효소들은 인간의 섬유질에서 type 1형 콜라겐을 분해한다. 본 연구에서는 블루베리(EEB)의 에틸 아세테이트 추출물의 항산화 및 항노화 효과를 평가했다. 블루베리의 항산화 실험은 DPPH 분석과 CCD-986sk 세포를 사용하여 ROS 생성을 평가했다. 블루베리의 주름방지 효능을 평가하기 위해 MMP-1 생성과 type 1형 procollagen 합성을 평가하고 Western Blot과 RT- PCR을 통해 MMP 1, 3의 발현량을 평가하였다. EEB는 2,2-diphenyl-1-picrylhydrazyl (DPPH) 라디칼의 소거능을 보이며 UVB에 유도된 ROS의 생성을 저해하였다. 또한 EEB는 procollagen 생성감소 및 MMP-1 생성량의 증가 등 광노화 및 피부암과 관련된 UVB로 야기되는 과정을 억제하였다. 더 정확히 말하면, EEB (50µg/ml)는 MMP-1과 -3의 mRNA와 단백질 발현을 현저히 억제하였다. EEB의 항노화 효과는 항산화 작용에 기인한다. 이러한 연구 결과는 EEB가 type 1형 procollagen, MMP-1, MMP-3의 수준을 조절함으로써 인간 섬유아세포에서 UVB가 유발하는 노화로부터 보호 효과를 가지고 있음을 나타낸다.