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Photoprotective Effects of Minerals from Korean Indigenous Ores on UVA-irradiated Human Dermal Fibroblast

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Abstract

The photoprotective effects of minerals from Korean indigenous ores, consisting mainly of sericite, on UVA-irradiated human dermal fibroblast (HDF) were examined. Zymographic analysis showed that the treatment of the minerals significantly reduced the UVA-enhanced MMP-1 activity and mRNA level. The minerals also showed strong inhibitory effect on MMP-2 activity and mRNA expression. Moreover, the minerals were better than polyphenol in reducing MMP-1 and MMP-2 expressions. Notably, the minerals significantly enhanced collagen biosynthesis in the HDF. Inhibition of the elastase activity and protection against the oxidatively damaged HDF cell were also found in the presence of the minerals. Taken together, the ore minerals may be used as the potent photo-protective and anti-skin-aging ingredients which can prevent skin cell damage by UVA.

Keywords: Ore minerals, Human dermal fibroblast, Photoprotection

The recognition that an intimate relationship exists between the environment, particularly the geologic materials, and human health has led to the recent development of a new field of science called medical geology[1-4]. Man and ore minerals as natural inorganic solids have common chemical compositions, and the minerals are added in cosmetic and medicine formulations, not in their elementary form but as inorganic or organic salts. The ore minerals which have been used as natural remedies are composed of either a single mineral or a mixture of more than one mineral from ores. The first written reference upon the use of ores and the beneficial effects on the human health is in "*De Materia Medica*" in 60 BC[5,6]. The use of 92 ore medicines were also described in the *Dongeuibogam*, the most celebrated Korean material medica [7].

Ore minerals such as sericite, talc, kaolinite, palygorskite etc. have been used in pharmaceutical formulations for therapeutic purposes, because they have high absorptive capacity, chemical inertness and low or no toxicity for the human[4,5]. These minerals are also used as active principles in gastrointestinal protectors, osmotic oral laxatives and antidiarrhoeaics. Moreover, they are also applied topically as dermatological protectors and cosmetics and are especially recommended for treating the inflammatory processes, namely acne and boils, due to their strong adsorptive properties[4,5,8,9]. There have been notable advances in mineralogical studies so far; however, information on safety, stability and chemical inertia is very limited. Moreover, further research is necessary on the mechanisms determining the health applications of ore minerals. Especially, biochemical data on the reaction mechanism of the medicinal ores in the cells are not available.

In this investigation, photoprotective effects of the minerals derived mainly from Korean indigenous sericite on UVA-irradiated human dermal fibroblast were examined at the molecular and cellular levels.

SEM Image and MTT Assay

The SEM image of the ore mineral powder is shown in Figure 1. The MTT assay was used to determine the maximum concentration at which approximately all human dermal fibroblasts survive at least 24 hrs without alteration in the morphology after incubation with serially diluted minerals[10,11]. All experiments on the cells were conducted with 0.244 μg /mL of the soluble fraction at which the mineral did not affect the fibroblast cell at all (Figure 2).

Effect of the Minerals on UVA-induced MMP-1 Expression

Ultraviolet (UV) irradiation was reported to damage human skin and cause photo-aging through the activation of matrix metalloproteinases (MMPs) which are responsible for the degradation of collagen, gela-

