J. Soc. Cosmet. Scientists Korea
Vol. 40, No. 3, September 2014, 279-287
pISSN 1226-2587
eISSN 2288-9507
http://dx.doi.org/10.15230/SCSK.2014.40.3.279

피부 섬유아세포에서 다이드제인의 파이토에스트로겐 효과

김 미 선† · 홍 찬 영 · 이 상 화

(주)LG생활건강 기술연구원

(2014년 2월 28일 접수, 2014년 3월 19일 수정, 2014년 7월 2일 채택)

The Phytoestrogenic Effect of Daidzein in Human Dermal Fibroblasts

Mi-Sun Kim†, Chan Young Hong, and Sang Hwa Lee

R&D Center, LG Household & Healthcare Ltd., 175 Gajeong-ro, Yuseong-gu, Daejeon 305-343, Korea

(Received February 28, 2014; Revised March 19, 2014; Accepted July 2, 2014)

요 약: 폐경 이후 여성에서 발생되는 에스트로겐의 감소는 피부노화와 밀접한 관련이 있으며, 피부의 정상적 상태와 기능을 저하시키게 된다. 지난 10여 년간 보고된 많은 연구결과를 살펴보면, 에스트로겐은 폐경 이후 여성의 피부에서 콜라겐 감소를 막아주고, 탄력을 회복시키며, 건조한 피부를 개선시키는 등의 피부 안티에이징 효능을 가지는 것을 알 수 있다. 에스트로겐과 유사한 구조로 인해 파이토에스트로겐이라 알려진 이소플라본은 자외선에 의해 유도된 피부 손상을 보호하는 기작이 널리 알려져 왔으나 피부세포에서 에스트로겐과 유사한 안티에이징 효능을 가지는지에 대해서는 많은 연구가 진행되지 않았다. 이에 본 연구에서는 콩류 등에 많이 함유된 이소플라본인 다이드제인이 에스트로겐과 유사한 활성 및 효능을 가지는지 밝히고자 하였다. 먼저 에스트로겐 수용체와의 결합을 통한 transcriptional activity에 미치는 효과를 luciferase assay를 통해 살펴본 결과, 다이드제인은 대조군에 비해 농도 의존적으로 estrogen receptor−dependent transcriptional activity를 유의하게 증가시켰다. 다음으로 사람의 피부 섬유아세포를 이용하여 다이드제인이 세포외 기질단백질 성분들의 발현에 미치는 효과를 조사한 결과, 다이드제인은 콜라겐 타입 I, 콜라겐 타입 IV, 엘라스틴 및 피브릴린−1의 발현을 유의하게 증가시키는 것을 확인할 수 있었다. 모든 실험조건에서 에스트로겐 단독 효능과의 비교 분석을 통해 다이드제인은 에스트로겐과 유사한 효능을 가진다는 것을 확인할 수 있었다. 본 연구 결과를 통해 다이드제인은 기존에 알려진 이소플라본의 광보호 효능과 더불어 파이토에스트로겐 효능을 가짐으로써 갱년기 여성의 피부 안티에이징을 위해 활용할 수 있을 것이라 제안한다.

Abstract: Estrogen deficiency results in a reduction of skin quality and function in postmenopausal women. Over the past decade, many studies have supported that estrogen provides anti-aging effects as a result of the ability of estrogen to prevent skin collagen decline, restore skin elasticity, and increase skin hydration in postmenopausal women skin. Due to their structural similarity with estrogen, isoflavones have been called phytoestrogens. Photoprotective effects of isoflavones are well established while their estrogenic-like activities are not fully understood in human skin. In this study, we investigated whether daidzein, an effective isoflavone, has phytoestrogenic activity and induces transcriptional change of extracellular matrix components in dermal fibroblasts. We examined the luciferase activity of daidzein and β-estradiol using transiently transfected NIH3T3-ERE cells. The estrogenic receptor-dependent transcriptional activity was increased in a dose-dependent manner when treated with daidzein, with a maximum of 2.5-fold induction at 10 μg/mL of daidzein compared with non-treated control. In addition, daidzein significantly in creased the expressions of collagen

