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In vitro and in vivo evaluation of topical delivery and potential dermal use of soy isoflavones genistein and daidzein

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ABSTRACT

Genistein, daidzein, and glycitein are soy isoflavones. These compounds can be used to protect the skin from oxidative stress induced by UVB radiation. To this end, the feasibility of skin absorption of soy isoflavones was evaluated in the present study. As assayed by flow cytometry, UVB-induced H_2O_2 production in keratinocytes was inhibited by genistein and daidzein, confirming that these two compounds can act as free radical scavengers when keratinocytes are photodamaged. Glycitein showed no protective activity against photodamage. The effects of vehicles on the in vitro topical delivery from saturated solutions such as aqueous buffers and soybean oil were investigated. The isoflavones in a non-ionized form (pH 6) showed higher skin deposition compared to the ionized form (pH 10.8). Soybean oil reduced the isoflavone amount retained in the skin, especially for genistein. Genistein generally exhibited greater skin absorption than did daidzein. However, daidzein permeation was enhanced when an aglycone mixture was used as the active ingredient. An eutectic effect was proposed as the enhancing mechanism. In vivo skin deposition showed a linear correlation with the in vitro results. The safety profiles suggested no or only negligible stratum corneum disruption and skin erythema by topical application of soy isoflavones. It was concluded that topical delivery may serve as a potent route for soy isoflavones against photoaging and photodamage.

1. Introduction

Nonmelanoma skin cancer (NMSC) is the most common human malignancy, and it is estimated that over 1.3 million such cancers are diagnosed each year in the US alone (Neville et al., 2007). Moreover, malignant melanoma has exhibited the most rapid increase in incidence compared to any other type of cancer (Moore et al., 2006). However, limited options are available for prevention. Chronic exposure to solar UV radiation, in particular its UVB component, is the primary cause for the vast majority of cutaneous malignancies (Afaq et al., 2007). The development of preventive and therapeutic agents against photocarcinogenesis has become an important subject in dermatological research. Topical application of photoprotective compounds possessing antioxidant properties can act to ablate the immediate effects of inflammation, erythema, and cutaneous neoplastic potentiation by UVB exposure.

Genistein, a major isoflavone in soybeans and a specific inhibitor of protein tyrosine kinase, was shown to inhibit UV-induced skin carcinogenesis (Shyong et al., 2002; Kang et al., 2003; Brand and Jendrzejewski, 2008). This compound is known to exhibit chemopreventive, cardioprotective, and antiosteoporosis activities (Zielonka et al., 2003). The aglycones of soy isoflavones consist of genistein, daidzein, and glycitein (Fig. 1). Besides genistein, other soy isoflavones also show potent pharmacological activities (Song et al., 1999; Chun et al., 2005). Soy isoflavones can also be used in the dermocosmetic field because of their role as a phytoestrogen and enhancer of hyaluronic acid production (Song et al., 1999; Miyazaki et al., 2002). Therefore, topically applied soy isoflavones may be good candidates for protective agents against photodamage. Topical administration may also be a suitable route for soy isoflavones to attain systemic bioavailability because of their rapid clearance from plasma (Busby et al., 2002). A previous study proved the feasibility of transdermal isoflavone permeation across the skin (Minghetti et al., 2006). However, no evidence currently exists to support the notion that significant amounts of soy isoflavones can be delivered into the skin for topical use. There is also a lack of well-defined correlations between skin absorption and physicochemical characteristics of these isoflavones.

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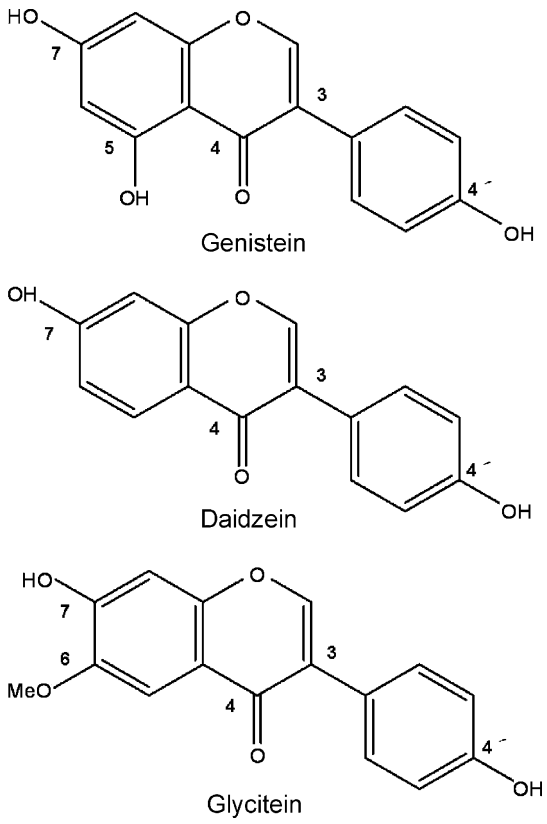

Fig. 1. Chemical structures of genistein, daidzein, and glycitein.

The aim of this study was to establish basic profiles of soy isoflavone permeation into the skin. Another purpose was to determine the protective effects of isoflavones on UVB-induced keratinocyte damage by analyzing H_2O_2 generation. The amounts of compounds retained within the skin reservoir were determined by in vitro and in vivo methods. The optimal pH and type of vehicles for better absorption of isoflavones were investigated. Possible pathways of the compounds via the skin were explored using skin treated by delipidation, α-terpineol, and oleic acid to indicate the specific routes for skin absorption.

2. Materials and methods

2.1. Materials

Genistein, daidzein, soybean oil, α-terpineol, and oleic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Glycitein and an aglycone mixture of soy isoflavones (consisting of 55% genistein, 43% daidzein, and 1.8% glycitein) were supplied by Kishida Chemical (Osaka, Japan). All other chemicals and solvents were analytical grade and used as received.