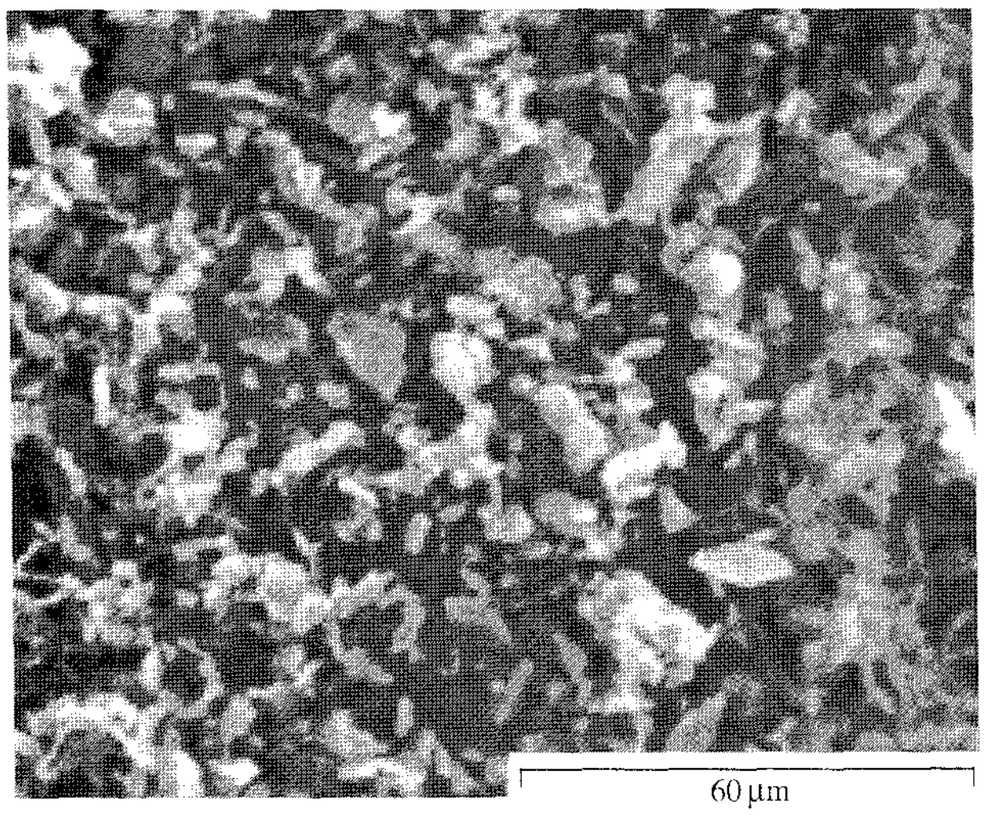

Figure 1. Scanning electron microscopic image of ore minerals.

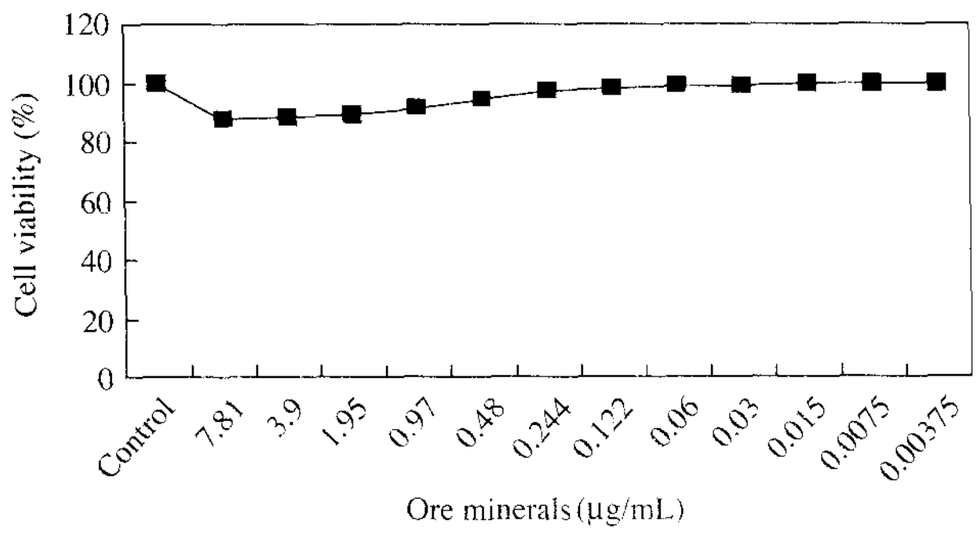

Figure 2. Cell viability in response to ore minerals in human dermal fibroblast.

tin and other components of the extracellular matrix (ECM)[12-14]. Inhibition of induction of MMPs has been reported to alleviate UV-induced photo-aging by preventing collagen destruction.

To estimate the effect of the minerals on MMP-1 (fibroblast-type collagenase) activity in UVA-irradiated HDF cell, zymography was performed to examine MMP-1 collagenase activity in the culture medium of HDF (Figure 3). The UVA irradiation increased the collagenase activity by approximately 50% than the normal as measured on the zymographic gel; however, the treatment of the minerals (0.244 μg/mL) significantly reduced the collagenase activity as compared to the UVA-treated HDF cell. Polyphenol treatment also decreased the collagenase activity, however, the minerals suppressed MMP-1 collagenase activity more markedly than polyphenol, the reported photo-aging resistant.

