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Effect of Porcine Placenta Extract from Subcritical Water Extraction on Photodamage in Human Keratinocytes

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Abstract

The objective of this study was to evaluated the photoprotective effects of porcine placenta extract (PPE) on ultraviolet B (UVB)-induced oxidative stress in human keratinocytes (HaCaT) to evaluate its functional activities as a skin food ingredient. PPE prepared by subcritical water extraction was termed SPE, and subsequently digested by enzymes to prepare E-SPE. Increased intracellular reactive oxygen species (ROS) levels (192.0%) induced by UVB were decreased by SPE and E-SPE. SPE had more effective ROS scavenging activity than E-SPE treatment. UVB treatment increased expression of tissue inhibitor of metalloproteinase 1 (TIMP-1), and this elevated expression was decreased by E-SPE treatment. High-dose treatment with E-SPE (50 and 100 μg/mL) reduced TIMP-1 expression levels of UVB-C (control) to 33.5 and 34.6%, respectively. In contrast, at low SPE doses (1 and 10 μg/mL), the treatment slightly decreased TIMP-1 expression levels to 73.3 and 71.3% of UVB-C, respectively. In conclusion, the present study demonstrated the protective effect of SPE and E-SPE against UVB damage in keratinocytes *via* ROS scavenging, down-regulating MMP-2 expression and up-regulating TIMP-1 expression. This highlights the potential for SPE as an ingredient in the preparation of functional food against photoaging.

Key words: porcine placenta extract, subcritical water extraction, UVB, ROS, MMP-2, TIMP-1

Introduction

Dairy products are a very commonly used food type in daily life. The huge demand on dairy products has led to the outgrowth of livestock. As the livestock business expands, concerns are being raised over the risk of environmental contamination by animal by-products, such as the placenta. Placenta extraction (PE) mix has been used in traditional Chinese medicines. The placenta contains and produces a wide variety of biologically active compounds, namely, proteins and peptides (Gude *et al.,* 2004; Muralidhar and Panda, 1999). In addition, it is a rich source of hormones, cytokines, chemokines, and growth factors such as hepatocyte growth factor, epidermal growth factor, and transforming growth factor subtypes α and β for which receptors have been identified in human placenta (Pal *et al.,* 2002; Tonello *et al.,* 1996).

In Chinese folk medicine, PE has been used for wound healing, and the medical uses and dermatological benefits of PEs have been reported. Recently, the benefits of topical application of PE on chronic and non-healing wounds have also been reported (Tiwary *et al.,* 2006). Human PE also features as a component of various skin ointments and is used for skin revitalization, nourishment, melanocyte growth, and pigment-inducing effects (Pal *et al.,* 2002). It is also used for the treatment of skin hypersensitivity conditions like dermatitis and psoriasis. Some typical human PEs contain keratinocyte growth factor (Failla *et al.,* 2000; O'Keefe *et al.,* 1985), or stimulators of endothelial cell growth (Presta *et al.,* 1985).

It is interesting to note that animal-derived PEs are available commercially and regularly used in various ointments and lotions for the purpose of improving skin properties. Although the effects of human PE have been

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largely studied, only a few scientific studies report the effects of PE derived from animals such as pig, sheep, and horse. In rats with thermal injury, treatment with porcine PE (PPE) resulted in wound healing (Wu *et al.*, 2003). However, the photoprotective effect of porcine PE on keratinocytes irradiated by ultraviolet B (UVB) has not been investigated.

Subcritical water extraction has been used successfully to selectively extract different classes of compounds. The process involves using hot water under pressure sufficient to maintain water in the liquid state. More polar compounds are extracted at lower temperatures, while less polar compounds are extracted at higher temperatures (Ibanez *et al.*, 2003). One disadvantage associated with subcritical water extraction is the potential to inadvertently degrade the desired organic compounds during an extraction (Lindquist, 2011). Unexpected degradation of bioactive components can occur at the high temperatures and pressures using during subcritical water extraction. Therefore, the objective of this study was to evaluated the photoprotective effects of PPE on UVB-induced oxidative stress to evaluate its functional cosmetic activities as a skin ingredient.

