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Sun-Young Kim MA, Su-Jong Kim MA, Jin-Young Lee MA, Wan-Gi Kim MA, Won-Seok Park MA, Young-Chul Sim PhD & Sang-Jun Lee PhD

Pharmaceutical & Health Research Institute, AmorePacific Corporation R&D Center, Yongin, Kyonggi, KOREA

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Original Research

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Sun-Young Kim, MA, Su-Jong Kim, MA, Jin-Young Lee, MA, Wan-Gi Kim, MA, Won-Seok Park, MA,
Young-Chul Sim, PhD, Sang-Jun Lee, PhD
Pharmaceutical & Health Research Institute, AmorePacific Corporation R&D Center, Yongin, Kyounggi, KOREA
Key words: isoflavones, UV, skin, photoaging, hairless mouse, fibroblasts

Objective: This study investigated the anti-aging effects of dietary isoflavones on photoaged hairless mouse skin.

Methods: Female hairless mice were administered soy isoflavone extract orally and irradiated with UV light for four weeks. The effects of the isoflavones on the skin appearance, collagen deposition and epidermal thickness in the UV-damaged mouse skin were measured using bioengineering and histochemical methods. In addition, the influence of the isoflavones on the collagen metabolism in the UVB-irradiated human skin fibroblasts was also investigated.

Results: In the isoflavone treated group, the skin had a better appearance and less wrinkling than that of the control group. Additionally, the amount of collagen deposition was higher in the isoflavone group. In the human fibroblast cells, the amount of procollagen de novo synthesized did not increase after isoflavone treatment and/or UV irradiation. However, the increase in the expression of the metalloproteinases (MMPs) as a result of UV irradiation was suppressed by the isoflavone treatment.

Conclusions: It appears that isoflavones had an anti-aging effect on the UV-damaged hairless mice model, which is partly due to the inhibitory effects on UV-induced MMP-1 expression and the subsequent collagen degradation.

INTRODUCTION

Chronic exposure of human skin to UV radiation is known to damage the structure and function of the skin. These changes are referred to collectively as photoaging, which is characterized by wrinkles, laxity, roughness and irregular pigmentation [1]. Photodamaged skin displays prominent alterations in the cellular component and extracellular matrix of the connective tissues such as an accumulation of disorganized elastin fibers (elastosis), a marked increase in glycosaminoglycans (GAGs), and a loss of interstitial collagens [2].

The unifying pathogenic agents responsible for these changes are UV-induced reactive oxygen species (ROS) that deplete and damage the non-enzymatic and enzymatic antioxidative defense systems of the skin, leading to oxidative damage of the cellular and non-cellular components and ultimately skin cancer, immunosuppression and premature skin aging [3]. ROS are believed to activate the cytoplasmic signal transduction pathways in the resident fibroblasts, which are related to growth, differentiation, senescence and connective tissue degradation, as well as causing permanent genetic changes [4]. Considering that UV induces oxidative stress-mediated adverse effects in the skin, the regular intake of antioxidants and antioxidant nutrients as well as an antioxidant topical treatment is suggested to be a useful way to reduce the harmful effects of UV radiation [5].

In recent years, isoflavones have attracted increased attention owing to their health-related beneficial aspects [6]. In addition to its diphenolic structure-based estrogen-like effect, isoflavones are associated with a broad range of biological activities that include antioxidant properties and inhibitory effects on the several enzymes of the estrogen receptor-independent signal pathways [7]. Genistein, the primary isoflavone from soy products, is known to enhance the antioxidant enzyme activity...
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activities such as superoxide dismutase (SOD), catalase and glutathione reductase in various mouse organs [8], and it has also been shown to inhibit tyrosine kinase and topoisomerase [9,10]. Therefore, an investigation into the protective effects of dietary soy isoflavones on UV-damaged skin in the nutritional aspect is warranted. Although several experimental studies have suggested that the topical treatment of soybean isoflavone genistein inhibits UVB-induced skin tumorigenesis in a hairless mice model [11], there has been no study on the influence of soy isoflavones on the photoaging aspects in vivo and in vitro.

