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Inhibition of inflammatory nitric oxide production and epidermis damages by Saccharomycopsis Ferment Filtrate

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KEYWORDS
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Summary

Background: Yeast extracts have been shown to perform anti-inflammatory and cytoprotective activities. However, the effects of yeast extracts on lipopolysaccharide (LPS)-induced nitric oxide (NO) production and epidermal damages are still unclear.
Objective: To investigate the effect of Saccharomycopsis Ferment Filtrate (SFF) on LPS-induced NO production in RAW264.7 macrophages and epidermal damages.
Method: RAW264.7 cells are incubated with LPS (25 ng/mL) and different concentrations of SFF. The amount of NO production is detected by Griess reaction. Additionally, the expression of inducible nitric oxide synthase (iNOS) and heme oxygenase-1 (HO-1) are detected by Western blotting. Artificial epidermis is also used to mimic the in vivo condition to investigate the protective effects of SFF on LPS- or ultraviolet radiation (UVR)-induced damages by histology and electron microscopy.
Results: The results show that SFF addition inhibits LPS-induced NO production and iNOS protein expression in a concentration-dependent manner without notable

Abbreviations: HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; SFF, Saccharomycopsis Ferment Filtrate; UVR, ultraviolet radiation

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cytotoxicity in RAW264.7 cells, and induction of HO-1 protein expression by SFF was observed. Interestingly, both HO-1 inducers, hemin and $CoCl_2$, significantly attenuated LPS-induced NO production and iNOS protein expression. The addition of $CoCl_2$ potentiated the inhibitory effect of SFF on LPS-induced NO production. It seems that HO-1 protein participates in SFF inhibition of LPS-induced NO production. Furthermore, SFF exhibits significant protective effect on LPS- or UVR-induced damages in the artificial epidermis via histological and electron microscopic observations.
Conclusion: This study provided the first evidence to indicate the beneficial effects of SFF in preventing NO production in macrophages and damages in epidermis, respectively. It suggests that SFF possesses potential to be further developed.

1. Introduction

Inflammation associated with reactive oxygen species (ROS) production has been shown in the aging process of human skin [1—3]. Skin is particularly vulnerable to the damages caused by ROS due to its richness in unsaturated fatty acids, and an exposure to high oxygen tension, ultraviolet radiation (UVR), and several environmental insults such as infestation and irritation was identified as the cause of skin diseases. Macrophage activation participates in skin damages and inflammation. Infiltrated macrophages in inflamed skin produce a large amount of ROS and cause damages and inflammatory responses in the skin [4]. Therefore, effective anti-oxidants and anti-inflammatory agents have been predicted to possess beneficial activities in prevention or treatment of skin damages.

Saccharomycopsis is a family of yeast which has been used to help fermentation of traditional beverages. Saccharomycopsis Ferment Filtrate (SFF), a kind of yeast extracts, was derived from filtration of saccharomycopsis fermented medium. Wildiers [5] and Crowe et al. [6] proposed that the alcohol-extract isolated from yeast culture contains some factors to promote the growth of yeast as well as animal cells. In addition, SFF has been used extensively in cosmetics for whitening and anti-aging. Reduction of the symptoms of burns, cutting wounds, and hemorrhoids by SFF also have been proposed [6—9]. Although SFF has been commonly used in health care, the supporting scientific evidences are still insufficient.

Nitric oxide (NO) has been shown to exert either anti-inflammatory or pro-inflammatory action, and is produced by conversion of L-arginine to L-citrullin catalyzed by nitric oxide synthases (NOSs). There are at least three types of NOS including inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) which have been identified [10,11]. In inflammatory process, activation of iNOS was detected to produce a large amount of NO molecules after exposure to exogenous inducers such as lipopolysaccharide (LPS) from Gram-negative bacteria infection, and it is generally accepted that NO derived from iNOS in LPS-stimulated macrophages is pro-inflammatory and deleterious [12—14]. Therefore, an agent exhibiting ability to block inflammatory response such as NO production in macrophages induced by LPS was predicated for anti-inflammation. Moreover, heme oxygenases (HOs) are enzymes for catalyzing the conversion of heme to bile pigments such as bilirubin and biliverdin [15], and at least two types including HO-1 and HO-2 have been identified [16]. Activation of HO-1 has been considered to protect cells from ROS-induced damages [17,18], and an increase in the sensitivity of cells to ROS was observed in HO-1 knockout cells [19,20]. Our previous studies indicated that HO-1 induction was able to prevent macrophages from LPS-induced NO production via reducing iNOS protein expression [21,22]. However, it is still unclear if induction of HO-1 gene expression occurs in the action mechanism of SFF.