Materials and Methods

Preparation of PPE by subcritical water extraction

Porcine placentas obtained following physiological delivery were washed thoroughly with sodium hydroxide (NaOH) solution, cut into small pieces (<1 cm^2), and rinsed in phosphate buffer solution as described previously (Georgieva *et al.*, 1995). The tissue was then homogenized using a blender (HR-2084, Philips Electronics N.V., Netherlands) for 10 min and centrifuged. The precipitate was then mixed with water (700 mL) and extracted using a subcritical extractor (DIONEX ASE 100, Dionex Corporation, USA). During the extraction process, the pressure was maintained at 375 bar for 3 h. Then, the extract was centrifuged at 2800 *g* for 20 min. The supernatants were concentrated by using a vacuum evaporator at 40°C and lyophilized to produce PPE. Hereafter in the manuscript, PPE produced by the subcritical water extraction method will be referred to as SPE.

Cell culture and UVB irradiation

Human keratinocytes (HaCaT) cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and antibiotics. HepG2 cells were purchased from JCRB (Japan). Cells were incubated at 5% CO$_2$/95%-humidified air at 37°C. When the cells reached confluence, the medium was exchanged with phenol-free DMEM containing 2% fetal bovine solution. UVB irradiation was performed at 40 mJ/cm^2 (445 µW for 1.5 min) using a UVB lamp (GL20SE, Sankyo, Denki, Japan).

Cell cytotoxicity was determined by using a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay to measure mitochondrial activity in viable cells using the method described by Mosmann (1983), but with minor modifications. Intracellular reactive oxygen species (ROS) was measured after 6 h of UVB irradiation, cell cytotoxicity after 24 h, mRNA levels of matrix metalloproteinases (MMPs) after 12 h, and protein levels of MMPs after 24 h.

Intracellular ROS

HaCaT keratinocytes were placed in 24-well plates at a density of 2×10^5 cells per well for determination of ROS levels. After 6 h of UVB irradiation, the production of intracellular ROS was determined by DCFH-DA (2',7'-dichlorofluorescin diacetate) assay (Shukla *et al.*, 2006). DCFH-DA (5 µg/mL) was introduced to the cells, and DCFH-DA was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a spectrofluorometer (VICTOR3TM, PerkinElmer, USA).

Determination of mRNA level by semiquantitative real time polymerase chain reaction (RT-PCR)

RNA isolation and RT-PCR analysis were performed as previously described (Ryu *et al.*, 2009). Primer sequences used to amplify the desired cDNA were as follows: MMP-2 forward and reverse primers: 5'-GGCCAAGTGGTC-CGTGTG-3' and 5'-GAGGCCCCATAGAGCTCC-3'; MMP-9 forward and reverse primers: 5'-CACTGTCCAC CCCTCAGAGC-3' and 5'-GCCACTTGTCGGCGATA AGG-3'; HPRT forward and reverse primers: 5'-ATTCT TTGCTGACCTGCTGGATT-3' and 5'-CTTAGGCTTT GTATTTTGCTTTTC-3'.

Statistical analysis

Data are expressed as mean±standard deviation (SD). Statistical analysis was performed using the Statistical Package for Social Sciences (Version 12.0, SPSS Inc., USA). Unpaired Student's *t*-test and one-way analysis of variance (ANOVA) were used to assess statistical significance. For post hoc comparisons, one-sided Duncan's multiple range tests were used. Differences were considered statistically significant when $p < 0.05$.

