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ORIGINAL ARTICLE

Skin-Healing Properties of Ginsenoside Rd against Ultraviolet-B-Induced Photooxidative Stress through Up-Regulation of Antioxidant Components in HaCaT Keratinocytes

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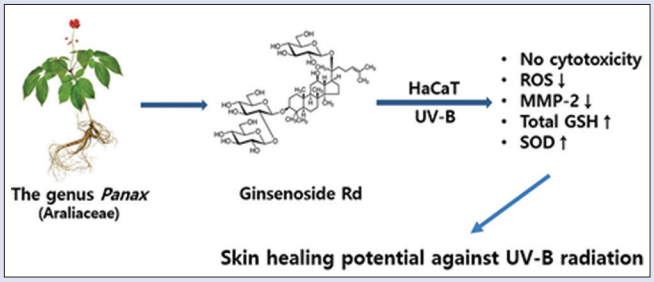
ABSTRACT

Background: Korean ginseng (*Panax ginseng* Meyer) is a traditional herbal medicine used worldwide today. Ginsenoside Rd (Rd), one of its main ginsenosides, has been ascertained to have various pharmacological efficacies including neuroprotective and anti-inflammatory activities. **Objectives:** This work assesses the antioxidant and protective potentials of Rd against ultraviolet (UV)-B-induced skin photooxidative stress in HaCaT keratinocytes. **Materials and Methods:** Cell viability was detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Intracellular reactive oxygen species (ROS) were measured using dichlorodihydrofluorescein diacetate. Promatrix metalloproteinase-2 (proMMP-2) activity and protein were detected using gelatin zymography and western analysis. Total glutathione (GSH) content and total superoxide dismutase (SOD) activity were spectrophotometrically determined. **Results:** Rd, at varying concentrations non-toxic to HaCaT keratinocytes, attenuated the UV-B-induced ROS generation. Rd at 5, 12, and 30 μM attenuated the UV-B-induced proMMP-2 gelatinolytic activities to 59.3% ± 9.5%, 41.3% ± 9.5%, and 13.1% ± 8.9%, respectively, of those of the non-treated control cells. It could also diminish the UV-B-induced proMMP-2 protein levels. Rd at 5, 12, and 30 μM augmented the UV-B-reduced total SOD activities to 1.6 ± 0.2-, 2.4 ± 0.3-, and 3.2 ± 0.2-fold of those of the non-treated control cells, respectively. Rd could up-regulate the UV-B-reduced total GSH. **Conclusion:** Rd has counteracting properties against elevated ROS and proMMP-2 and attenuated GSH and SOD under UV-B irradiation, implying that it possesses a protective activity against photoaging possibly through up-regulating antioxidant components. These findings suggest that Rd can be considered as a novel natural resource for anti-photoaging functional cosmetics.

Key words: Ginsenoside Rd, glutathione, promatrix-metalloproteinase-2, reactive oxygen species, superoxide dismutase, ultraviolet-B radiation

SUMMARY

- Ginsenoside Rd diminishes the ultraviolet (UV)-B-induced elevations of reactive oxygen species and Promatrix metalloproteinase-2 in HaCaT keratinocytes
- Ginsenoside Rd augments the UV-B-reduced total glutathione and superoxide dismutase in HaCaT keratinocytes
- Ginsenoside Rd has no cytotoxicity on HaCaT keratinocytes
- Ginsenoside Rd possesses anti-photoaging properties through up-regulating antioxidant components.



Abbreviations used: Rd: Ginsenoside Rd; ROS: Reactive oxygen species; GSH: Glutathione; proMMP-2: Promatrix metalloproteinase; SOD: Superoxide dismutase; PPD: Protopanaxadiol; PPT: Protopanaxatriol; PBS: Phosphate-buffered saline; GR: Glutathione reductase; GSSG: Oxidized glutathione; GAPDH: Glyceraldehyde 3-phoaphate dehydrogenase; BSA: Bovine serum albumin; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum.

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INTRODUCTION

Korean ginseng (*Panax ginseng* Meyer, family *Araliaceae*) has been regarded as a panacea-like herbal medicine to many people in Asian countries and its therapeutic and supplementary use have popularly increased worldwide. Ginsenosides are its principal active ingredients to which most of its beneficial pharmacological effects are ascribed to. They are categorized into the four types, protopanaxadiol (PPD)-, protopanaxatriol-, oleanolic acid and ocotillol-type ginsenosides, according to the chemical structures of their aglycones. The PPD-type ginsenoside Rd [Rd; (3 β, 12 β)-20-(β-D-glucopyranosyloxy)-12-hydroxydammar-24-en-3-yl 2-*O*-β-D-glucopyranosyl-β-D-glucopyranoside, Figure 1], a relatively newly-discovered ginsenoside, has been considered to have stronger pharmacological activities, especially in neuroprotection, anticancer, and anti-inflammation therapies, than other ginsenosides.[1]