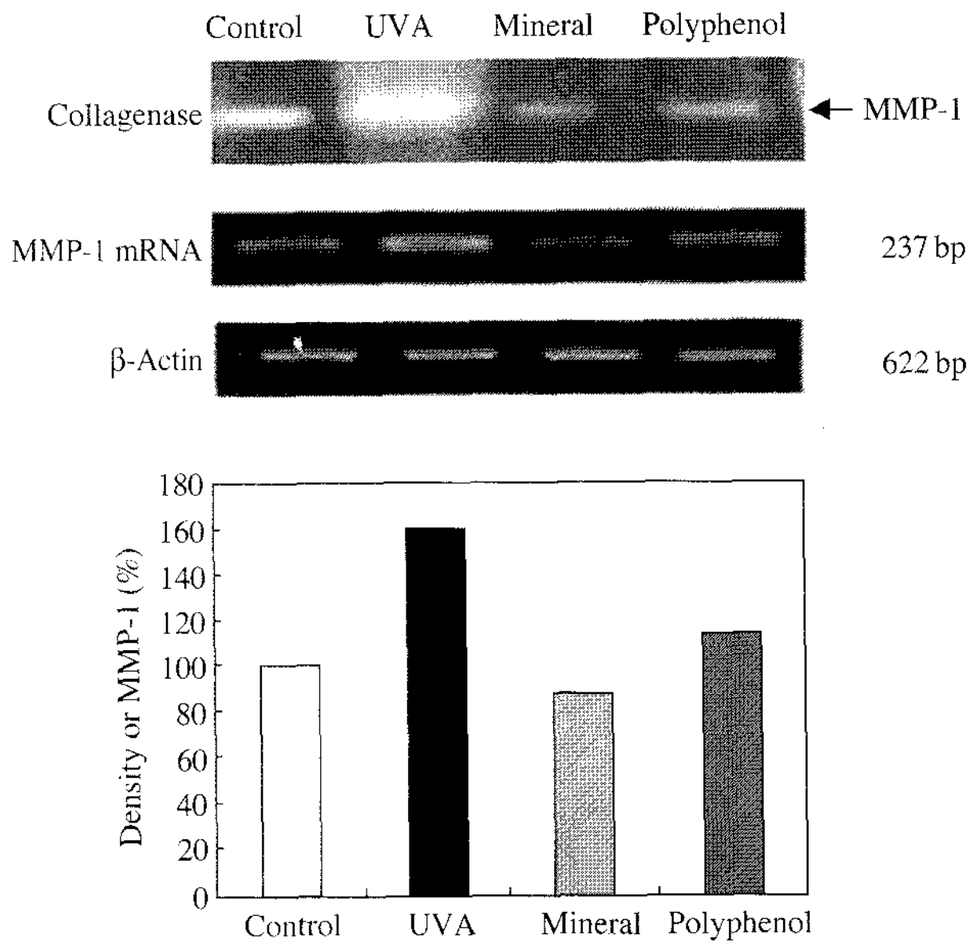

Figure 3. Effect of ore minerals on MMP-1 collagenase activity and MMP-1 mRNA expression in UVA-irradiated human dermal fibroblast.

Also, the effect of minerals on the steady-state MMP-1 mRNA level in relation to β-actin mRNA level was examined in Figure 3. Expression of MMP-1 mRNA in UVA-irradiated HDF was significantly reduced by the minerals, while β-actin mRNA was constant. The HDFs exposed to the UVA had a 60% increase in MMP-1 mRNA as compared to the normal not exposed to the UVA. The polyphenol treated group showed approximately 30% less MMP-1 mRNA than that of the control cell treated with UVA only. But, when UVA-irradiated HDF cell was treated with the minerals, an approximately 47.5% lower mRNA level was found compared to that treated with the UVA alone. These results indicate that the ore minerals reduced more markedly the MMP-1 enzyme activity and mRNA expression than polyphenol in the UVA-applied HDF cell.

Effect of the Minerals on UVA-induced MMP-2 Expression

The skin is the organ most susceptible to damage by UV irradiation as it is directly exposed to UV light. Molecular mechanisms of skin wrinkles are probably due to the loss of macromolecules making up the dermal matrix, among which collagen is the major component[12-14]. In UV-irradiated skin, the level of MMPs that are important enzymes for the proteolysis of extracellular matrix proteins is elevated. Among them, MMP-2 (gelatinase A, 72 kDa) secreted as proenzyme, plays an important role in degrading type IV colla-