2.2. Protective effect of soy isoflavones against UVB-induced damage of keratinocytes

Spontaneously immortalized human keratinocytes (HaCaT cell line) were a gift from Dr. Yee-Jin Lee, Fu-Jen Catholic University. HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were cultured in a humidified incubator at 37 °C in an atmosphere containing 5% CO_2. Cells reaching a 90–95% of confluency were starved in DMEM at 37 °C for 24 h. Intracellular production of H_2O_2 from keratinocytes was assayed as previously described with minor modifications (Wu et al., 2006). Confluent keratinocytes starved with DMEM on 1.5-cm culture dish were pretreated with isoflavones at 10 mM for 12 h. Cells were washed with phosphate-buffered saline (PBS) and DMEM, and then treated with dihydrorhodamine 123 (10 μg/ml) in DMEM for 30 min. After a brief washing, cells were irradiated by UVB (Bio-Sun System Illuminator, Vilber Lourmat, France) with a UV peak at 312 nm. UVB was supplied by a closely spaced array of two UVB lamps, which delivered uniform irradiation at a distance of 10 cm. The UVB irradiation dose was 70 mJ/cm^2. Cells were then collected by scraping and centrifugation after an irradiation duration of 1 min. The cell pellets were resuspended in 1 ml PBS and then immediately analyzed by Partech CyFlow ML flow cytometry (Partech, Münster, Germany) at excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals of 10,000 cells were collected to calculate the mean fluorescence intensity of a single cell.

2.3. Determination of soy isoflavone-saturated solubility in vehicles

An excess amount of soy isoflavones was added to 1 ml of the selected buffers or oil, and shaken reciprocally at 37 °C for 24 h. The suspension was centrifuged at 10,000 rpm for 10 min, and the compound concentration in the supernatant was determined by high-performance liquid chromatography (HPLC) after an appropriate dilution.

2.4. HPLC analytical method

The HPLC system for genistein, daidzein, and glycitein included an L-7110 pump, an L-7200 sample processor, and an L-7400 UV/visible detector all from Hitachi (Tokyo, Japan). A 25-cm-long, 4-mm inner diameter stainless RP-18 column (Merck, Darmstadt, Germany) was used. The mobile phase was a methanol-aqueous solution with 1% phosphoric acid (50:50) at a flow rate of 1 ml/min. The UV/visible detector was set at 254 nm. The log K' values (capacity factor) of the isoflavones were determined isocratically using HPLC. The retention time of each compound was measured, and K' values were calculated from the following equation:

$$\log K' = \log \frac{t_r - t_0}{t_0};$$

where t_r is the retention time of each compound and t_0 is the retention time of the non-retained solvent peak (methanol).

2.5. Determination of the melting points of the soy isoflavones by differential scanning calorimetry (DSC)

DSC analysis was performed using a Q10 DSC calorimeter (TA Instruments, New Castle, DE, USA). Powdered compounds (2.5–3.5 mg) were put into aluminum pans. The thermal analysis profiles were obtained as the temperature was increased from room temperature to 350 °C at a rate of 10 °C/min under nitrogen.

2.6. Preparation of skin membranes

Female nude mice (8 weeks old) were sacrificed, and full-thickness skin was excised from the dorsal region. To obtain delipid skin, the stratum corneum (SC) side was pretreated with chloroform–methanol (2:1) for 1 h. Five percent α-terpineol or oleic acid in a 25% ethanol/water vehicle was used to pretreat skin mounted on Franz cells for 2 h, followed by the in vitro skin absorption experiment. The dorsal skin of a specific pathogen-free (SPF)

pig (1 week old) was supplied by the Animal Technology Institute Taiwan (Miaoli, Taiwan).

2.7. In vitro skin absorption of soy isoflavones

The skin with or without treatments was mounted on the receptor compartment of a Franz cell with the SC side facing upwards into the donor compartment. Five and a half milliliters of 3:7 (v/v) ethanol-pH 7.4 buffer was used as the receptor medium. The donor compartment was occluded by parafilm and filled with 0.5 ml vehicle containing isoflavones at a dose of saturated solubility. The available diffusion area between compartments was 0.785 cm^2. The stirring rate and temperature were kept at 600 rpm and 37 °C, respectively. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and immediately replaced with an equal volume of fresh medium.

At the end of the in vitro experiment (24 h), the skin was removed from the cell and the skin surface was cleaned by cotton wool swab immersed in water and methanol for three times each. The skin was then weighed, cut with scissors, positioned in a glass homogenizer containing 1 ml of methanol, and homogenized for 10 min at 300 rpm. The resulting solution was centrifuged for 10 min at 10,000 rpm and then filtered through a polyvinylidene difluoride (PVDF) membrane with a pore size of 0.45 μm. All samples were analyzed by HPLC.

2.8. In vivo skin absorption of soy isoflavones

For the in vivo experiment, an 8-week-old nude mouse was used. A glass cylinder with an available area of 0.785 cm^2 was placed on the dorsal skin with glue (Instant Super Glue®, Kokuyo, Japan). An aliquot of 0.2 ml of vehicle with soy isoflavones was added to the cylinder. The application time of the vehicle was 8 h. The application region of the skin was excised at the end of the experiment. The procedure for wash procedure and extraction of the compound from the skin was the same as for the in vitro experiment.