In the present study, we try to investigate the inhibitory effect of SFF on inflammation and epidermal damages. The effect of SFF on modulating the expression of iNOS protein, HO-1 protein, and NO production was elucidated.

2. Materials and methods

2.1. Reagents

Saccharomycopsis Ferment Filtrate (Pitera) was a kind gift from Kobe Technical Center, Procter & Gamble, Japan. A concentrated SFF (200×) stock solution was prepared by freeze drying technology. Briefly, 1000 mL of SFF solution was frozen and dried to powders, and re-dissolved in sterilized PBS buffer (5 mL). LPS, *N*-nitro-L-arginine (NLA) and *N*-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (Sigma Chemical Co.).

2.2. Cell culture

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection. Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/mL of penicillin and 100 U/mL of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco-BRL) and maintained at 37 °C in a humidified incubator containing 5% CO_2.

2.3. Nitrite assay

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction [23]. Briefly, RAW264.7 cells were seeded into 24-well tissue culture plates at a density of 9×10^5 cells per well. After incubation for 12 h, the cells in quadruplicate wells of 24-well plates were co-treated with 25 ng/mL of LPS and different concentrations of SFF. After incubation at 37 °C for 12 h, 100 μL of cell-free supernatant was mixed with 100 μL of Griess reagent (1% sulfanilamide, 0.1% napthyl ethylenediamine dihydrochloride, 5% H_3PO_4) to determine nitrite production. The absorbance at 530 nm was determined with an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories). The nitrite concentration was calculated from a $NaNO_2$ (Sigma) standard curve. Background levels of nitrite were determined in cell-free DMEM with or without the additives and were subtracted from the total amount of nitrite formed.

2.4. Indirect NOS activity assay

Indirect NOS activity assay was performed as previously described [24]. To measure the iNOS enzyme activity in intact cells, RAW264.7 cells were plated in 100 mm tissue culture dishes (4×10^6 cells) and pre-treated with LPS (25 ng/mL) for 12 h. The cells were washed twice with PBS, then were harvested and plated into a 24-well plate (9×10^5 cells per well) and incubated in the absence or presence of tested compounds for further 12 h without LPS in medium. The nitrite produced in the cell-free supernatants was determined by Griess reaction as mentioned above. In this study, L-NAME and NLA are L-arginine analogs, used as positive control.

2.5. Western blot analyses

Total cellular extracts (30 μg) were prepared according to our previous papers [25], separated on 8% SDS-polyacrylamide minigels, and transferred to Immobilon polyvinylidenedifluoride membranes (Millipore). The membrane was blocked at room temperature for 30 min with 3% bovine serum albumin and then incubated at 4 °C overnight with anti-iNOS, anti-HO-1, or anti-α-tubulin monoclonal antibodies (Transduction Laboratories), followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG antibody for 1 h. Protein was visualized by incubating with the colorimetric substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as described in our previous report [22].

2.6. Artificial epidermis tissue culture

EpiDerm™ engineered human skin (MatTek Corp., Ashland, MA) containing human epidermal keratinocytes was grown on collagen-coated Millicel CM membranes. Before testing, the tissue cultures were processed as previous report [26]. Briefly, the cultures were placed in 12-well plates with defined media supplied by the manufacturer (which contains no bovine pituitary extract). On day 4, the epidermal tissue cultures were lifted to the air—liquid interface and then cultured for another 4 days according to the instructions by the manufacturer to generate horny layer. Twenty-four hours after airlifting of the tissue cultures, the medium was changed to medium without epidermal growth factor (EGF). After the horny layer developed, the tissue cultures were used for further testing.