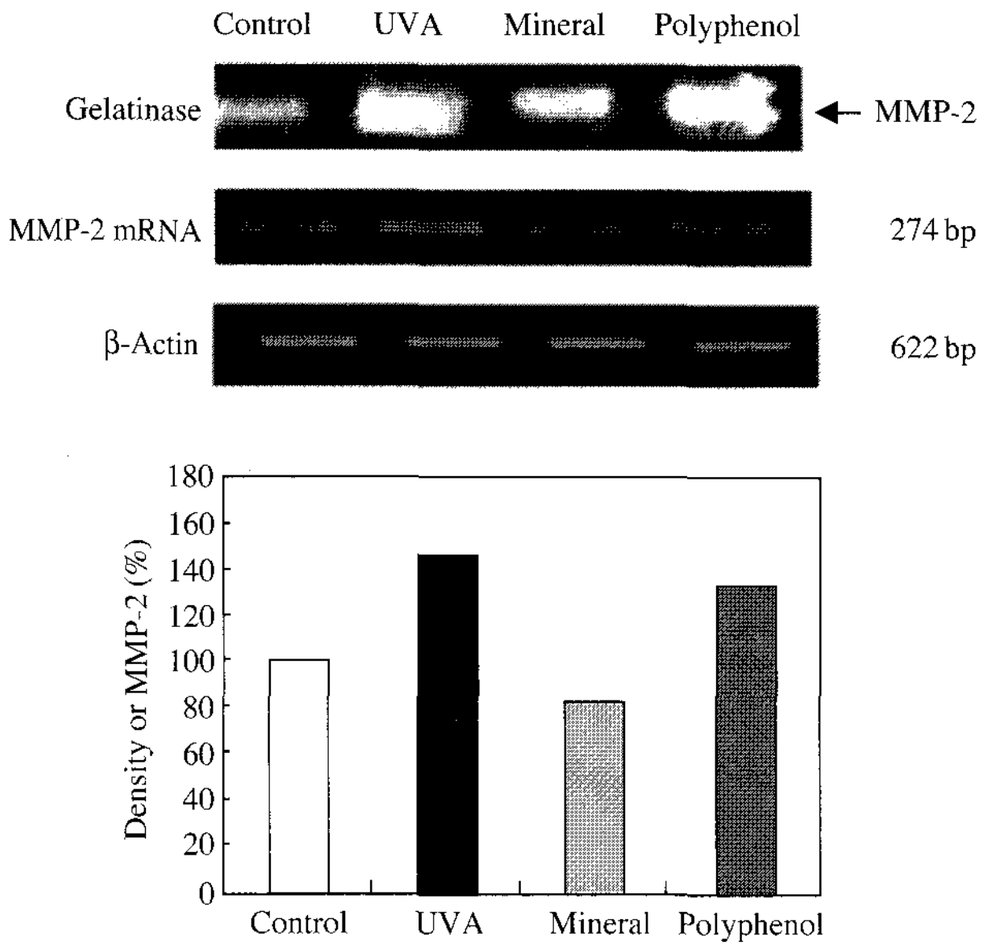

Figure 4. Effect of ore minerals on MMP-2 gelatinase activity and MMP-2 mRNA expression in UVA-irradiated human dermal fibroblast.

gen[15-18].

The suppression of MMP-2 expression by the minerals is shown in Figure 4 through the zymography. The effects of the minerals on MMP-2 gelatinase activities were examined using the secreted medium from the UVA irradiated HDF cell (Figure 4). The UVA irradiation enhanced MMP-2 gelatinase activity notably as evaluated by the band intensity on the zymographic gel; however, the ore minerals suppressed MMP-2 activity in UVA-irradiated cell more markedly than the polyphenol.

We also tested the efficacy of the minerals on the expression of MMP-2 mRNA level in HDF cell exposed to the UVA (Figure 4). The MMP-2 mRNA of the HDFs exposed to the UVA showed a 53% more increased level, compared with the control group not exposed to the UVA. The polyphenol group indicated 12% less MMP-2 mRNA than the group treated with the UVA only. On the other hand, upon adding the minerals, HDFs showed about 42% less MMP-2 mRNA level than that treated with UVA alone. Taken together, the results indicate that the minerals from Korean indigenous ores are outstanding inhibitors of the MMPs, suppressing greatly not only MMP-1 but also MMP-2 at the protein and mRNA level.