2.9. In vivo skin irritation test

A 0.6-ml aliquot of pH 6 buffer with isoflavones was spread uniformly over a sheet of non-woven polyethylene cloth (1.5 cm × 1.5 cm), which was then applied to the back area of a nude mouse. The polyethylene cloth was fixed with Tegaderm® adhesive dressing (3M, USA) and Fixomull® stretch adhesive tape (Beiersdorf AG, Germany). After 24 h, the cloth was removed, and the treated skin area was swabbed clean with a cotton wool swab. After withdrawal of the vehicle for 30 min, transepidermal water loss (TEWL), colorimetric parameters, and the pH of the applied skin were measured. TEWL was recorded using a Tewameter® (TM300, Courage & Khazaka, Köln, Germany). Measurements taken at a stable level were performed 30 s after application of the TEWL probe to the skin. The TEWL was automatically calculated and expressed in g/m^2/h. A spectrocolorimeter (CD100, Yokogawa Electrical, Tokyo, Japan) was used to measure the skin erythema (a*). The instrument recorded color reflectance three-dimensionally (L*, a*, b*) as recommended by the CIE (Commission Internationale de l'Eclairage). When recording the color values, the measuring head was held perpendicular to the dorsal skin of the mouse, and the aperture was fitted with an applicator, to avoid compression of the subcutaneous capillaries. The reading was obtained within a few seconds on the display. The skin surface pH was determined by Skin-pH-Meter® PH 905 (Courage & Khazaka). An adjacent untreated site was used as a baseline standard for each determination. The temperature and relative humidity in the laboratory were kept at 26 °C and 55%, respectively. The sample number for each experiment is six ($n = 6$).

2.10. Statistical analysis

Statistical analysis of differences between different treatments was performed using unpaired Student's *t*-test. A 0.05 level of probability was taken as the level of significance.

3. Results

3.1. Protective effect of soy isoflavones against UVB-induced damage to keratinocytes

In order to confirm the protective effect of soy isoflavones against UVB-mediated damage to keratinocytes, the H_2O_2 level inside the cells was determined. H_2O_2 is a well-accepted marker of oxidative stress. The intracellular H_2O_2 during cell adhesion was detected using a specific dye, dihydrorhodamine 123, following determination by flow cytometry. We examined the protective activity of four groups of soy isoflavones, including genistein, daidzein, glycitein, and an aglycone mixture as shown in Fig. 2. The results are presented as histograms of cell number versus fluorescence intensity in Fig. 2A. The flow cytometric analysis showed that the mean fluorescence increased in UVB-treated cells (Fig. 2A, panels a and b). As shown by data in Fig. 2B, UVB irradiation of cells resulted in a significant induction of H_2O_2 (*t*-test, $p < 0.05$). The increase in intracellular H_2O_2 was inhibited by treatment with soy isoflavones (Fig. 2A, panels c to f).

Quantitative analysis of the data clearly showed that genistein, daidzein, and the aglycone mixture inhibited UVB-induced H_2O_2 production (*t*-test, $p < 0.05$, Fig. 2B). The H_2O_2 levels of daidzein and the mixture were lower than that of genistein, although these did not reach statistical significance (*t*-test, $p > 0.05$). The application of glycitein prior to UVB exposure resulted in a non-significant decrease in the amount of H_2O_2.

3.2. Physicochemical characteristics of soy isoflavones

The physicochemical properties of genistein and daidzein are summarized in Table 1. The pH of the vehicle has been shown to be one of the major variables influencing the diffusivity of compounds. In this work, pH 6 and pH 10.8 buffers were selected as the vehicles to exhibit the non-ionized and anionic forms, respectively. The solubility of genistein was low at pH 6, and it increased from pH 6 to 10.8, which was above the pK_{a2} (Table 1). Soybean oil, a lipophilic vehicle, was also used as a vehicle for genistein. Genistein showed a higher solubility in soybean oil compared to the pH 6 buffer (*t*-test, $p < 0.05$). Daidzein was more soluble (*t*-test, $p < 0.05$) than genistein in both pH 6 and 10.8 buffers. Contrary to the aqueous solubility, the solubility of daidzein in oil was relatively low. This indicates that daidzein is more hydrophilic compared to genistein. The hydrophilicity ranking was evaluated by measuring the capacity factor ($\log K'$), which indicates the relative retention of a compound in the HPLC system. The $\log K'$ result confirmed the greater hydrophilicity of daidzein than genistein (Table 1). Because of the low protection afforded by glycitein, it was excluded from the following study of physicochemical characterization and skin absorption.

3.3. In vitro skin absorption of genistein and daidzein

The compound amount in skin was calibrated by the saturated solubility in the donor compartment (calibrated skin deposition) as shown in Fig. 3. The isoflavone solution at saturation was used for