In the LPS-stimulation study, the medium was changed to contain LPS at a concentration of 200 ng/mL. The tissue cultures were then treated with different concentrations of SFF for 12 h, followed by fixation in 10% formalin and paraffin-embedded for histological examination under hematoxylin and eosin stain.

In the UVR-stimulation study, before UVB irradiation, medium was changed to serum-free medium containing different concentrations of SFF for 30 min. Then the tissue cultures were exposed to 800 mJ cm^{-2} of UVB (312 nm). The source of UVB was UV crosslinker (TCX-20M, Vilber Lourmat, France). After UVR, the tissue cultures were cut into 1 mm^3 pieces before being fixed in 2.5% gluteraldehyde in 0.1 M Cacodylate buffer, and then sent for scanning electron microscopy (SEM) (Hitachi S2400).

2.7. Statistics

All experiments were performed in triplicate. ANOVA was used to determine the difference among groups. If significant difference is found, *t*-test was used to determine the location of difference; values of $P < 0.05$ and <0.01 were considered statistically significant.

3. Results

3.1. Inhibition of LPS-induced NO production by SFF in RAW264.7 cells

In order to examine if SFF addition is able to inhibit LPS-induced NO production, RAW264.7 macrophages were used as a model in the present study. In the presence of LPS (25 ng/mL), an increase in the amount of NO production in medium to 30 μM was detected by Griess reaction. As illustrated in Fig. 1, SFF addition exhibits a dose-dependent inhibition on LPS-induced NO production in RAW264.7 cells (Fig. 1A). SFF alone showed no effect on NO production in cells (data not shown). We further examine if SFF inhibition of NO production via affecting iNOS enzyme activity by an indirect NOS enzyme activity assay. Data of Fig. 1B showed that SFF at the concentration of 4× and 8× significantly reduces NO production. Addition of NOS enzyme inhibitors L-NAME and NLA significantly inhibited NO production in the assay as a positive control here.

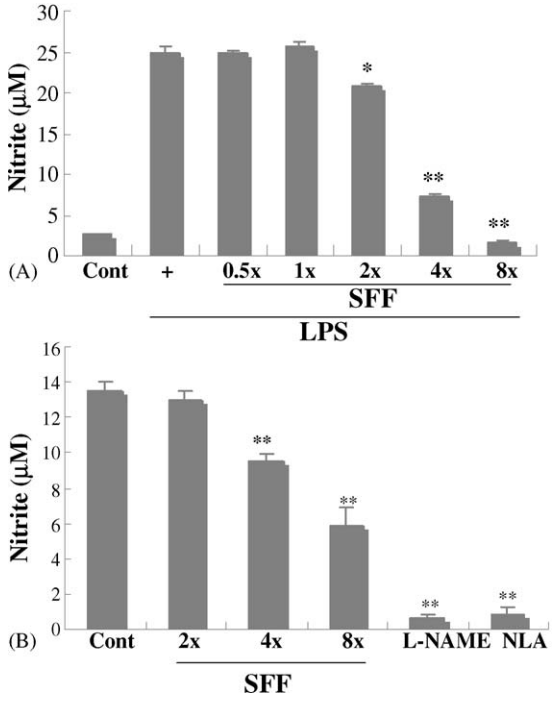

Fig. 1 Inhibition of LPS-induced NO production by SFF in RAW264.7 cells. (A) The amount of NO production induced by LPS in the medium of RAW264.7 cells in the presence or absence of SFF stimulation was determined by Griess reaction. RAW264.7 cells were treated with indicated concentrations of SFF for 30 min followed by adding LPS (25 ng/mL) for 12 h. The amount of NO produced in the medium was detected by Griess reaction. (B) Inhibitory effect of SFF on NOS enzyme activity by an indirect NOS activity assay. Cells were pre-treated with LPS for 12 h, followed by incubating with indicated concentrations of SFF or two NOS inhibitors L-NAME and NLA for a further 12 h. The amount of NO was analyzed by Griess reaction. $^{*}P < 0.05$; $^{**}P < 0.01$ indicates the signficance between control and treated groups. Error bars correspond to 95% confidence intervals.