Results

SPE and E-SPE inhibit photodamage

The cytotoxic effects of SPE and E-SPE were determined in a cell culture system. Serial concentrations of these extracts (50-250 μg/mL) were added to the medium and cells were incubated for 24 h. As shown in Table 1, SPE was not associated with cytotoxicity at a concentration of 250 μg/mL in the absence of UVB treatment. However, cells survival rate was 93.1% under these conditions. HaCaT cells were exposed to UVB at an intensity of 40 mJ/cm², and cellular activity was detected for a following 24 h. The survival rate of cells exposed to UVB irradiation was decreased to 91.2% and 92.6% in the absence of SPE and E-SPE, respectively. However, the addition of SPE and E-SPE partially improved cellular activity. The concentration of both extracts that proved to have a protective effect on cells ranged between 1 and 250 μg/mL. Furthermore, the protective concentrations of SPE and E-SPE were significantly different. In light of these findings, 100 μg/mL was selected as the maximum concentration in order to study the effects of SPE and E-SPE in HaCaT cells.

Intracellular ROS scavenging effects

The intracellular ROS scavenging effects of SPE and E-SPE were assessed in UVB-irradiated cells using the oxidant sensitive fluorescent probe, DCFH-DA. As shown in Fig. 1, UVB irradiation induced a 192.0% increase in intracellular ROS levels compared with control. SPE and E-SPE treatment lessened the effect of UVB on intracellular ROS levels. SPE treatment at concentrations greater than 10 μg/mL lowered ROS levels by 32.5% compared with UVB exposure alone. E-SPE treatment was shown to decrease intracellular ROS levels to a similar extent. However, more effective ROS scavenging activity was
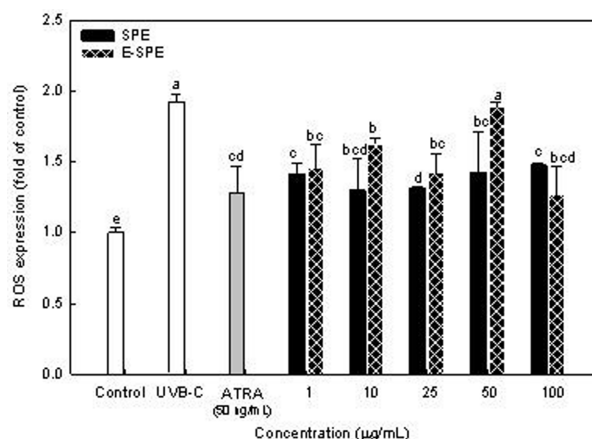

Fig. 1. Preventive effects of SPE and E-SPE on ROS generation in UVB-irradiated HaCaT cells. Data are expressed as % of control without UVB irradiation. Values are expressed as mean±SD (n=3). Different letters at each measurement indicate differences among groups ($p<0.05$), and values were analyzed with repeated measures ANOVA. Control indicates control cells not exposed to UVB irradiation; UVB-C indicates cells exposed to UVB irradiation but not samples. All-trans retinoic acid (ATRA) was used as a positive control. E-SPE, porcine placental extract treated with digestive enzyme; HaCaT, human keratinocytes; ROS, reactive oxygen species; SPE: porcine placental extract prepared by subcritical water extraction; UVB, ultraviolet B; HaCaT, human keratinocytes.

demonstrated by SPE than E-SPE. In our previous study (Han *et al.*, 2013), the SPE showed a noticeable activity in terms of antioxidant capacity for both measurement methods, FRAP and ABTS.

Effects on MMP-2 expression

MMP is the enzyme complex responsible for promoting degradation of skin membrane. Specifically, MMP-2 has gelatinase and collagenase activities. The mRNA levels and activity of MMP-2 were detected following 12-h

Table 1. Cell viability of SPE and E-SPE with or without UVB irradiation

Conc. (μg/mL)	Cell viability (% of normal control)			
	Control		UVB irradiated	
	SPE	E-SPE	SPE	E-SPE
0	100.0±3.2[ns]	100.0±5.47[ab]	91.2±4.3[a]	92.6±5.43[a]
1	103.5±0.8	104.0±7.08[b]	96.7±0.3[ab]	104.2±4.42[ab]
10	106.1±1.9	108.8±3.89[b]	98.0±3.5[ab]	107.3±6.55[b]
50	105.1±1.8	108.1±1.73[b]	96.2±1.0[ab]	106.7±4.61[ab]
100	102.7±1.5	102.3±4.98[ab]	93.5±2.1[ab]	106.9±5.92[b]
250	106.0±1.5	93.1±1.04[a]	96.1±1.8[ab]	97.8±3.42[ab]