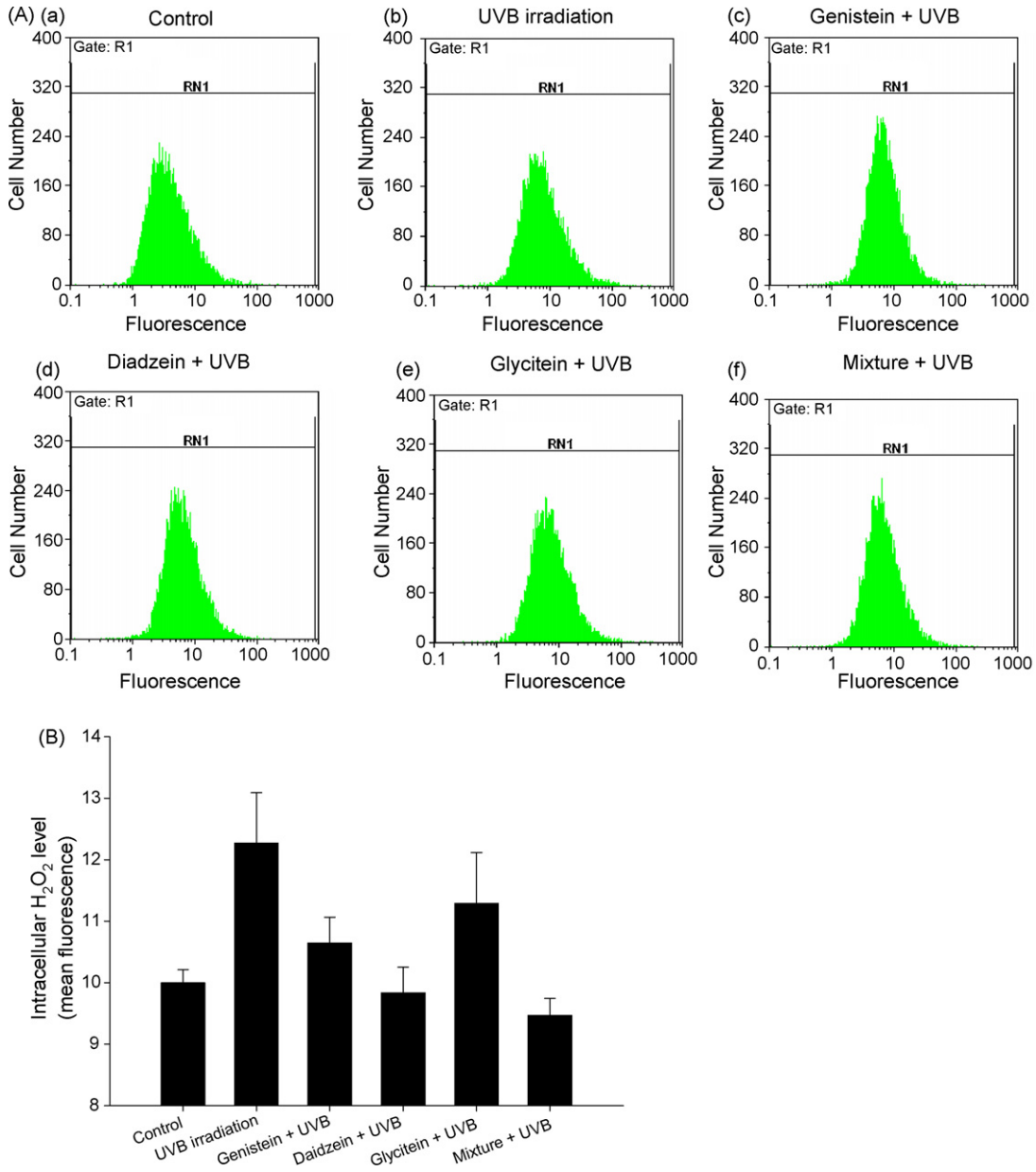

Fig. 2. Soy isoflavones inhibited UVB irradiation-induced intracellular H_2O_2 production in human keratinocytes. (A) Intracellular H_2O_2 production (denoted by the mean fluorescence) expressed as a histogram and (B) analysis of results from four independent experiments. All data are presented as the mean of four experiments ± S.D.

the experiment to ensure that there was uniform thermodynamic activity and thus common activity for the compound in each formulation. This value is independent of the donor concentration, unlike the skin deposition (nmol/g), so this parameter is important for comparing absorption levels between genistein and daidzein which showed the same thermodynamic activity. The uptake of isoflavones within nude mouse skin was determined as shown in Fig. 3A. The pH of the vehicle had a significant effect on the transport of genistein and daidzein (*t*-test, $p < 0.05$). The calibrated skin deposition increased in the order of pH 6 > pH 10.8. Although there are similarities in the structures of these isoflavones, the skin absorption of these compounds showed discrepancies. The skin uptake of daidzein was significantly lower (*t*-test, $p < 0.05$) than that of genistein from the aqueous buffers. A contrary result was observed when using soybean oil as the vehicle. Pig skin was also utilized as a skin model to examine the skin accumulation of soy isoflavones (Fig. 3B). A similar trend was detected between pig skin and nude mouse skin. However, daidzein accumulation was comparable

Table 1
Physicochemical parameters and solubility (nmol/ml) of genistein and daidzein in various vehicles

Compound	Molecular weight (Da)	Log *K'*	Solubility in pH 6 buffer	Solubility in pH 10.8 buffer	Solubility in soybean oil
Genistein	270.2	0.23	12.94 ± 0.53	659.70 ± 10.96	243.14 ± 2.04
Daidzein	254.2	−0.05	18.76 ± 0.33	1875.89 ± 292.01	10.88 ± 2.73

The solubility value represents the mean ± S.D. ($n = 6$).

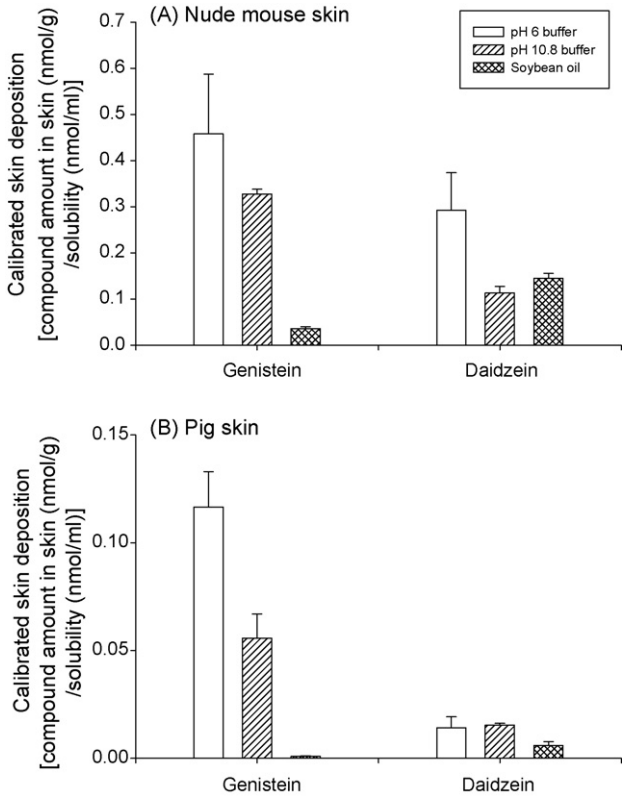

Fig. 3. In vitro-calibrated skin deposition of genistein and daidzein after a 24-h application from aqueous buffers and soybean oil in a saturated solubility condition. (A) Skin absorption into nude mouse skin and (B) skin absorption into pig skin. All data are presented as the mean of four experiments ± S.D.