3.2. SFF inhibition of LPS-induced iNOS protein expression in accordance with inducing HO-1 protein expression in RAW264.7 cells

We examine if SFF suppression of LPS-induced NO production is through blocking endogenous iNOS protein expression by Western blotting using specific antibodies. Results of Fig. 2 show that elevation of iNOS protein is detected in LPS-treated RAW264.7 cells, and the 4× and 8× SFF significantly reduces iNOS protein elevation induced by LPS. In addition, SFF dose-dependently induced HO-1 protein expression in RAW264.7 macrophages. Similar amount of α-tubulin protein in each treatment was detected and described as an internal control.

3.3. HO-1 inducers hemin and $CoCl_2$ inhibition of LPS-induced NO production and iNOS protein expression in accordance with stimulating HO-1 protein expression

In order to elucidate if HO-1 induction was able to block NO production induced by LPS, both HO-1 inducers including hemin and $CoCl_2$ were applied in the present studies. As illustrated in Fig. 3(upper panel), addition of hemin or $CoCl_2$ inhibits LPS-induced NO production by Griess reaction in a dose-dependent manner. As the same part of experiment, hemin and $CoCl_2$ dose-dependently inhibit LPS-induced iNOS protein expression with elevating HO-1 protein expression in cells (Fig. 3, lower

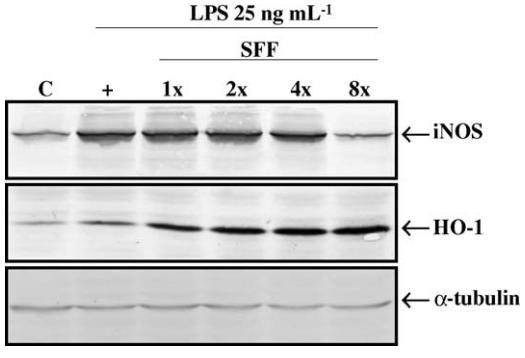

Fig. 2 Inhibition of iNOS protein expression and induction of HO-1 protein expression by SFF in LPS-treated RAW264.7 cells. Cells were treated with indicated concentrations of SFF for 30 min followed by incubation with LPS for a further 12 h. The expression of indicated protein was analyzed by Western blotting using specific antibodies.

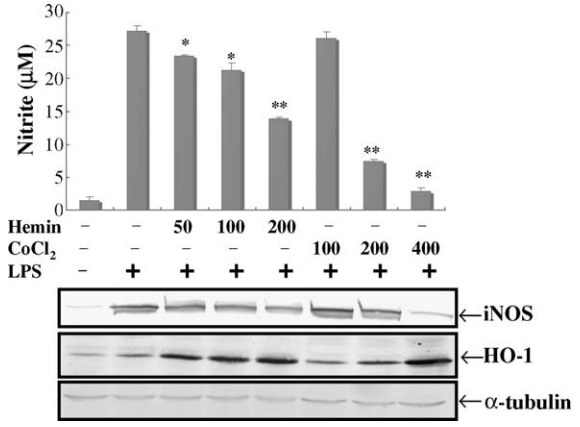

Fig. 3 Induction of HO-1 protein by HO-1 inducers hemin and $CoCl_2$ associated with attenuation of LPS-induced NO production and iNOS protein expression. Cells were treated with different concentrations of hemin or $CoCl_2$ for 30 min, followed by adding LPS for a further 12 h. (Upper panel) The amount of NO in the medium under different treatments was detected by Griess reaction. (Lower panel) The expression of iNOS, HO-1, and α-tubulin protein in cells under different treatments was detected by Western blotting using specific antibodies. $^{*}P < 0.05$; $^{**}P < 0.01$ indicates the significance between control and treated groups.

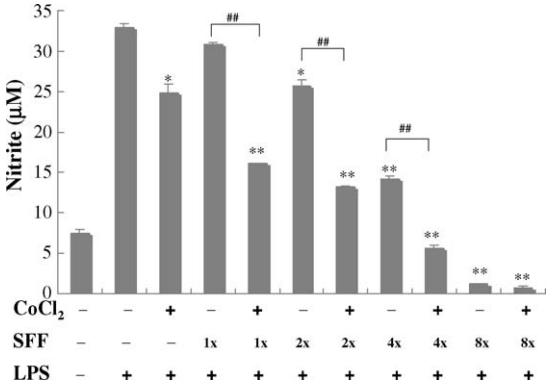

Fig. 4 Addition of $CoCl_2$ enhances the inhibitory effect of SFF on NO produciton and iNOS protein expression with increasing HO-1 protein expression. (Upper panel) Cells were treated with $CoCl_2$ (100 mM) with or without SFF for 30 min, followed by incubation with LPS for a further 12 h. The amount of NO production was analyzed by Griess reaction. $^{*}P < 0.05$; $^{**}P < 0.01$ indicates the significance between control and treated groups. $^{\#\#}P < 0.01$ indicates the signficance between indicated groups.