† 주 저자 (e-mail: misunkim@lgcare.com)

type I, collagen type IV, elastin, and fibrillin-1 in human dermal fibroblasts. By comparing with the effects of β-estradiol through out all the experiments, we confirmed that daidzein had estrogenic activity and function in fibroblasts. These results suggest that daidzein-based application, having both photoprotective and phytoestrogenic effects, may be a powerful approach for skin anti-aging of postmenopausal women.

Keywords: *daidzein, estrogenic activity, extracellular matrix, postmenopausal women*

1. Introduction

Skin aging is a combined result of intrinsic and extrinsic factors. Intrinsic (chronologic) skin aging represents the biological passage of time of skin and is characterized of smoothness, paleness, low elasticity, and fine wrinkles[1]. There has been growing interest in hormonal involvement in chronological skin aging processes. To our knowledge, there is a direct or indirect connection between hormonal deficiency and skin aging[2]. Patients with hormone deficiency present signs of early skin aging such as dry, thin, and wrinkled skin. A decrease in steroid hormones induces a reduction of skin functions that are under hormonal control in the skin.

Women spend one third or more of their lifetime in the postmenopausal period. A sudden hormonal change occurs such as loss of estrogen production in the period[3]. The estrogen deficiency may be a common and important factor for climacteric symptoms and skin aging in postmenopausal women[4]. Many women report a sudden onset of skin aging several months after menopausal symptoms begin. Decrease of circulating estrogens is associated with increased skin dryness and slackness and decreased skin elasticity, thickness, and collagen content[5,6]. Clinical studies have shown that systemic estrogen treatment improves skin quality in postmenopausal women[7,8]. In addition, it has been reported that topical estrogen application has recovery effect on signs of aging in postmenopausal women skin[9,10]. Kainz C et. al. showed that estrogen ointment applied to the face of postmenopausal women could not induce significant change in serum hormone levels[11]. These studies demonstrate that estrogen might be a powerful anti-aging candidate without the potential safety issues in postmenopausal women skin. However, estrogen can not be used as a general cosmetical ingredient because it is a hormone.

On the basis of their structural similarity with estrogen, isoflavones have been called phytoestrogens. There are three main classes of phytoestogens - isoflavones, lignans, and coumestans. Among them, isoflavones are well known for their effectiveness on reducing radical oxygen species as a natural antioxidant. Because of their photoprotective effect, they are considered a good ingredient against UV-induced photodamage in skin[12]. Numerous studies have explained the anti-inflammatory and antioxidant effects of isoflavones[13,14]. Isoflavones are non-steroidal substances of vegetal origin that can bind estrogen receptor (ER) and exert estrogenic or anti-estrogenic effects. ER exists as two subtypes, ERα and ERβ, that are related but distinct nuclear hormone receptors. Recently, it has been suggested that ERβ is a key target for anti-aging in the skin[15]. Chang et. al. reported that ERβ-selective compounds significantly reduced UV-induced wrinkle formation in an animal model of photoaging[16]. Genistein and daidzein have a higher binding affinity for ERβ than ERα in competition binding assays[17]. The effect of topical application of genistein on postmenopausal women skin has been reported. Topical application of genistein stimulated production of hyaluronic acid[18] and increases of epidermal thickness as well as blood vessels[19]. Despite the effective action of phytoestrogens on aged human skin, the precise action mechanism of daidzein, another well-known phytoestrogen, in skin cells is still unknown.

In this study, we show the effect of daidzein on ER-dependant estrogenic transcriptional activity. Furthermore, we investigate whether daidzein would induce transcrip-

tional changes of extracellular matrix (ECM) components such as collagen, elastin, and microfibril in human dermal fibroblasts. We finally propose that a non-steroidal plant isoflavone, daidzein, has estrogen-like biological activity and will help to improve skin aging in postmenopausal women's skin.