(t-test, $p > 0.05$) in the pH 6 and 10.8 buffers. Pig skin deposition was extremely low from soybean oil compared to nude mouse skin.

Although the examination of drug permeability across the skin is less advantageous when targeting skin tissue, an understanding of the permeability may be helpful to elucidate the mechanisms involved in the skin absorption of the drugs. Table 2 summarizes the permeability coefficients (K_p) of genistein and daidzein across various skin membranes from pH 6 buffer. The flux value at the steady-state was determined and expressed per unit of diffusion area in nmol/cm^2/h by a linear regression calculation from the slope of the linear portion of the permeated amount-time profiles. The K_p value (cm/h) was calculated from the flux divided by the saturated isoflavone concentration in the donor compartment. The data indicate that the K_p of genistein across delipid skin was 1.44-fold higher (t-test, $p < 0.05$) than the K_p across intact skin. Daidzein showed a 6.11-fold increase (t-test, $p < 0.05$) in K_p after SC lipid extraction.

To further explore the permeation mechanisms of genistein and daidzein, α-terpineol and oleic acid were used to pretreat the skin. Permeation data across the pretreated skin from pH 6 buffer are given in Table 2. Pretreatments with α-terpineol and oleic acid did not further increase genistein permeation in the non-ionized form (t-test, $p > 0.05$). On the other hand, the K_p of non-ionized daidzein across pretreated skin increased by ~3-fold (t-test, $p < 0.05$). The permeation data of isoflavones in an ionized form (pH 10.8) are shown in Table 3. Lipid extraction of the skin enhanced the K_p of daidzein to a greater extent than genistein. The enhancement ratio (ER) of genistein was ~1.3 with pretreatment of α-terpineol and oleic acid, although the statistical analysis revealed no significant difference (t-test, $p > 0.05$) compared to intact skin. α-Terpineol and oleic acid pretreatment showed an ER higher than intact skin but lower than the delipid skin for ionized daidzein, which had the same trend as the non-ionized form.

3.4. In vivo skin absorption of genistein and daidzein

As shown in Fig. 4, the in vivo uptake of genistein was greater than that of daidzein, which was similar to the results of in vitro skin absorption. The skin deposition from pH 10.8 buffer was much lower (t-test, $p < 0.05$) than that from pH 6 buffer. This discrepancy between various pH conditions was larger than in the in vitro experiment. Bioengineering methods such as TEWL, colorimetry, and pH value for evaluating the preliminary safety of genistein and daidzein were also conducted in vivo. The Δ values (the value of the treated site minus the value of an adjacent untreated site) of TEWL, a*, and pH were determined after a 24-h application as shown in Fig. 5. No significant skin irritation can be determined when the bar of the standard deviation (S.D.) passes across the zero line in Fig. 5. Genistein produced minor enhancement of ΔTEWL and pH values. No skin erythema was detected after topical genistein delivery. This suggests tolerable changes in the skin with topically applied genistein. Only a slight increment in the skin pH value was observed in the case of daidzein with no change in TEWL or a* compared to the untreated site.

3.5. Skin absorption of genistein and daidzein from the aglycone mixture

The solubility of genistein and daidzein in the aglycone mixture using pH 6 buffer as the medium was measured as shown in Table 4. The solubility of genistein and daidzein in the mixture increased by 5.2- and 3.3-fold compared to the pure compounds. The in vitro calibrated skin deposition of daidzein in the mixture also increased

Table 2
Permeation data of genistein and daidzein from pH 6 aqueous solutions across various skin types

Compound	Skin type	Flux (nmol/cm^2/h)	K_p ($\times 10^{-2}$ cm/h)[a]	Enhancement ratio (ER)[b]
Genistein	Intact skin	0.34 ± 0.02	2.65 ± 0.17	–
	Delipid skin	0.49 ± 0.03	3.83 ± 0.26	1.44
	α-Terpineol treatment[c]	0.34 ± 0.03	2.60 ± 0.22	0.98
	Oleic acid treatment[c]	0.33 ± 0.02	2.55 ± 0.19	0.96
Daidzein	Intact skin	0.05 ± 0.003	0.28 ± 0.02	–
	Delipid skin	0.32 ± 0.04	1.71 ± 0.22	6.11
	α-Terpineol treatment[c]	0.17 ± 0.001	0.91 ± 0.004	3.25
	Oleic acid treatment[c]	0.18 ± 0.04	0.94 ± 0.20	3.36

Each value represents the mean ± S.D. ($n = 4$).
[a] K_p (cm/h), permeability coefficient = flux (nmol/cm^2/h)/solubility (nmol/ml).
[b] Enhancement ratio (ER), K_p across treated skin/K_p across intact skin.
[c] The concentration of α-terpineol or oleic acid in a 25% ethanol/water was 3% (v/v).

Table 3
Permeation data of genistein and daidzein from pH 10.8 aqueous solutions across various skin types

Compound	Skin type	Flux (nmol/cm^2/h)	K_p ($\times 10^{-2}$ cm/h)[a]	Enhancement ratio (ER)[b]
Genistein	Intact skin	8.22 ± 1.27	1.25 ± 1.10	–
	Delipid skin	20.30 ± 0.95	3.08 ± 0.14	2.46
	α-Terpineol treatment[c]	10.38 ± 1.06	1.57 ± 0.16	1.26
	Oleic acid treatment[c]	10.96 ± 1.22	1.66 ± 0.18	1.33
Daidzein	Intact skin	5.45 ± 1.03	0.29 ± 0.06	–
	Delipid skin	36.87 ± 1.75	1.97 ± 0.09	6.79
	α-Terpineol treatment[c]	24.22 ± 3.19	1.29 ± 0.17	4.45
	Oleic acid treatment[c]	20.75 ± 3.70	1.11 ± 0.20	3.83

Each value represents the mean ± S.D. ($n = 4$).
[a] K_p (cm/h), permeability coefficient = flux (nmol/cm^2/h)/solubility (nmol/ml).
[b] Enhancement ratio (ER), K_p across treated skin/K_p across intact skin.
[c] The concentration of α-terpineol or oleic acid in a 25% ethanol/water was 3% (v/v).