panel). The expression of α-tubulin protein was described as an internal control to identify similar amount of protein loaded in each lane.

3.4. Addition of HO-1 inducer $CoCl_2$ enhances the inhibitory effect of SFF on LPS-induced NO production in RAW264.7 cells

We further provide evidence to support that HO-1 may participate in NO inhibition of SFF by co-treatment of cells with HO-1 inducer $CoCl_2$ and SFF. Results of Fig. 4 show that $CoCl_2$ (100 μM) slightly but significantly inhibits NO production, and addition of $CoCl_2$ with SFF (1×, 2×, and 4×) significantly potentiates the inhibitory effect of SFF on NO production induced by LPS. No significant additive effect was observed in SFF (8×)-treated cells due to SFF at the dose of 8× performed almost complete inhibition on NO production induced by LPS. It suggests that induction of HO-1 protein expression may potentiate NO inhibition of SFF in LPS-treated macrophages.

3.5. SFF prevention of damages induced by LPS and UVR in 3D skin model

EpiDerm™ has been used as a tool to evaluate cytotoxicity and irritancy of the skin [26,27]. The EpiDerm™ consists of normal human-derived epidermal keratinocytes which have been cultured to form a multilayered, highly differentiated model of the human epidermis. Microscopically, EpiDerm™ consists of organized basal, spinous, granular, and cornified layers analogous to those found in vivo (Fig. 5A, control). In the presence of LPS treatment, an occurrence of dyskeratotic cells in the epidermis and hypergranulosis with coarsening of the keratohyaline granules in the granular layer was observed (Fig. 5B). Those abnormal events can be significantly prevented by incubation of EpiDerm™ with SFF (4×) (Fig. 5C). SFF at the dose of 1× performed less protective effect on LPS-induced damages in EpiDerm™ (Fig. 5D). Similar results were confirmed in UV-irradiated (UVR) EpiDerm™ in the presence or absence of SFF incubation. Results of Fig. 6 showed that a smooth surface was observed in control EpiDerm™ under SEM (Fig. 6A), and blebbing keratinocytes with irregular morphology were detected in UV-irradiated EpiDerm™ (Fig. 6B). Interestingly, incubation of EpiDerm™ with SFF (4×) blocks the occurrence of blebbing keratinocytes in UV-irradiated EpiDerm™ (Fig. 6C). Less protective effect of SFF at the dose of 1× was detected on UV-induced damages in our study (Fig. 6D).

4. Discussion

In the present study, we provide evidences to indicate that SFF exhibits ability to inhibit LPS-induced