Effect of the Minerals on the Collagen Biosynthesis

Type I collagen is the major collagen of bone, tendon, as well as soft tissue such as skin and lung. The

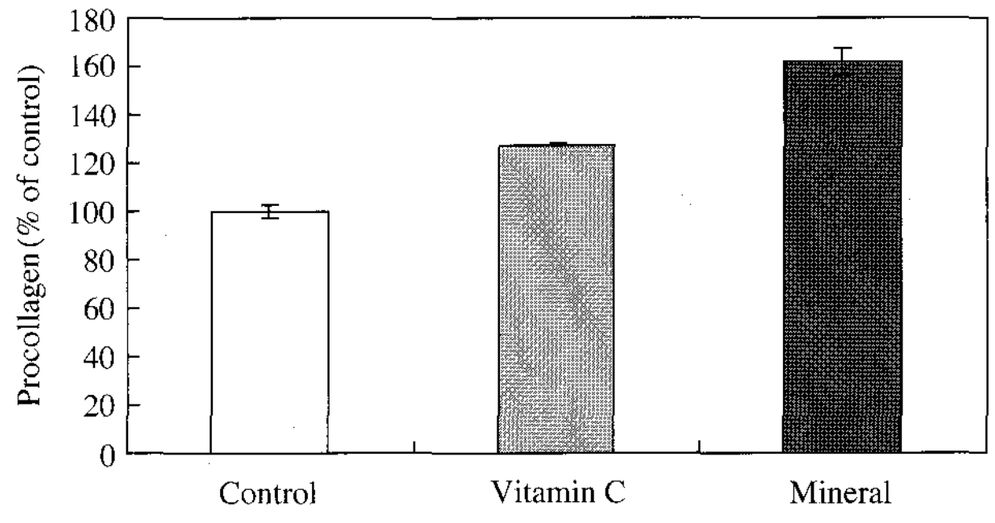

Figure 5. Effect of ore minerals on type I collagen production in human dermal fibroblast.

amount of type I collagen, synthesized by a given cell that varies, was known to be determined by the differentiated phenotype of the cell[16,17]. Vitamin C has been known to be required as a cofactor for the correct hydroxylation of prolyl and lysyl residues of the procollagen polypeptides[19,20] allowing their triple helical conformation in the cells[15]. Vitamin C stimulated collagen biosynthesis[21] not only by promoting the activity of the hydroxylases, but also by increasing the steady state level of the procollagen mRNA [22,23]. This activity is dependent upon the increased transcription of the main types of collagen (I and III) present in skin.

The effect of the minerals on the collagen biosynthesis was tested in Figure 5. The amount of the procollagen generated after 48 hour culture was measured through ELISA method. About 60% more procollagen was biosynthesized by the mineral treatment than the control (Figure 5). The result showed that, even if the mineral was treated in a concentration lower than that of the vitamin C, it generated more amount of procollagen. Therefore, the minerals might have stronger stimulatory effect on the collagen biosynthesis in the HDF than vitamin C.

Inhibitory Effects of the Minerals on the Elastase Activity

Elastase is the rate-limiting enzyme in elastin synthesis, and some elastin production-inhibiting agents such as polyphenols are known to inhibit the elastase activity[24]. Elastase is capable of degrading many structural proteins namely elastin, collagen and fibrinogen, and is thought to be involved in endothelial cell, vascular and cardiac damages[25]. Figure 6 shows the inhibitory effect of the minerals on the elastase *in vitro*. The minerals suppressed about 40% of the elastase activity. The inhibitory effect of the minerals was dose-dependent (Data not shown).

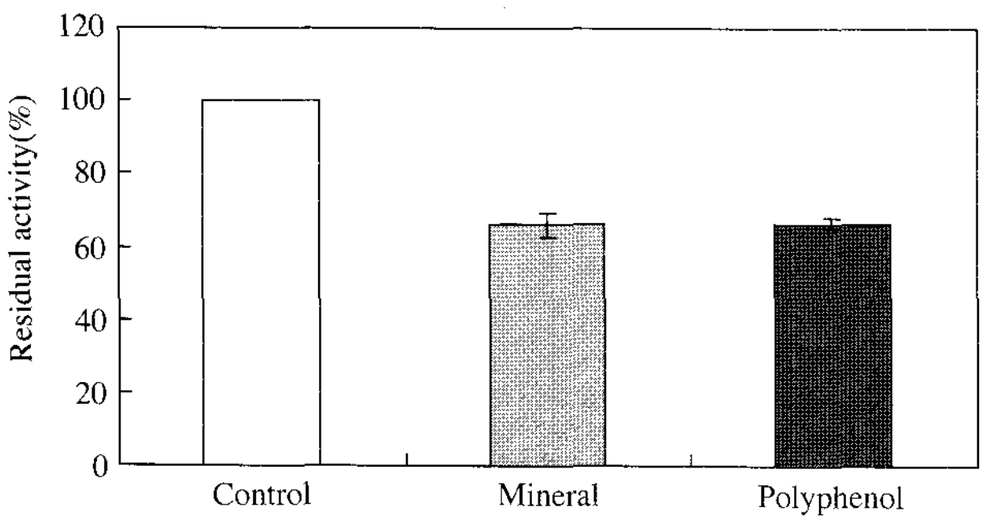

Figure 6. Inhibitory activity of ore minerals on the human elastase.