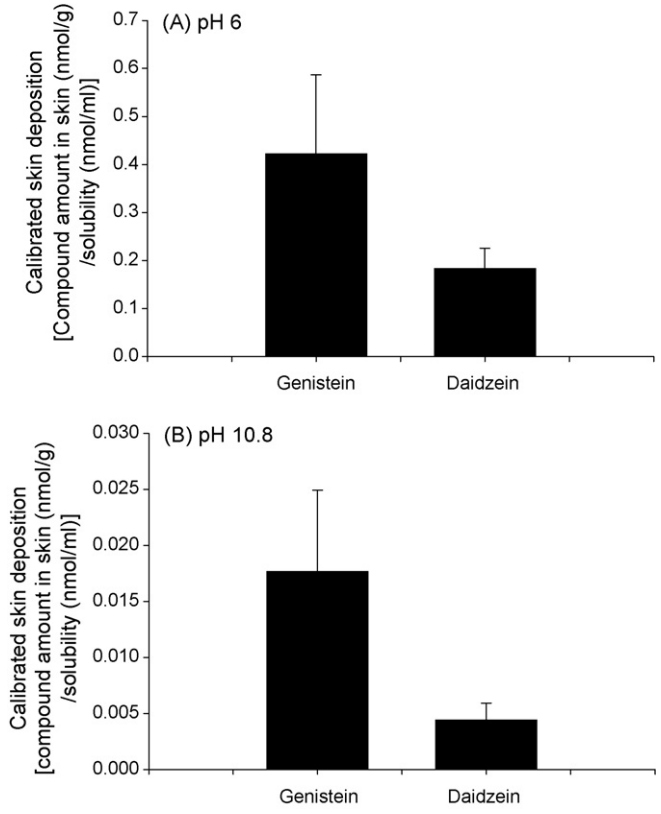

Fig. 4. In vivo-calibrated skin deposition of genistein and daidzein after an 8-h application in a saturated solubility condition. (A) pH 6 and (B) pH 10.8 buffer as the compound vehicle. All data are presented as the mean of six experiments ± S.D.

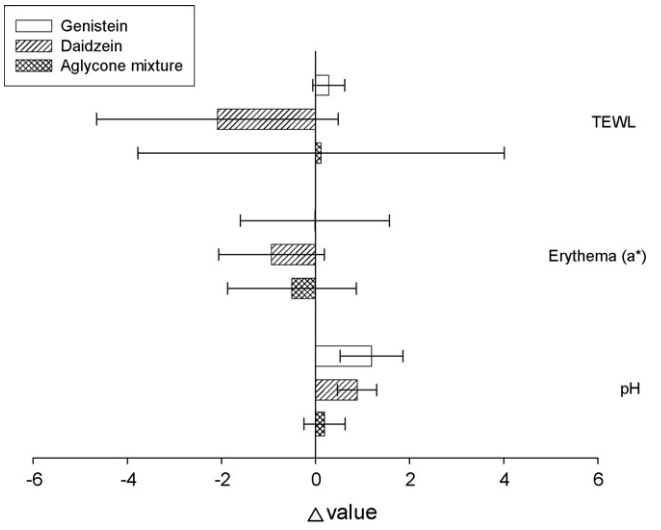

Fig. 5. In vivo skin irritation examination determined by transepidermal water loss (TEWL), erythema (a*), and the pH value after a 24-h application of topically applied genistein, daidzein, and an aglycone mixture from pH 6 buffer. The Δ value indicates the value of the treated site minus the value of an adjacent untreated site. All data are presented as the mean of six experiments ± S.D.

Table 4
Solubility and in vitro skin absorption data of genistein and daidzein in the aglycon mixture

Compound	Solubility in pH 6 buffer (nmol/ml)	Calibrated skin deposition[a]	K_p ($\times 10^{-2}$ cm/h)[b]
Genistein	67.34 ± 2.68	0.50 ± 0.11	2.23 ± 0.36
Daidzein	61.75 ± 10.35	0.85 ± 0.10	9.33 ± 1.55

Each value represents the mean ± S.D., $n = 6$ for solubility; $n = 4$ for skin absorption data.
[a] Calibrated skin deposition = compound amount retained in skin (nmol/g)/solubility (nmol/ml).
[b] K_p (cm/h), permeability coefficient = flux (nmol/cm^2/h)/solubility (nmol/ml).

by 2.6-fold. The skin uptake of daidzein even surpassed that of genistein in the aglycone mixture. There was no significant difference (t-test, $p > 0.05$) in the skin deposition of genistein between the aglycone mixture and the pure compound. The same phenomenon was shown for the K_p profiles (Table 4). In vivo topical delivery of the aglycone mixture was also evaluated. The in vivo calibrated skin deposition values of genistein and daidzein in the mixture were 0.50 ± 0.16 and 0.74 ± 0.22, respectively. The preliminary safety evaluation showed no or negligible skin irritation for the topically applied aglycone mixture (Fig. 5).

4. Discussion

Despite various reports linking many of the beneficial properties of soy isoflavones to their dermal use, no comprehensive study has been conducted investigating the skin absorption ability of these compounds. The aim of this work was to compare the topical delivery of various soy isoflavones. The preliminary safety of isoflavones on skin was also examined. The results showed that genistein exhibited considerable absorption into the skin, especially in the non-ionized form. Daidzein absorption was enhanced by incorporation with other isoflavones (in the aglycone mixture). The isoflavones showed negligible irritation of the skin after

in vivo topical application, indicating their feasibility for dermal use.