2. Materials and Methods

2.1. Cell culture

NIH3T3 cell line, a fibroblast and suitable transfection host (ATCC CRL-1658), was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum, penicillin (400 units/mL) and streptomycin (50 g/mL). Normal human dermal fibroblasts (NHDFs) derived from the dermis of normal adult skin were obtained from Lonza (USA) and cultured in fibroblast basal medium (FBM, Lonza) supplemented with growth factors, fetal bovine serum, gentamicin, and amphotericin B. Cells were maintained in humidified 5% CO_2 atmosphere at 37 ℃.

2.2. Reagents

Cell culture media were purchased from Gibco BRL (USA). Daidzein and β-estradiol were from Sigma-Aldrich (USA). Estrogen receptor (ER)-responsive luciferase construct was from Qiagen (Germany) and dual-luciferase reporter assay detection system was from Promega (USA). For RT-PCR, ERα, ERβ, and GAPDH primer sets were synthesized by Bioneer Co. (Korea). Primer/probe and reagents for real-time q-PCR were obtained from Applied Biosystems (Life Technology Co., USA). All other chemicals were from Sigma-Aldrich.

2.3. Dual-luciferase reporter assay

NIH3T3 cells (1×10^5 cells/well) were seeded in 24-well plate. The following day, cells were transfected with ER-responsive luciferase construct having tandem repeats of estrogen response elements (ERE, Qiagen). The used ERE reporter plasmid is a mixture of an inducible ER-responsive Firefly luciferase construct and constitutively expressed Renilla construct as an internal control (40 : 1). Transfection was carried out using Lipofectamin 2,000 reagent (Invitrogen, USA). The transfected cells were treated with daidzein or β-estradiol for 24 h. We monitored the ER-mediated activity using dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). After stopping reaction with final substrates, the samples were read by luminometer Victor 3 (PerkinElmer, Finland). Experiments were performed at least three times and the data were assessed as folds of firefly luciferase activities normalized to the renilla luciferase control activities from individual wells.

2.4. Semiquantitative RT-RCR

Daidzein-treated for 24 h or non-treated NHDFs (5×10^5 cells) were rinsed with PBS and harvested and total RNA was extracted using RNeasy Mini Kit (Qiagen). One μg of total RNA was converted to cDNA using reverse transcriptase at 45 ℃ for 1 h using cDNA Synthesis Kit (PhileKorea, Korea). PCR reactions were performed using 1 - 2 μL of cDNA and aliquoted PreMix PCR master mix (Bioneer Co.) with Mycycler PCR instrument (Bio-Rad, USA). Optimal semi-quantitative conditions were set to fall in the linear PCR product range. PCR profiles were as follow; 94 ℃ for 30 s, 55 ℃ (ERα) or 53 ℃ (ERβ) for 30 s and 72 ℃ for 30 s. In parallel, GAPDH gene was amplified in each RNA sample as an internal control. The used ERα primers were 5'-TAC TGC ATC AGA TCC AAG GG-3' (forward) and 5'-ATC AAT GGT GCA CTG GTT GG-3' (reverse). ERβ primers were 5'-GCA AGA TCG CTA GAA CAC-3' (forward) and 5'-TTA TGT CCT TGA ATG CTT C-3' (reverse). GAPDH primers were 5'-ATT GTT GCC ATC AAT GAC CC-3' (forward) and 5'-AGT AGA GGC AGG GAT GAT GT-3' (reverse). The primer sets yielded PCR products of 650 bp, 215 bp and 565 bp for ERα, ERβ, and GAPDH, respectively.