Values are expressed as mean±SD (*n*=3). Different letters at each measurement indicate differences among groups ($p<0.05$), and values were analyzed with repeated measures ANOVA. SPE: porcine placental extract prepared by subcritical water extraction; E-SPE, porcine placental extract treated with digestive enzyme; UVB, ultraviolet B.
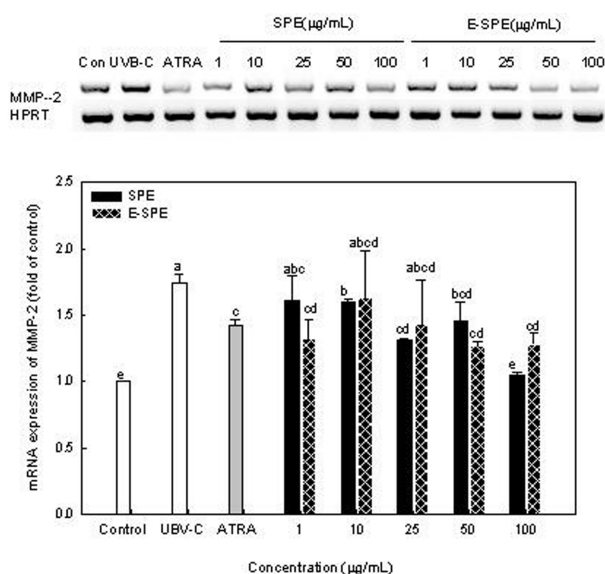
Fig. 2. Effect of SPE and E-SPE on MMP-2 mRNA expression. Values are expressed mean±SD (n=3). Different letters at each measurement indicate differences among groups (*p*<0.05), and values were analyzed with repeated measures ANOVA. ATRA, all-trans retinoic acid used as a positive control; Con, control; E-SPE: porcine placental extract treated with digestive enzyme; HPRT, hypoxanthine-guanine phosphoribosyltransferase; MMP-2, metalloproteinase-2; SPE, porcine placental extract prepared by subcritical water extraction; UVB-C, ultraviolet B control.

UVB irradiation of HaCaT cells at an intensity of 40 mJ/cm^2. As shown in Fig. 2, MMP-2 activity was greater in the presence of UVB treatment than in the control group. However, further treatment of HaCaT cells with SPE or E-SPE significantly decreased MMP-2 activity in a dose-dependent manner. Expression of MMP-2 in the group treated with 100 µg/mL SPE was similar to that of the control group. MMP-2 expression in the group treated with 100 µg/mL E-SPE was also slightly lower than the all-*trans* retinoic acid (ATRA, 50 ng/mL) positive control group. And other expressions of MMPs (MMP-1, 9 and 13) were not showed significantly differences between control and the group treated with SPE and E-SPE (data were not shown). At high concentrations (50 and 100 µg/mL), SPE- and E-SPE–treated groups showed especially significant decreases in MMP-2 expression levels compared to the UVB control (UVB-C) group.

Effects on tissue inhibitor of metalloproteinase expression

Tissue inhibitor of metalloproteinase (TIMP) acts as an inhibitor of MMPs. Fig. 3 shows the effects of SPE and
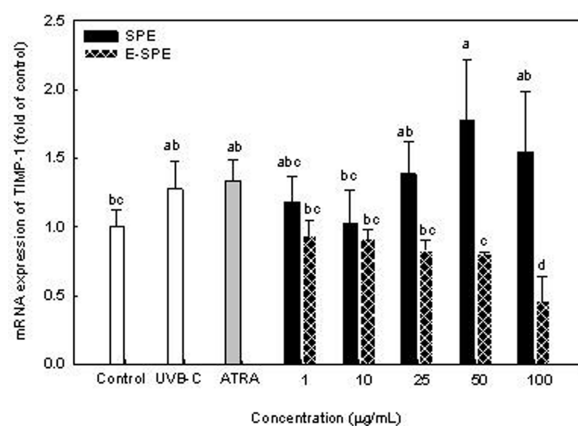