UVB is known to directly and indirectly generate DNA damage through oxidative stress by increasing levels of reactive oxygen species (ROS) (Nazim et al., 1999). The term ROS includes free radicals such as O_2^- and $OH^\bullet$, as well as non-radical intermediates such as H_2O_2 and $^1O_2$. Soy isoflavones as antioxidants have been considered to ameliorate the adverse effects of ROS. It was shown that H_2O_2 was generated in keratinocytes during UVB irradiation (Fig. 2). Therefore we determined whether the soy isoflavones affected UVB-induced intracellular H_2O_2 production. Genistein decreased the H_2O_2 increment caused by UVB by 71%. Genistein may act through a free radical-scavenging-dependent pathway. Because of its polyphenolic structure, genistein can donate hydrogen atoms to deleterious oxy radicals and form less-reactive phenoxyl radicals in the process. A previous study suggested that the mechanisms of action of genistein involve protection of oxidative and photodynamically damaged DNA, downregulation of UVB-activated signal transduction cascades, and antioxidant activities (Wei et al., 2003).

Although the protective activity of genistein against UVB irradiation has been reported in detail, related information about other soy isoflavones is lacking. We examined the protective effect of other isoflavones for comparison. Daidzein and an aglycone mixture almost completely inhibited H_2O_2 produced by UVB. Daidzein, which lacks the C-5 hydroxyl group of genistein, was expected to be less effective as an antioxidant. However, this was not the case in the present study. Based on a previous study, it can be stated that polyhydroxyflavones show the following sequence of deprotonation: 7-OH > 4′-OH > 5-OH (Zielonka et al., 2003). The 5-OH group showed a high deprotonation energy of 493 kcal/mol, indicating difficulty in deprotonating 5-OH in genistein. Hence the 5-OH group might not effectively function in UVB-protective activity. Glycitein exhibited much lower activity compared to the others in the H_2O_2 assay. This suggests that blocking hydroxyl by the carboxyl group of genistein or daidzein affected the reduction in antioxidant activity.

Genistein's structure has multiple protonation sites. The dissociation constants were determined to be $pK_{a1} = 7.2$, $pK_{a2} = 10.0$, and $pK_{a3} = 13.1$ by the spectrophotometric method (Zielonka et al., 2003). The pK_a values of genistein indicated that at pH 6 genistein exists in water in a neutral form, as it had low aqueous solubility. Genistein is predominantly in an ionic form in pH 10.8 buffer, which is beneficial to aqueous solubilization as depicted in Table 1. The same phenomenon was observed for daidzein. Genistein has an additional hydroxyl group in the 5-position of the structure compared to daidzein (Fig. 1). The extra moiety may contribute to the higher aqueous solubility of genistein than daidzein. However, the facts were contrary to this expectation. A possible explanation is that the hydrogen in the 5-position of genistein may link to the oxygen in the 3-position to form hydrogen binding in the presence of water. An additional ring structure is thus developed for genistein. This ring structure attenuates the hydrophilicity of genistein. Xu et al. (1994) also suggested lower water solubility of genistein than daidzein. The capacity factor ($\log K'$) confirms the hydrophilic trend of both isoflavones (Table 1). Rothwell et al. (2005) indicate a higher octanol–water partition coefficient ($\log P$) of genistein (3.4) than daidzein (2.5). Further study for elucidating the actual mechanisms is needed.

For topical formulations, the compound skin content is considered an important parameter, and in the present study, the skin deposition was determined at the end of the in vitro experiment. The isoflavones were extracted from the skin to examine the isoflavone accumulation. In the skin absorption studies, the selection of a skin model is an important prerequisite. The most reliable skin absorption data are collected from human studies. However, such studies are generally not feasible during the initial development of a novel dosage form or system. The availability of such systems is also limited. The skin of rodents is most commonly used for in vitro and in vivo skin permeation studies. There are a number of hairless species (e.g., nude mice and hairless rats) in which the absence of a hairy coat mimics the human skin better than hairy skin (Godin and Touitou, 2007). Hence nude mice were used as an animal model in this study. Although nude mouse skin is more permeable than human skin (Catz and Friend, 1990; Fang et al., 1999), it is still a good model for examining the skin transport of permeants because of the limited variability among individuals and similar hair follicle density to human skin.

Because of the known pH effect on drug skin permeation, we decided to determine the influence of pH on the skin absorption of isoflavones. Genistein in a completely neutral condition (pH 6) showed higher skin accumulation compared to the ionized condition (pH 10.8). The same result was observed for permeation profiles of daidzein. The ionized form of a compound always shows lower permeation compared to the non-ionized form because of the lipophilic characteristics of the SC (Cole and Heard, 2007; Lee et al., 2007). Skin absorption of a compound is determined by its physicochemical properties, in particular its lipophilicity and molecular weight (MW), which play major roles in the skin permeation process (Marti-Mestres et al., 2007). The higher retained amount of genistein in the skin with respect to daidzein may have been due to the higher lipophilicity that favors the partitioning of molecules into the lipophilic SC. One may expect lower permeation to be associated with higher-MW compounds. Nevertheless, this was not the case with soy isoflavones since their molar volumes do not greatly differ. The MWs of genistein and daidzein are 270 and 254 Da, respectively, both of which fall in the appropriate range for topical delivery (<500 Da) (Doh et al., 2003).

Soybean oil exhibited low genistein absorption into the skin. The increasing genistein solubility in oil hindered the partitioning of the compound into the SC. As the oil is relatively lipophilic, the affinity of genistein would be expected to favor the vehicle. This phenomenon is more significant by using pig skin as the barrier. Thus the partitioning of genistein from soybean oil to pig SC was more difficult as compared to mouse SC. Another explanation is its slow release from soybean oil. This may have been due to the higher viscosity of the oil relative to the aqueous solution, making it difficult for the compound to diffuse within the oil system (Hung et al., 2008). It cannot be neglected that pig skin and rhesus monkey skin are the best substitutes for human skin (Riviere and Papich, 2001; Wang et al., 2007). Hence pig skin was also used to mimic actual conditions. A similar trend was seen between pig skin and nude mouse skin, indicating the likely predictability of human skin results by nude mouse skin profiles.