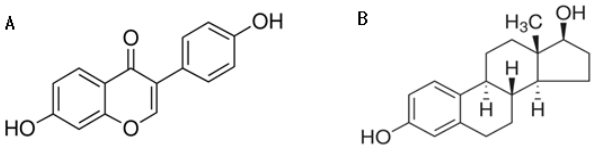

Figure 1. The structure of (A) daidzein and (B) β-estradiol.

2.5. Quantitative real time RT-RCR

NHDFs (5×10^5 cells) were treated with daidzein or β-estradiol for 24 h. Cells were washed and total RNA was isolated. Extracted total RNA (1 μg) was converted to cDNA (aforementioned in RT-PCR section). Gene expression was analyzed using StepOnePlus™ real-time PCR system (Applied Biosystems). All reactions were performed in triplicate for 40 cycles. The relative fold expression levels of collagen type I α1 (COL1A1), collagen type IV α1 (COL4A1), elastin (ELN), fibrillin-1 (FBN1), ERα, and ERβ were calculated. The threshold cycle (Ct) was determined and normalized to the average of housekeeping gene's (GAPDH) level (⊿Ct). The ⊿Ct of chemical-treated cells was then subtracted from non-treated control cells (⊿⊿Ct) and the relative fold expression was calculated using equation $2^{-\Delta\Delta Ct}$.

2.6. Statistics

Results are expressed as means ± SD of three independent experiments. The significance of differences between two independent groups was analyzed using the Mann-Whitney U-test. For all tests, p-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Effect of daidzein on ERE-mediated transcriptional activity

Estrogens mediate their effects by interacting with specific their receptors, ERs, which act as ligand-activated nuclear transcriptional factors. Activated ERs bind to specific regions in target gene promoters and modulate the target gene expression. We first examined the luciferase activity of about 300 chemical compounds which belong to the derivatives of triterpene or flavonoid of natural origin. In transiently transfected NIH3T3-ERE cells, daidzein was the most effective. The ER-dependent transcriptional activity was increased in a dose-dependent manner, with maximal 2.5-fold induction at 10 μg/mL (Figure 2). β-estradiol also induced significant increase (2-fold) in the ER-ERE-mediated luciferase activity at 0.1 μg/mL compared to non-treated control (p < 0.05).

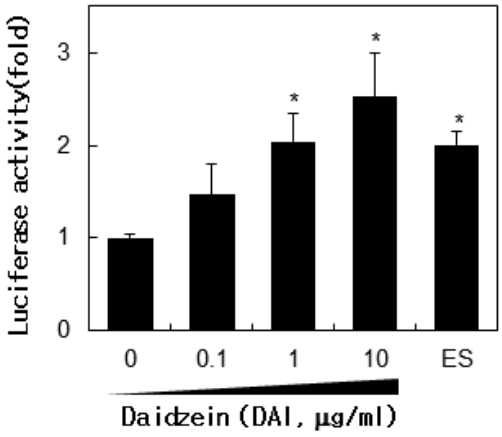

Figure 2. Effects of daidzein on transcriptional activity through estrogen receptor. NIH3T3 cells were transfected with plasmids containing tandem repeats of estrogen responsive elements (ERE). The transfected cells were treated with various concentrations of daidzein at 0.1, 1, and 10 μg/mL or β-estradiol (0.1 μg/mL). After 24 h treatment, the luciferase activities of the cells were analyzed using dual-luciferase reporter assay system. *, p < 0.05 compared with non-treated control cells. DAI, daidzein; ES, β-estradiol.