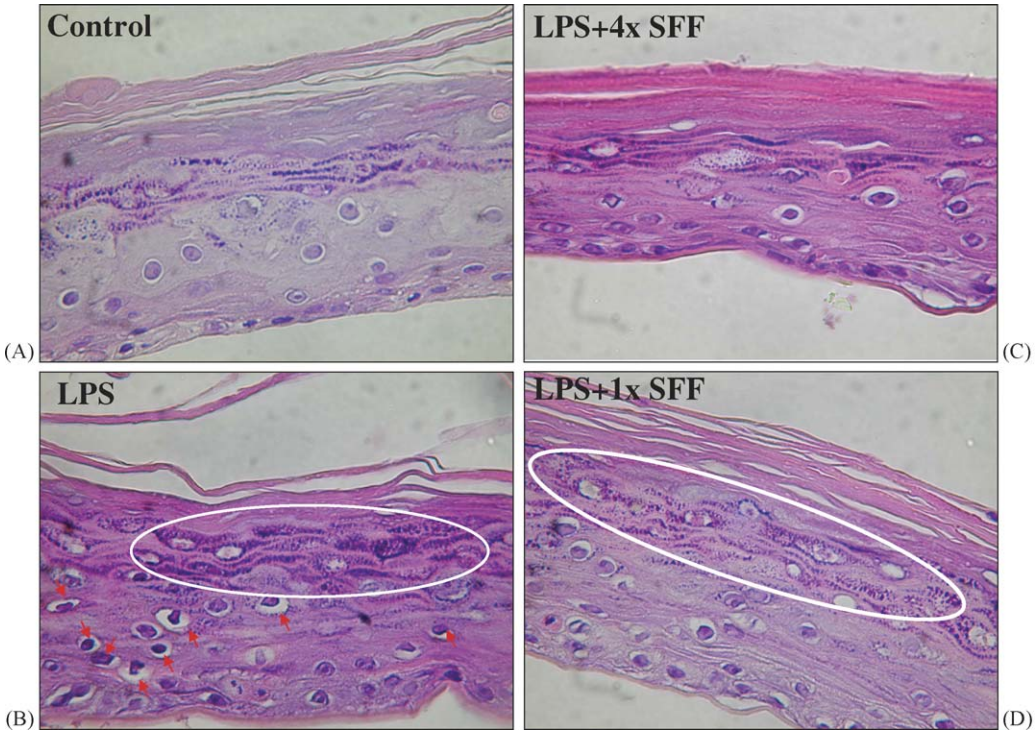

Fig. 5 SFF protection of LPS-induced damage in EpiDerm™. The morphology of cells in 3D skin models was detected under microscopic observation via H&E staining. (A) Control group. (B) Tissues were treated with LPS (200 ng/mL) incubation for 12 h. (C and D) The skins were pre-treated with SFF (C, 4×; D, 1×) for 30 min followed by LPS treatment for a further 12 h. Arrow: dyskeratotic cells; white circle: hypergranulosis with coarse granules.

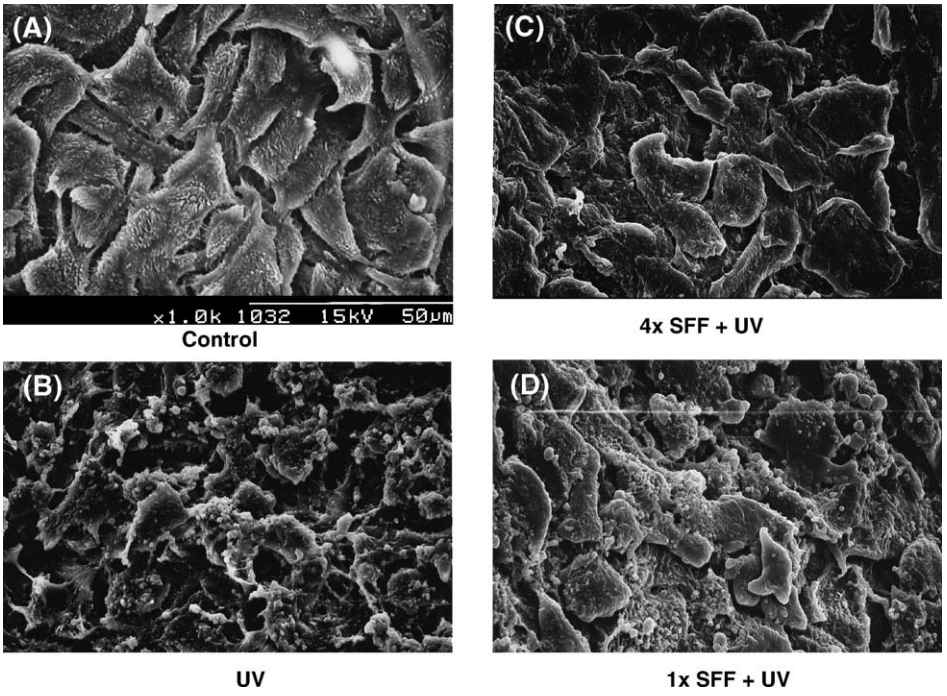

Fig. 6 SFF protection of UVR-induced damages in EpiDerm™. The alternative compositions of EpiDerm™ under different treatments were detected by scanning electromicroscopy. (A) Control group. (B) Tissues were irradiated with UVB (312 nm, 800 mJ cm^{-2}), and incubated for 12 h. The rough surface of keratinocytes cell membrane with blebbing was observed. (C and D) The skins were pre-treated with SFF (C, 4× D, 1×) for 30 min followed by UVR treatment and incubated for a further 12 h. Magnitude of amplification: 1000×.