The predominant route for most permeant transport into or across skin is the intercellular region of the SC. The delipidation process can remove the intercellular lipids in the SC. The SC is principally lipophilic in nature and far more resistant to polar than non-polar compounds. This speculation is consistent with the permeation profiles of isoflavones with the removal of the lipids which largely increased daidzein but not genistein permeation. This indicates that the SC is a major contributor to the barrier function against the skin absorption of daidzein. α-Terpineol and oleic acid have been used as permeation enhancers for transdermal drug delivery. Terpenes are known to act at the lipid polar heads of ceramides (Jain et al., 2002; Panchagnula et al., 2004), while fatty acids act at the lipidic tail portion of intercellular lipid bilayers (Gwak and Chun, 2002; Jain and Panchagnula, 2003). Neither enhancer could elaborate their permeation-enhancing activity on genistein permeation. Oleic acid has an effect on the compound's partitioning into the SC (Moser et al., 2001). This suggests that

genistein is easily partitioned into the SC with no need to increase the partitioning by enhancers. The results confirm the success of topical delivery of genistein because of its excellent absorption in the absence of enhancers. Daidzein permeation showed greater enhancement when the skin was pretreated with both enhancers. This suggests that the lipid bilayers are the main barrier blocking the transit of daidzein in both the neutral and ionic forms. Both routes of the polar head and non-polar tail of the lipids contribute to daidzein's absorption.

A good in vitro–in vivo correlation was observed for isoflavone skin absorption from various systems. This suggests that the amount of soy isoflavones retained in the skin in the in vitro status can predict the in vivo compound accumulation within the skin reservoir. However, the in vivo skin deposition was less than the in vitro results, especially in the ionized form (pH 10.8). This was possibly due to the diffusion and distribution of isoflavones from the skin to the systemic circulation or other tissues in the in vivo status. TEWL is utilized to assess the degree of SC disruption, and a good correlation between the chemical damage to the skin barrier and TEWL increment has been demonstrated (Zhao and Singh, 1999; Fang et al., 2007). Genistein caused a slight increase in ΔTEWL. Genistein may have produced higher perturbation of the lipid bilayers in the SC due to the higher amount of this chemical within the skin. It has been shown that the complete removal of lipids from the SC leads to a 100-fold increase in water permeability (Serup and Agner, 1990). The safety of genistein may be acceptable because of its limited increase in ΔTEWL. A previous report (Moore et al., 2006) also corroborates that neither carcinogenicity nor genotoxicity was observed in mouse skin chronically treated with topical genistein.

One cannot straightforwardly rationalize the topical delivery of multiple species from complex mixtures, as the behavior of each species potentially influences that of another (Heard et al., 2006). The saturated solubility and topical absorption of genistein and daidzein in the aglycone mixture exhibited an uncommon difference compared to the pure form. A eutectic mixture formed of two or more compounds is a well-known melting technique for preparing homogeneous solutions. In comparison to monotectic dispersions, eutectic solid dispersions can reduce the particle size to ultrafine crystals, and thus induce higher solubility of the compounds in the carrier (Leuner and Dressman, 2000; Vippagunta et al., 2007). The major effect of eutectic formation is to lower the melting point of a substrate mixture. In order to explore the mechanism, the melting points of the isoflavones and mixture were determined by differential scanning calorimetry (DSC) as shown in Fig. 6. The melting points of genistein, daidzein, and the aglycone mixture were 305, 338, and 283 °C, respectively. It is apparent that the melting point was greatly reduced in the mixture, confirming the eutectic effect. The advantages of eutectic dispersions result in the improved mobility of the compounds and high driving force for topical permeation (Ke et al., 2005; Kim et al., 2005). Touitou et al. (1994) indicated that compounds in a eutectic form have enhanced solubility in the SC, thus increasing skin permeation. The enhancing mechanisms of topically applied mixtures should be elucidated by further investigations.

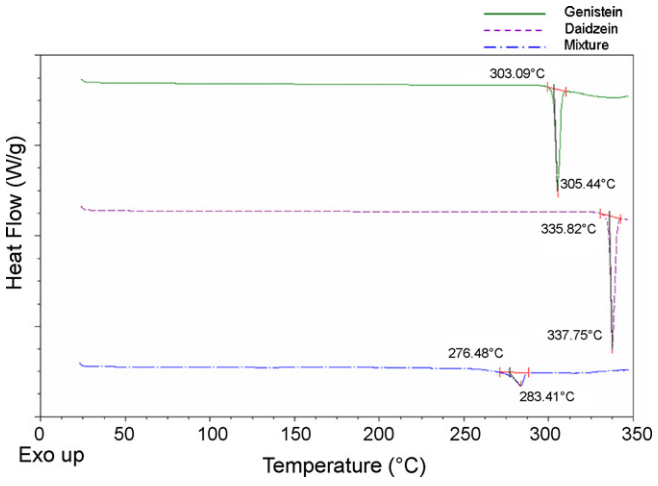

Fig. 6. Melting points of genistein, daidzein, and an aglycone mixture determined by differential scanning calorimetry.

5. Conclusions

As demonstrated in this study, the pure compounds and a mixture of soy isoflavones inhibited UVB-induced intracellular H_2O_2 production and consequently protected keratinocytes against UVB radiation. Reduction of photodamage in skin by the topical application of antioxidants could be an efficient way to enrich the endogenous cutaneous protection system. The results in this report suggest that soy isoflavones are moderately absorbed by the skin. Genistein showed higher skin absorption than daidzein. Daidzein absorption can be increased if incorporated in an aglycone mixture of soy isoflavones. Isoflavone transport via the skin was closely related to the vehicle in which it was formulated, with an aqueous buffer at a lower pH exhibiting good skin deposition. The preliminary safety examination of the skin also showed an acceptable skin tolerance to soy isoflavones. The present study indicates the promise of further in vivo or clinical applications of soy isoflavones delivered via the skin.

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