Fig. 3. Effect of SPE and E-SPE on TIMP-1 mRNA expression. Values are expressed mean±SD (n=3). Different letters at each measurement indicate differences among groups (*p*<0.05), and values were analyzed with repeated measures ANOVA. ATRA, all-trans retinoic acid used as a positive control; Con, control; E-SPE: porcine placental extract treated with digestive enzyme; SPE, porcine placental extract prepared by subcritical water extraction; TIMP-1, tissue inhibitor of metalloproteinase 1; UVB-C, ultraviolet B control.

E-SPE on TIMP-1 gene expression. UVB treatment increased expression of TIMP-1 compared with control, but its elevated expression was decreased by E-SPE treatment. High-dose treatment of E-SPE (50 and 100 µg/mL) reduced TIMP-1 levels to 33.5% and 34.6%, respectively, of UVB-C values. In contrast, low-dose SPE treatment (1 and 10 µg/mL) slightly decreased TIMP-1 levels to 73.3% and 71.3% of UVB-C values, respectively. TIMP-1 mRNA expression was not influenced by high-dose treatments of SPE. TIMP-1 mRNA expression levels were generally lowered in the presence of SPE and E-SPE treatment, except for the high-dose E-SPE group. The positive effects of SPE and E-SPE on skin health are believed to be mediated through regulation of MMP-2 and TIMP-1 expression.

Discussion

Subcritical water extraction is a technique based on the use of water as an extractant, and uses temperatures between 100°C and 374°C, and pressures high enough to maintain water in the liquid state. The technique is associated with greater selectivity, lower cost, shorter extraction times, and increased safety because it does not employ toxic organic solvents (Herrero *et al.,* 2006; Ozel *et al.,* 2003). A major advantage of using pressurized hot water for extraction is that the dielectric constant of water

is reduced with increasing extraction temperatures, which decreases its polarity to facilitate the extraction of more nonpolar compounds (Rodríguez-Meizoso *et al.,* 2006). In addition, the high pressures can further enhance the extraction of analytes trapped in matrix pores (Güçlü-Üstündağ *et al.,* 2007).

The critical point at which the water ionization constant increases is 374°C and 22 MPa; hence, water ionizes readily to hydrogen and hydroxide ions (Watchararuji *et al.,* 2008). Hydrogen ions disrupt peptide bonds thereby resulting in hydrolysis of peptides (Brunner, 2009). Information on the effects of subcritical water for the hydrolysis of soybean and rice bran protein is available from previous reports (Sunphorka *et al.,* 2012; Watchararuji *et al.,* 2008). Although changes in the dielectric constant are often cited to justify this increase in solute solubility, loss of hydrogen-bonding capacity, polarizability, and cohesional energy density more closely reflect the molecular level changes occurring in water as its temperature is increased above boiling point (Srinivas *et al.,* 2009). Thus, biologically active proteins or other large molecules in the placenta can be degraded or inactivated, and it is thought that these factors affect safety and biological activity.

SPE showed lower protein content than placenta homogenate, and was associated with decreasing antioxidant activity as the extraction time increased. Placenta homogenate and SPE demonstrated inhibitory effects on elastase activity; the half-maximal inhibitory concentration values were 46.1 and 34.7 µg/mL, respectively. SPE has greater skin permeability properties than placenta homogenate (Han *et al.,* 2013). PPE has also been shown to affect inflammatory responses (Jash *et al.,* 2011). Han *et al.* (2013) also reported that PPE and its constituents regulate protein-energy malnutrition-induced fatigue through improving levels of immunity and decreasing fatigue-related factors. PPE may have potential use as an agent for recovery from fatigue.