NO production, and LPS- or UVR-induced epidermal damages. Blocking iNOS protein expression in accordance with elevating HO-1 protein expression was identified in SFF-treated cells. It suggests that induction of HO-1 protein is involved in SFF inhibition of NO production induced by LPS.

HO-1 is a stress-inducible protein which catalyzes the degradation of heme and leads to the production of free iron, carbon monoxide (CO), and biliverdin [28]. Several previous studies have shown the cytoprotective effect of HO-1 in response to various oxidative insults both in vitro and in vivo [29—31]. Inhibition of iNOS protein expression by HO-1 has been shown in macrophages and smooth muscle cells, however, the inhibitory mechanisms of HO-1 on iNOS gene expression is still unclear [32—36]. Lee et al. [33] reported that HO-1 inhibited LPS-induced phosphorylation of IκBα protein and nuclear translocation of p65 subunit of NF-κB [33]. It suggests that interrupting intracellular signal cascade is involved in HO-1 inhibition of iNOS gene expression. Additionally, heme, a substrate of HO-1, has been reported as a key component of iNOS protein, and the amount of NO production catalyzed by iNOS enzyme is reduced in the condition without heme [37,38]. Stuehr indicated that formation of heme—iNOS complex played an important role in maintaining the stable iNOS protein via dimmers formation [39]. It suggests that blocking iNOS gene expression via suppressing intracellular signal cascade or reducing iNOS enzyme activity via decreasing intracellular heme level may contribute to the inhibitory mechanism of HO-1 on NO production in cells.

Two possible mechanisms may be included in SFF inhibition of NO production. One is SFF addition blocks iNOS protein expression and the other is SFF performs inhibitory effect on iNOS enzyme activity. In relation to SFF inhibition of iNOS protein expression has been elucidated in the previous paragraph. Results of NOS enzyme activity assay showed that SFF addition also significantly suppressed NO production. It suggests that NO production inhibited by SFF may be mediated by blocking both iNOS protein expression and enzyme activity. The reason for why SFF reserves ability to affect iNOS protein expression and enzyme activity at the same time is still unclear. In order to make this point clear, we have tried to purify and isolate the components in SFF, and the effects of isolated components on iNOS protein expression, iNOS enzyme activity, and NO inhibition is under investigation.

Although SFF has been used extensively in skin cosmetics, the protective effect of SFF on skin damages is still unclear. Several previous studies showed that NO produced by LPS induced deleterous effects on cells [40,41]. Selleri et al. indicated that inhibition of iNOS gene expression by antisense oligonucleotide prevented haemopoietic cells from Fas-mediated apoptosis [42]. Choi et al. reported the attenuation of HO-1 gene expression by antisense oligonucleotides against HO-1 resistance to Fas-mediated apoptosis [43]. These data indicated that agents possessing ability to suppress iNOS gene expression or to induce HO-1 gene expression reserve potential need to be further developed. In the present study, EpiDerm™ was used to examine the protective effects of SFF on skin damages induced by LPS or UVR. Data of the present study support that SFF addition significantly attenuates LPS- or UVR-induced skin damages. The mechanism of SFF protection skin from LPS- or UVR-induced damages is still unclear. In our previous study, SFF dose-dependently inhibited 1,1-dipehnyl-2-picrylhydrazyl (DPPH) radical production in vitro (data not shown). It suggests that SFF prevention of LPS- and UVR-induced damages in EpiDerm™ system may attribute to its inhibitory effect on ROS and NO production.

In conclusion, we provide first scientific evidences to indicate that SFF possesses several beneficial activities such as anti-inflammation and protection of skin from damages. Inhibition of NO production and iNOS protein expression with HO-1 protein induction was identified. Therefore, identification and purification of functional components are important topics for the application of SFF in the future.

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