This study investigated the protective effects of dietary isoflavones on UV-induced skin aging in a hairless mice model. The effects of the isoflavones on the skin appearance, collagen and epidermal thickness in the UV-damaged mouse skin were examined. The effects of isoflavones on collagen synthesis/degradation and the metalloproteinases (MMPs) were also investigated in UVB-damaged fibroblast cultures.

MATERIALS AND METHODS

Animal Study

Animals. Six week old female albino hairless mice (Skh: hr-1) were obtained from the Charles River Laboratories (Wilmington, MA). The animals were housed eight per cage in a controlled temperature (23 ± 2°C), humidity (55 ± 10%) and light (12 hours light/12 hours darkness, without any ultraviolet emission). They were allowed ad libitum access to water and food ad libitum. The integrated UV irradiance was measured by 70 mJ/cm² (1 week), then 80 mJ/cm² (2 weeks) to afford a total dose of 600 mJ/cm² over the four weeks. During the period of exposure the mice were group-housed in a stainless steel irradiation chamber, and the animals could move around freely in the chamber. The integrated UV irradiance was measured with a Waldmann UV meter (Waldmann Lichttechnik GmbH, Germany). A non-irradiated group of animals was included as control. The animals were killed three days after the final irradiation to allow the recovery from the acute UV effects.

Skin Surface Physiology. The transepidermal water loss (TEWL: mg/cm²/hour) was regularly measured with Tewameter TM210 (Courage + Khazaka Electronic, Köln, Germany). At the end of the study, prior to killing, skin replicas were made from the back skin of the hairless mice using a SILFLO impression material (Flexico, England). The SkinVisiometer SV500® (Courage + Khazaka Electronic, Köln, Germany) was used to assess the roughness of the skin. The following parameters for the skin roughness were calculated: Rt (depth of roughness), Rz (mean depth of roughness), Rm (maximum roughness) and Ra (mean roughness).

Histological Evaluation. On the day after the final sample administration, the mice were killed, and biopsies were obtained from the central dorsal skin, perpendicular to the long axis of the trunk. The biopsies were fixed in 10% buffered formalin and prepared for optical microscopy.

Immunohistological analysis of type I pN collagen was performed using the methods described previously [12]. Type I pN collagen was detected using the mice monoclonal IgG1 antibodies raised against the aminoprepeptide region of the human type I procollagen. Haematoxylin-eosin (H&E) staining was conducted for a routine examination of the tissue and to measure the epidermal thickness.

Quantitative analysis of the histology sections was carried out using two contiguous histological sections, which were each photographed at the midpoint of the section (H&E stain: X100 magnification). The thickness of the epidermis was evaluated by taking 10 representative sample measurements per tissue section on the microscope.

In Vitro Study

Cell Culture. Human fibroblasts Hs68 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL, NY) supplemented with fetal bovine serum (10%, v/v) and antimycotic-antibiotics (1%). The cells were incubated at 37°C in a 5% CO₂, and 90% air humidified
incubator. The cells were grown on 100 mm culture dishes to subconfluence (about 80%–90%).

Irradiation of Fibroblast Culture and Measurement of Secreted Interstitial Collagenase. The fibroblast cells (Hs68) were plated on a 48-well plate (5 x 10^4 cells per well). After 24 hour serum deprivation, the cells were washed with phosphate-buffered saline (PBS). The fibroblast cells were irradiated with a UVB (15 mJ/cm^2) source (Dermlight cube 401 equipped with UVB filters, Uvatec, Inc., Germany) through a thin layer of PBS in the wells. After irradiation, the fibroblasts were re-incubated with serum-free DMEM containing either retinoic acid (1 μM), transforming growth factor-β (TGF-β: 10 ng/mL) or the isoflavones (1 ppm, 10 ppm). The supernatant from the fibroblast cell culture was collected 24 hours later, and the interstitial collagenase was determined using a MMP-1 human enzyme-linked immunosorbent assay (ELISA) Kit (#PRN2610, Amersham, Sweden) [13]. The cellular protein concentrations were measured with a commercial BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. The concentrations of MMP-1 in the fibroblast cultures are shown as ng/gram of the cellular protein. Each sample was tested in triplicated wells.

Statistics

The results are expressed as a mean ± SD. An analysis of variance (ANOVA) and a Student’s t-test for paired samples were conducted. The data was analyzed using the SPSS package (SPSS, Chicago, IL). Statistical significance was defined as p < 0.05.