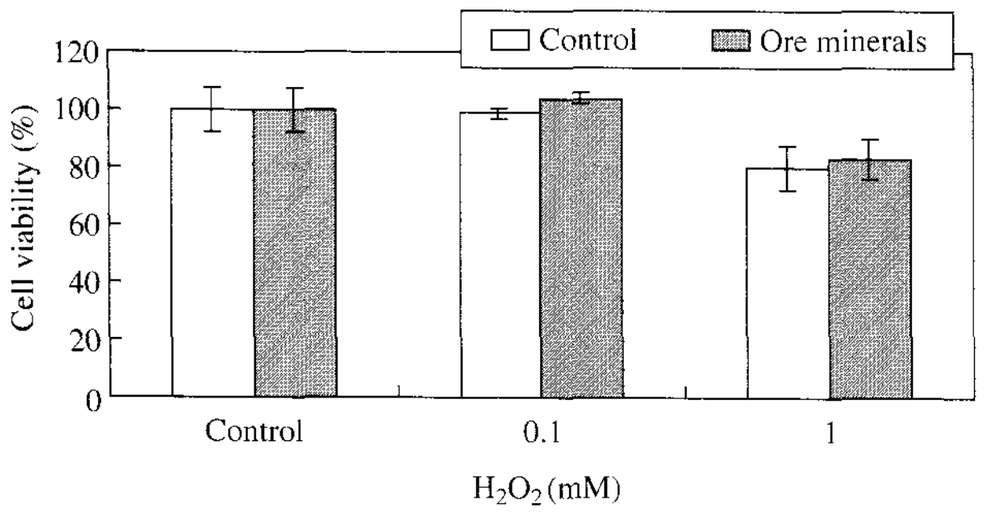

Figure 7. Protective effects of ore minerals on the H_2O_2-induced oxidative damage of human dermal fibroblast.

Protective Effect of the Minerals on the H_2O_2-Induced Oxidative Damage

UV irradiation may induce oxidative stress in the cell possibly by generating free radicals and reactive oxygen species (ROS). ROS includes superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$). H_2O_2 is hostile and damaging to various biomolecules, including oxidative damage of protein, tissue loosening, genetic damage and the promotion of disease and aging[26-28]. A lot of antioxidants that possess oxygen radical scavenging properties[29,30] have been tested as potentially beneficial photoprotective agents from these extrinsic factors. Oxidation-protecting substances named tocopherols, flavonoids, ascorbic acid and carotenes appear to diminish the undesired effects caused by oxidation processes in organisms[29,30].

Figure 7 represents the protective effects of the minerals on the H_2O_2-induced oxidative damage in HDF cell. The minerals enhanced the cell viability by protecting the HDF against H_2O_2-induced oxidative damage, indicating ROS scavenging activity of the minerals.

Discussion

Ore minerals have been used as the active principles in cosmetics and medicines due to their high grease and toxin absorption capacity[1-5]. They are also beneficial for inflammation-related acnes and ulcers, and are used in creams, powders and emulsions as antiperspirants to remove shine in the skin. According to the *Dongeuibogam*, which had described 92 medicine ores by Jun-Heo in 1613, minerals from the ores have been used as mineral drug in oriental medicine to cure catharsis, dysentery, dry breast, poor micturition, chronic indigestion, etc[6,7].

Among them, talc is finely powdered native hydrous magnesium silicate with great oil and grease absorption capacity[8,9]. Sericite is layered silicate mineral, generally recognized as white fine powders of muscovite in form, widely used in the alkali flux and cosmetics[8,9]. Silicate is considered to be fundamental for the synthesis of collagen[4,5], and it is known that the Si content in the human body decreases with aging. Halloysite is ubiquitous in soils and rocks, and has a remarkable ability to absorb toxins and impurities from the surface of the skin; thus, it is especially beneficial for acne-prone skin and drawing dead skin cells away from the skin surface[1-5].