3.2. Effect of daidzein on ER expression

Secondly, the mRNA expression of ER was detected in NHDF cells and the effect of daidzein on ER expression was examined. To date, two isoforms of the nuclear ER, ERα and ERβ, have been identified, cloned, and characterized from several species[20,21]. ERα predominates in reproductive tissues whereas ERβ is more highly expressed in peripheral non-reproductive tissues. Two predominant ERs were detected in human skin. According to reports using specific antibodies to ERα and ERβ, ERβ is widely expressed in human skin while ERα is only

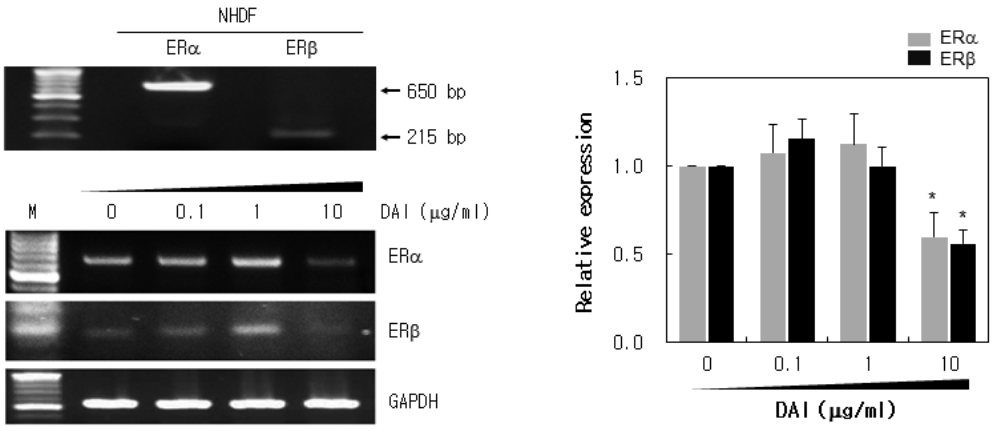

Figure 3. Effects of daidzein on the mRNA expressions of ERα and ERβ in normal human dermal fibroblasts (NHDF). (A) Total RNA was extracted from non-treated and daidzein-treated NHDFs (5×10^5 cells). The mRNA expressions of ERα and ERβ were determined by semiquantitative RT-PCR. (B) The mRNA expressions of ERα and ERβ were measured by quantitative real-time RT-PCR in various daidzein-treated NHDFs. *, $p < 0.05$ compared with non-treated control cells. ERα, estrogen receptor α; ERβ, estrogen receptor β; GAPDH, housekeeping gene; DAI, daidzein.

found in very few subsets of structures[22]. Recently, ER β has been suggested as a novel target for prevention and treatment of anti-aging in skin[15,16].

To address the effects of daidzein and β-estradiol, we assessed basal gene expression of ERs in NHDFs. Similar to the previous report, we detected the expression of ERα and ERβ in NHDFs. Daidzein treatment (10 μg/mL) induced decrease in the mRNA expression of ERs in RT-PCR analysis (Figure 3A). As shown in Figure 3B, the mRNA levels of ERs upon daidzein treatment were confirmed using quantitative real-time RT-PCR. The expression of ERs was significantly decreased by 10 μg/mL daidzein treatment in NHFBs. It demonstrates that the production of ERs decreases when the ligand level is high. Our data are in agreement with previously published studies that demonstrated that estrogen treatment induces downregulation of ER expression. Downregulation of receptors by their ligands is a fundamental process to control sensitivity against stimuli. It is reported that transcriptional and post-transcriptional events contribute to the estradiol-induced loss of ER expression in MCF-7 cells[23]. More recently, it was explained that decreases in ERα protein and mRNA levels in response to estrogen happened via ubiquitin-protease pathway and Sin3A action at the proximal promoter, respectively[24].

3.3. Effect of daidzein on the expression of ECM components

The classical mechanism of estrogen action in cells involves interaction with intracellular receptors and regulation of gene transcription. Once bound by a ligand, ER undergoes conformational change, allowing it to modulate the transcription of target genes. Most clinical studies demonstrated a beneficial effect of estrogen treatment on the collagen content of skin[25]. Estrogen induces increases of collagen as well as hyaluronic acid and fibroblast proliferation in postmenopausal skin[18,26].