RESULTS

Clinical Observation

During the study period, the body weight of the hairless mice was measured regularly. Mean body weights of the non-irradiated control group, UV-irradiated control group and isoflavone group were similarly increased throughout the period of study. There was no significant difference in the mean body weight among the groups (data not shown). Food consumption during the experimental period was not different among the three groups (data not shown). Therefore, it seems that body weight gain and food consumption were not affected by UV irradiation and isoflavone treatment.

The transepidermal water loss (TEWL) was also regularly measured. UV irradiation induced an increase in TEWL both in the control and isoflavone groups. However, there was a larger increase in TEWL in the control group compared to the isoflavone treated group although this was not statistically significant due to the large variance of the TEWL values (p < 0.05) (data not shown).

During the period of UV exposure, the hairless mice were observed for fine wrinkling of the skin in both groups. However, the isoflavone treated group appeared to have less wrinkles than the UV control group. Skin replicas were taken from the normal age-matched mouse skin, the UV-irradiated mouse skin given the vehicle as a control and the UV-irradiated mouse skin given the isoflavones. Fig. 1A indicates that the UV irradiation induced more wrinkles on the hairless mouse skin than the age-matched normal mouse skin. However, a skin replica of the control mice had more coarse wrinkles than the isoflavone treated mice. To quantitatively assess the skin roughness, a skin visiometer was applied through the replicas. Fig. 1B represents the mean values of the skin roughness of parameters; Rz (mean depth of roughness), Ra (mean roughness), Rm (maximum roughness) and Rt (depth of roughness). Except for the Ra, all the roughness parameters showed significantly higher values in the UV irradiated control group than in the unirradiated group. The Rt, Rz, and Rm values of UV-irradiated skin were 36%, 45% and 48% higher, respectively, than those of in the age-matched normal mice. However, in the isoflavone group, the Ra, Rm and Rt values were significantly 24%, 23% and 19% lower, respectively, than in the UV irradiated control group.

Histological Observations

UV irradiated hairless mice showed greater changes in the epidermis than in the age-matched normal mice (Fig. 2A). Fig. 2B shows the histological measurements of the epidermal thickness of the hairless mouse skin. Measurements of the epidermal thickness showed significant increases in both the...
UV control group and the isoflavone group compared to the age-matched normal group. UV irradiation induced an 86% increase in the epidermal thickness relative to the age matched normal group after four weeks irradiation. However, the epidermal thickness of the isoflavone treated group was 80% of that of the UV irradiated control group. The mean epidermal thickness in the isoflavone group (23.73 ± 2.25 µm) was significantly thinner than in the UV control group (29.31 ± 2.04 µm).

Fig. 3 shows the immunostaining results of the type 1 procollagen in the UV-irradiated skin as well as in the normal hairless mouse skin. Collagen fibers appeared to be lower in the UV-irradiated control skin compared to the normal mouse skin, as estimated by histochemical analysis for procollagen. However, isoflavones increased the procollagen staining intensity in the epidermis/dermis junction and the dermis.

DISCUSSION

Recently, efforts to reveal the relationship between food intake and human health including skin condition have been increasing [14]. It is well known that the skin function and appearance is affected by the nutritional status. Dietary supplementation in the animals with vitamins, minerals, or essential fatty acids results in improved skin conditions. On photodamaged skin, it has been reported that some nutrients such as vitamin A, E, C and herbal extracts including green tea exhibit protective activities due to their antioxidant effects [15–18]. In this respect, the importance of the dietary source for photoprotection has gained a great deal of interest.

Isoflavones are hormone-like compounds from a dietary source, which have beneficial health-related effects due to their diphenolic structure [19]. In addition, it is also believed that...
many of the protective effects of the isoflavones are related to their antioxidant activities [20]. Despite the many reports linking the beneficial properties of isoflavones to their antioxidant activities, no study investigating the anti-aging efficacies of the dietary isoflavones on UV-damaged skin, which represents an excellent and accessible model organ for examining environmental oxidative damage, has been conducted.