The dermis contains predominantly type I and type III collagen, elastin, proteoglycans and fibrinonectin; also, the alteration and disarrangement of collagen, the major structural component of skin, have been suggested to be a cause of skin wrinkling[22,30,31]. Moreover, as far as the major changes in photodamaged skin are concerned, the extracellular matrix turnover and deposition are required. The matrix degradation by UV-induced matrix metalloproteinases (MMPs) secreted by various cells, including keratinocytes, fibroblasts and inflammatory cells, contributes substantially to the connective tissue damage that occurs during photo-aging[15-18]. UVA can penetrate the dermis, while UVB radiation may reach only the epidermis and upper dermis. Photo-aging by UV radiation causes the unwanted age-associated changes in the skin's wrinkling, irregular pigmentation, and a variety of benign, premalignant, and malignant neoplasms [12,13].

The minerals from Korean indigenous ores used in this study mainly consisted of sericite, talc and halloysite. Their photoprotective and anti-skin-aging effects on the UV-irradiated human dermal fibroblast were examined. The minerals showed notable collagen biosynthesis ability in human dermal fibroblast. The minerals increased collagen type I synthesis in HDFs by about 60% compared to untreated control, while vitamin C increased it about 35%. The minerals were

used in a lower concentration level that of the vitamin C; hence, the minerals might have much superior efficacy in generating collagen in human skin than vitamin C, probably due to the presence of silicate in the ore minerals[4,5,31,32]. Moreover, enhancement of the UVA-induced MMP-1 and MMP-2 activity and mRNA expression in HDFs was remarkably reduced by the minerals. Although at present the exact molecular mechanisms for the inhibition of MMP -1 and MMP-2 protein and mRNA expression by the minerals are not clearly explained, one possible explanation could be its antioxidant activity. Accumulating evidences suggest that various antioxidants such as quercetin, β-carotene, EGCG (epigallocatechingallate) and vitamin E inhibit expression of MMP-1 or MMP-2[29,30,33], because reactive oxygen species may be important signaling mediators for MMP-1 and MMP-2 expression by exogenous stimuli including UV and cytokines. In support of this assumption, the mineral mixture has shown to inhibit the oxidative damages of the cell by H_2O_2. Complex mixture of green tea was reported to have far less CYP1A1 activity than the single catechin due to the synergistic anticancer effect of the mixture[34]. Therefore, the notable photo-protective and anti-skin-aging effect of ore minerals may not be attributed to the action of a single component but rather due to the synergistic effects of many components in the mixture. The ore minerals also inhibited human elastase activity, suggesting the anti-wrinkle effect of the minerals.

Taken together, the data here suggest that ore minerals are potent photo-protective and anti-skin-aging ingredients that prevent the skin cell damage by UVA irradiation. These results imply that the ore minerals from Korean indigenous ores may be useful as new ingredients for anti-aging cosmetics, drugs or health foods if safety of the ore minerals is verified.

Methods

Ore Mineral Preparation

The ore minerals used to investigate the efficacy of photoprotection against human dermal fibroblast were developed by NT & BT Co. Ltd (Hongsung, Chungnam, Korea). The manufacturing process of the minerals started with the mining of the raw materials, named sericite, talc and halloysite, etc. The mined material was broken down into the fine pieces. The pieces went through water washing and drying process, and then they were reduced to powder. The powder passed through the selection stage equipped with the selector using weight difference, and then the metallic elimination via magnetism was conducted. The ingredients of the materials were mixed, and then heat treated. Then, the mineral mixture was finally ready to be used in this investigation.

Mineralogical and Chemical Property

The mineral mixture powder was coated with carbon-film in an ion sputter apparatus (ShimadzuTM C-50) and the microstructures such as shape and surface were examined by using a scanning electron microscopy (LEO 135 VP SEM, Leica Electron Optics), at an acceleration voltage of 15 kV, a working distance of 18 mm, and a magnification of × 1,000.

Human Dermal Fibroblast Culture and UV Irradiation

Human dermal fibroblasts (HDFs) were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum (FBS) and kept in a humidified 5% CO_2 at 37°C. HDFs from passages 6 to 10 were used in this experiment. HDFs (1.5×10^5 cells/well) were seeded into 100Φ plates (Falcon, Becton Dickinson, USA) and cultured overnight. Prior to irradiation, when cells were 70-80% confluent, they were washed twice with phosphate buffered saline (PBS). UVA simulator (Spectroline, USA) filtered for the emission of UVA (320-400 nm), was used at a tube-to-target distance of 5 cm. The dose of UVA radiation, determined with a UV radiometer was set at 300 mJ/cm^2. During irradiation, control cells were treated identically, except for the exposure to UV light. After UV irradiation, fresh serum-free medium containing minerals were added to the cells at 37°C for 24 hrs.