Thus, we next examined whether daidzein affected the expression of the components of collagen fibers and elastic fibers. The mRNA expressions of collagen type I, collagen type IV, elastin, and fibrillin-1 were measured after daidzein treatment in NHDF. We first compared the effects with that of β-estradiol (Figure 4A). Daidzein showed similar effects as β-estradiol on the expression of ECM components at the same concentration (0.1 μg/mL). As shown in Figure 4B, daidzein significantly increased the mRNA expression of collagen type I, colla

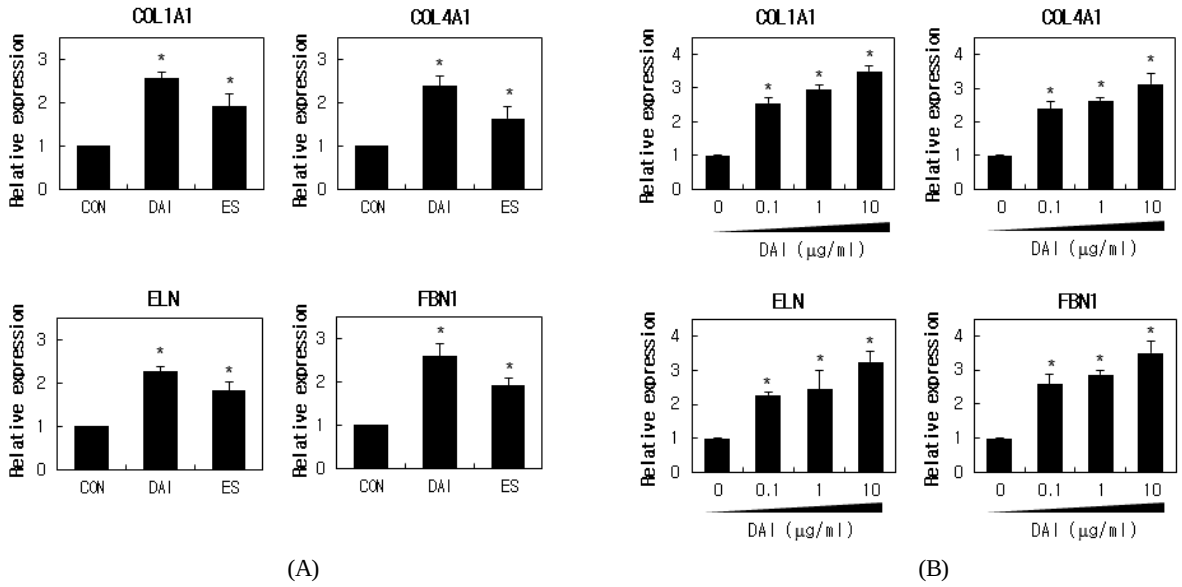

Figure 4. Effects of daidzein on the mRNA expressions of extracellular matrix (ECM) components in normal human dermal fibroblast (NHDF). (A) 5×10^5 NHDFs were treated with 0.1 μg/mL of daidzein or β-estradiol for 24 h. The gene expression levels of collagen type I, collagen type IV, elastin, and fibrillin-1 were measured and compared using quantitative real-time RT-PCR. (B) NHDFs were treated with various concentrations of daidzein at 0.1, 1, and 10 μg/mL for 24 h, and the expression levels of collagen type I, collagen type IV, elastin, and fibrillin-1 were analyzed using quantitative real-time RT-PCR. *, $p < 0.05$ compared with non-treated control cells. COL1A1, collagen type I α1; COL4A1, collagen type IV α1; ELN, elastin; FBN1, fibrillin-1; DAI, daidzein; ES, β-estradiol.

gen type IV, elastin, and fibrillin-1 in a dose-dependent manner. The treatment of 10 μg/mL daidzein increased the mRNA levels of collagen type I, collagen type IV, elastin, and fibrillin-1 by 3.5-, 3.1-, 3.3-, and 3.5-fold, respectively ($p < 0.05$ vs non-treated control). As aforementioned, ER is a ligand-activated transcription factor that can both activate and repress the expression of genes. Transcriptional regulation of ECM componets by daidzein might be independently mediated by ER transcription (Figure 3).