The placenta can be considered as a reservoir of cytokines, hormones, bioactive peptides, enzymes, growth factors, vitamins, and minerals (Togashi *et al.,* 2002). PE also contains many valuable bioactive compounds that have various bio-capabilities such as inhibition of the following: aging, inflammation, sunburn, gene mutation, anaphylaxis, and oxidation (Kim *et al.,* 2003).

Recently, benefits of the topical use of PE on chronic and non-healing wounds were reported (Tiwary *et al.,* 2006). Intramuscular injection of human PE commercially known as "Placentrex" also healed radiation mucositis that developed in squamous cell carcinoma patients during radiation therapy (Kaushal *et al.,* 2000). Human PE also features as a component of various skin ointments and is used for skin vitalizing, nourishment, melanocyte growth, and pigment-inducing activities (Pal *et al.,* 2002), as well as for the treatment of skin hypersensitivity conditions, like dermatitis and psoriasis. However, the components and medicinal efficacy of PE have not been clarified in detail.

Ultraviolet (UV) radiation, in particular the middle wavelength (UVB, range 280-320 nm), elicits many adverse effects towards the skin, including cancer (Lyons and O'Brien, 2002), suppression of the immune system (Yoshikawa *et al.,* 1990), and photoaging (Goihman-Yahr, 1996). UVB damage either directly or indirectly occurs through the production of ROS (Cunningham *et al.,* 1985; Vile and Tyrrell, 1995). In the present study, UVB-irradiation induced an increase in intracellular ROS levels. However, SPE treatment in the presence of UVB irradiation significantly lowered ROS levels (Fig. 1).

ROS are produced as a molecular response of skin against UV exposure. However, over-exposure to UV rays leads to unregulated production of ROS, causing damage of cellular components such as lipids, protein, and DNA (Afaq *et al.,* 2006). Particularly, UV-induced ROS production leads to DNA damage and subsequent apoptosis (Liu *et al.,* 2007). ROS up-regulates the expression of MMPs, which mediate extracellular matrix degradation during pathological processes such as arthritis, inflammation, cardiovascular diseases, and cancer (Kong *et al.,* 2010). Among them, MMP-2 (gelatinase A, 72 kDa) is capable of degrading basement membrane in the epidermis and are assumed to be involved in UV-mediated cutaneous damage (Park *et al.,* 2006). High levels of MMPs are known to cause damage to skin connective tissue by degrading collagen and reducing collagen synthesis (Kong *et al.,* 2010). We also demonstrated the increase of MMP-2 expression by UVB irradiation. Therefore, the inhibitory effect of SPE and E-SPE against UVB damage was confirmed by its ability to block MMP-2 expression. In our previous study, SPE showed radical scavenging activity on ABTS and ferric reducing capacity (Han *et al.,* 2013).

UVB exposure also affects the expression of TIMPs (Onoue *et al.,* 2003). Among the identified TIMPs, TIMP-1 and TIMP-2 are known as the major endogenous inhibitors that regulate the activity of MMP-2 (Corbel *et al.,* 2000; Kong *et al.,* 2010). The findings of the present study revealed that the presence of SPE essentially inhibited the effect mediated by UVB; this was achieved by suppress-

ing the expression levels of MMP-2 and inhibiting the decreased expression of TIMP-1. As with SPE, E-SPE had a similar effect on ROS, MMP-2, and TIMP-1 expression. These findings suggest that SPE itself may become biologically active through gastric and intestinal digestion.

Although several types of placenta are commercially available, the use of human and bovine placenta is prohibited in cosmetics. In contrast, porcine placenta is relatively safe and its immune effect is similar to that of human placenta, therefore it is used widely in cosmetic agents. Indeed, porcine placenta has been shown to have beneficial skin care effects, to improve basic metabolic function, and strengthen immune function (Kim *et al.,* 2011).

Notably, E-SPE showed a similar protective effect against UVB damage compared with SPE. Based on these properties, SPE is an effective natural component for preventing cellular damage induced by UVB exposure with potential dermatological and cosmetological value. SPE may provide benefits against photoaging when used as an ingredient for the preparation of functional cosmetic.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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