This study investigated the positive effects of orally administered isoflavones on UV-induced skin aging using a hairless mouse model for the first. These results demonstrated that the dietary isoflavones have photoprotective effects in a hairless mouse model.

As shown in the results, UV irradiation induced an increase in the number of wrinkles and a decrease in the resilience in the hairless mouse skin. However, the isoflavone treated group had a better skin appearance and less wrinkling than the control group according to the analysis using a skin visiometer as well as a visual observation. In addition, the mechanically measured roughness parameters including Ra, Rm, and Rt showed lower values for the isoflavone group than in the control group (Fig. 1B).

Hairless mice have been extensively used in histochemical studies on the effects of UV irradiation [21]. Continuous UV irradiation is known to induce a progressive thickening of the mouse epidermis, which histologically mimics the hyperkeratinization and hyperplasia associated with photoaging and sun-exposed skin in humans [22]. In this study, four weeks of UV irradiation caused the thickness of the epidermis of hairless mice compared to the age-matched, normal mice. However, the mean epidermal thickness in the UV/isoflavone group was significantly thinner than that of the UV irradiated group without an isoflavone supplement.

A great number of studies suggest that photoaging causes alterations in the composition, organization and structure of the collagen in the skin dermis [2] and that the reduction of collagen is responsible for the old and wrinkled appearance [23]. The histochemical assay conducted in this study showed that the amount of collagen was lower as a result of UV irradiation, as reported previously [2]. However, the isoflavone administered group had more collagen deposition in the dermis than the control group.

As to their proteolytic activity, various UVB-induced matrix-degrading metalloproteinases (MMP-1, MMP-3) in dermal fibroblasts contribute to the breakdown of dermal interstitial collagen and other connective tissue components and thus, initiate cutaneous photoaging [24]. It is well known that the amount of collagen in the dermis is controlled by type 1 procollagen synthesis and its degradation by MMP-1 [25]. We decided to examine the amounts of type 1 procollagen and MMP-1 in a UVB-irradiated fibroblast culture to determine whether or not the isoflavones increased procollagen de novo synthesis or prevent collagen degradation by MMPs. The results show that the isoflavones did not increase the amount of collagen synthesis in the UV-damaged fibroblasts (data not shown). Rather, the UVB-induced MMP-1 was reduced by

46% in the isoflavone treated fibroblast culture (Fig. 4). Overall, these findings suggest that the increased collagen fibers in the UV-damaged/isoflavone treated hairless mouse skin compared to the control group is due to the decrease in collagen breakdown by MMP-1, and not to the increase in collagen synthesis.

It was reported that UV-generated ROS results in the activation of the transcription of the MMP encoding genes [3]. The MMPs are a family of proteolytic enzymes that specifically degrade collagen, elastin and other proteins in the connective tissue and bone. The transcription of the genes for the MMPs is dependent on the transcription factor activation protein 1 (AP-1), and nuclear factor-kappa B (NF-κB), which is activated by UV irradiation [25]. It is possible that a free radical scavenger can prevent UV-induced dermal damage by inhibiting the induction of the MMPs. Retinoic acid, which is known for its ability repair photoaged skin, has been shown to interfere with the UV/ROS initiated signal transduction pathways of MMPs induction [26]. In a recent study, occlusive pretreatment of genistein blocked cJun protein and its driven enzyme (collagenase) induced after UV irradiation, which resulted from inhibiting the tyrosine kinase and anti-oxidant activities [27]. Therefore, isoflavones with antioxidant activities might at least partly prevent photoaging by working as oxygen radical scavengers. The effects of the isoflavones on the AP-1 and NF-κB signal pathways need to be studied.

It has been reported that estrogen replacement therapy results in an improvement in the skin appearance and an increase in the amount of collagen in menopausal women [28]. Although estrogen or estrogen-like compounds may reduce the skin aging caused by an estrogen deficiency resulting from chronological aging, there is insufficient evidence showing the photoprotective activity of the estrogens. Therefore, further studies are needed to determine the role of the isoflavones in the collagen metabolism of UV-damaged skin.

To our knowledge, this is the first study to describe the photoprotective effect of orally administered isoflavones in an animal model. However, more studies particularly related to the signaling pathways are needed in order to clarify the molecular mechanism of the isoflavone on anti-photoaging.

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