In aged skin, the number of keratinocyte and fibroblast decreases and results in skin thinning and reduction of connective tissue. Skin connective tissue is primarily comprised of fibrillar collagen bundles and elastic fibers that fill extracellular spaces[27]. Collagen and elastic fibers are responsible for the structural and elastic qualities of skin and gradually decrease with age. Collagen type I is the most abundant fibril-forming collagen in skin, and the massive loss of this protein with age is regarded as the primary cause of wrinkle formation. Collagen type IV is a major component of the dermal-epidermal junction and attaches to each other to form complex protein networks. In human skin, collagen type IV is a unique basement membrane component and declines with aging[28]. It is well known that menopause-associated estrogen decline affects the reduction of collagen production in postmenopausal women's skin, which accelerates the climacteric skin aging. Brincat et al. found that skin collagen level declined by 2.1% per postmenopausal year, accompanied by a 1.1% decrease in skin thickness every postmenopausal year[29]. Thus, the improvement of collagens is might important in skin anti-aging of postmenopausal women.

Elastin and microfibril (fibrillin-1) are two main constituents of elastic fiber and provide elasticity and resilience in skin. Histologic study showed a general reduction of elastin and elastic fiber disintegration in aged human skin[30]. The relationship between elastic fibers and

estrogen deprivation was suggested by Bolognia et al. Accelerated degenerative changes in dermal elastic fibers have been observed in young women with premature menopause. In comparative analysis using age-related ultrastructural changes of elastic fibers, the changes in women with premature menopause appeared at least 20 years older than similar age-related changes[31]. Topical application of estrogen improved a local increase in the concentration and size of elastic fibers in postmenopausal women's skin[32]. Son et al. reported an increase of tropoelastin and fibrillin-1 in aged human skin by topical 17 β-estradiol treatment[9]. In addition to estrogen, there are reports about the evaluation of cutaneous healing and improvement in postmenopausal women skin using topical selective ER modulators[33] or phytoestrogen treatment[19]. However, the effect of daidzein on the expressional changes of dermal matrix proteins such as collagen type IV, elastin, and fibrillin-1 has not been studied extensively. In our study, we investigated in detail the effect of daidzein on estrogenic transcriptional activity after binding to ER and on ECM expression. Although we have not fully understood how daidzein induces such ER-mediated signaling changes in skin cells, we believe this effective isoflavone may act positively in postmenopausal skin aged by estrogen deficiency. It was reported that estrogen-like actions of isoflavone might not be strong enough to increase the risks of hormone-related cancers[34]. However, further investigation is required to determine the optimal dose for local benefits. Taken together, we suggest that daidzein has beneficial effects on the increase of matrix proteins and it may help improve postmenopausal skin aging through its phytoestogenic activity.

4. Conclusion

In summary, estrogen deficiency influences reduction in skin quality and function of postmenopausal women. Despite the well documented effects of estrogen on skin physiology and aging, there is very limited data on the effects of daidzein, a phytoestrogen. In this study, we investigated estrogenic activity of daidzein and compared the activity with that of β-estradiol. Daidzein is an effective phytoestrogen and had an ER-dependent transcriptional activity in a dose-dependent manner. In addition, daidzein significantly increased the mRNA expressions of collagen type I, collagen type IV, elastin, and fibrillin-1 in NHDF compared with non-treated control. We believe that the action of daidzein would arise from both phytoestrogenic and photoprotective effects and that it would protect against both intrinsic chronological aging and extrinsic aging in skin. Thus we suggest that daidzein-based application provides promising dermal benefits in improving or maintaining a youthful appearance of postmenopausal aged skin.

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