Cell Viability Assay

Cell viability was measured by MTT assay[10,11]. HDFs were plated at a density of approximately 2×10^4 cells/well in 96-well plates. The minerals were treated for 24 hrs following H_2O_2 treatment for 3 hours. The cells were then incubated in MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution for 4 hrs at 37°C. The blue formazan produced was solubilized in dimethyl sulfoxide, and the optical density was read at 570 nm. The results were expressed in percentages relative to the control. All experiments were expressed as the mean ± S.E. of three separate experiments.

Determination of MMP Activity by Zymography

MMP (matrix metalloproteinase) enzyme activity was assayed by using gelatin zymography[34]. The conditioned media of 1.5×10^5 cells/well were separated by electrophoresis on 10% sodium dodecyl sul-

fate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% gelatin. The electrophoresed gel was washed twice with washing buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2.5% Triton X-100, followed by a brief rinsing in washing buffer without Triton X-100. Then, the gel was incubated with incubation buffer of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% NaN at 37°C. After incubation, the gel was stained with Coomasie brilliant blue G-250. The inhibitory effects of the ore minerlas on the MMP-1 and MMP-2 were compared with those of polyphenol, the reported photo-aging resistant.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from HDFs was extracted using RNeasy MiNi Kit (Qiagen, USA) according to the supplier's instruction. In order to synthesize cDNA, reverse-transcription polymerase chain reaction (RT-PCR) was performed using an Accupower RT Premix Kit (Bioneer, Korea) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed with oligonucleotide primers and Taq DNA polymerase in the presence of each cDNA of MMP-1, MMP-2 and β-actin gene. The forward primer used for MMP-1 cDNA amplification was 5′-AAAGGG AATAAGTACTGGGC-3′, and the reverse primer was 5′-AATTCCAGGAAAGTCATGTG. The forward primer for MMP-2 cDNA amplification was 5′-CACCCCTAAGAGATCCT-3′, and the reverse primer was 5′-GTGCATACAAAGCAAACTGC-3′. The reactions were incubated in an automatic heat-block DNA thermal cycler (Progene, Techne) for 28 cycles: denaturation for 30 sec at 94°C; annealing for 30 sec at 50°C; extension for 60 sec at 72°C. PCR products were electrophoresed on a 1.0% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and visualized by ethidium bromide staining. The intensity of each band was quantified using the ImageMaster™ 2D Elite software.

Determination of Procollagen Type I by ELISA

The expression of type I collagen was assayed by enzyme-linked immunosorbent assay (ELISA). HDFs (1.5×10^5 cells/well) were seeded into 100Φ plates and cultured overnight. The culture media were replaced with DMEM containing the minerals. After 48 hrs, the concentrations of procollagen type I in the media were measured by procollagen type I C-peptide ELISA kit according to the manufacturer's protocols (Takara BIO Inc). 160 ng/mL of PIP (procollagen type I carboxyl-terminal peptide) standard diluent was prepared, and then the PIP solution was added to each 100 μL of the Antibody-POD (horseradish peroxidase) conjugated solution. And then the sample was treated in the wells of an antibody coated microtiter plate at 37°C for 3 hours. The wells were aspirated and each well was washed 4 times with 400 μL of phosphate buffered saline. 100 μL of substrate solution was added into each well and incubated at room temperature for 15 min. After adding 100 μL of stop solution (1 N H_2SO_4), the optical density at 450 nm of each well was measured. All experiments were expressed as the mean ± S.E. of three separate experiments. The stimulatory effect of the ore minerals on the collagen biosynthesis was compared with that of vitamin C, the known stimulator of collagen biosynthesis not only by promoting hydroxylase activity, but also by increasing the steady state level of procollagen mRNA.

Elastase Inhibition Assay

The inhibitory effect of the minerals on the elastase was determined by using human neutrophil elastase (Sigma, USA), and N-succinyl-Ala-Ala-Ala-p-nitroanilide was used as a chromogenic substrate. The hydrolysis reaction was performed under buffered conditions (0.05 M Tris-HCl, pH 8.4) in 96 well microplates to which 50 μL of buffer solution containing elastase (1.0 unit/mL) and 50 μL of sample solution were added. After adding 100 μL of 0.5 mM chromogenic substrate for 10 min at 37°C, the formation of the substrate hydrolysis product (*p*-nitroaniline) was measured at 410 nm using a microplate reader[25]. All experiments were expressed as the mean ± S.E. of three separate experiments. The elastin production-inhibiting activity of the ore minerals was compared with that of polyphenol, the known elastase inhibitor.

Acknowledgements

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