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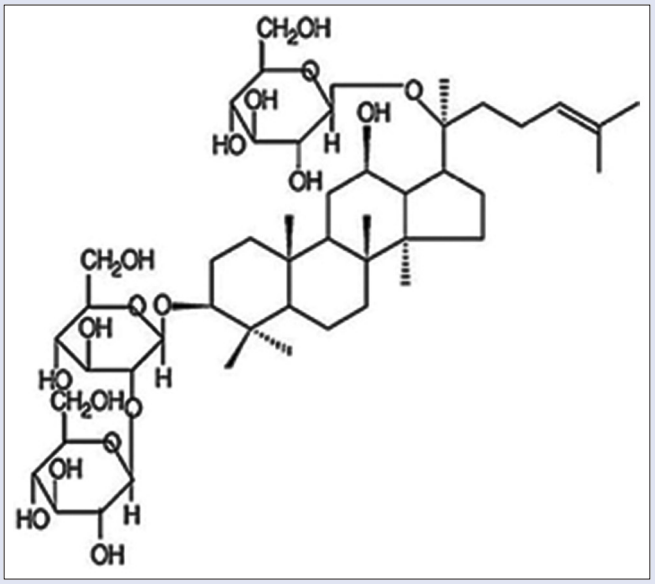

Figure 1: The chemical structure of ginsenoside Rd (Rd)

Rd has been verified to possess ameliorating abilities against neurological disorders, such as ischemic stroke and Alzheimer's and Parkinson's diseases.[1-5] Rd exerts a protective role in the transient focal ischemia of an aged brain through an early free radical-scavenging activity and a late anti-inflammatory effect.[2] It attenuates amyloid-β (Aβ)-induced pathological tau phosphorylation by altering the functional balance of glycogen synthase kinase 3 β and protein phosphatase 2A, protects against neuronal insults induced by Aβ (25–35) peptide in primary cultured hippocampal neurons and improves learning and memory ability in Aβ-protein precursor transgenic mice, implying its anti-Alzheimer's potential.[1,3,4] In experimental models of Parkinson's disease, it exerts a protective effect through its antioxidant effects and mitochondrial function preservation.[5]

The anti-tumor and anti-inflammatory properties of Rd attract other interests. Rd was suggested to have an antitumor property against human breast cancer through its anti-angiogenic activity.[6] It attenuates breast cancer metastasis in mouse mammary carcinoma 4T1 cells through depressing Smad2 expression regulation mediated by miR-18a, a member of the miR-17-92 cluster noted for its oncogenic potentials.[7] It also suppresses metastasis in human hepatocellular carcinoma HepG2 through reducing the expression of MMP-1,-2 and-7 and blocking mitogen-activated protein kinase signaling pathway involved in tumor cell migration.[8] Rd possesses a therapeutic property against 2,4,6-trinitrobenzenesulfonic acid-induced ulcerative colitis, characterized by oxidative and nitrosative stresses and neutrophil infiltration, through facilitating the recovery of pathologic changes in the damaged colonic tissue.[9]

Rd diminishes the frequency of γ-irradiation-induced apoptosis and enhances intestinal crypt survival, implying its radio-protective effect on animals under γ-irradiation.[10] It protects and rescues rat intestinal epithelial cells from γ-irradiation-induced apoptosis through a pathway requiring activation of phosphatidylinositol 3-kinase/protein kinase B, inactivation of mitogen-activated protein kinase and inhibition of a mitochondrial/caspase pathway.[11] Radio-protective effects of Rd against other types of radiations remain to be elusive.

Although some important pharmacological properties of Rd were well documented, its skin beneficial properties have not been elucidated in detail. In this work, the skin antioxidant and anti-photoaging properties of Rd were assessed by evaluating reactive oxygen species (ROS), Promatrix metalloproteinase-2 (proMMP-2), and some antioxidant components as well as cellular viability in the UV-B-irradiated HaCaT keratinocytes.

MATERIALS AND METHODS

Chemicals

Ginsenoside Rd (Rd, purity ≥98%) was from Ambo Institute (Seoul, Korea). Bovine serum albumin (BSA), Bradford reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), glutathione (GSH), GSH reductase (GR), catalase, xanthine, xanthine oxidase, cytochrome c and NADPH were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Cell lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM 1,2-diaminocyclohexane-*N*, *N*, *Nv*, *Nv*-tetraacetic acid, 2 mM dithiothreitol, 10% glycerol, 1% Triton X-100) was purchased from Promega Korea (Seoul, Korea). All other chemicals used were of the highest grade commercially available.

Cell culture

An immortalized HaCaT keratinocyte cell line (ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

Ultraviolet-B irradiation

An ultraviolet (UV) lamp (peak, 312 nm; model VL-6M, Vilber Lourmat, Marine, France) was used as a UV-B source together with a radiometer (model VLX-3W) with a sensor (bandwidth, 280–320 nm; model CX-312). Based on a preliminary work, HaCaT keratinocytes were irradiated with solar simulated UV-B radiation at the intensity of 70 mJ/cm^2 chosen to induce photooxidative stress.

Cell lysate preparation

Adherent cells were washed twice in phosphate-buffered saline and stored on ice for 5 min. The cells were collected by scraping off the bottom of the dish using a cell scraper. After centrifugation at 15,000 g for 10 min, the cell pellets were resuspended in cell lysis buffer and stored for 30 min on ice. Cellular lysate was taken after centrifugation at 15,000 g for 15 min.

Protein determination

Protein contents in cellular lysates were determined according to the procedure of Bradford (1976)[12] using BSA as a reference protein.

Quantitation of intracellular reactive oxygen species

To determine intracellular ROS in HaCaT keratinocytes, an ROS-sensitive probe DCFH-DA, which generates the fluorescent 2′,7′-dichlorofluorescein (DCF; $\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 530 nm) upon enzymatic reduction and subsequent oxidation by ROS, was used.[13] After cells were treated with Rd and/or 20 μM DCFH-DA for 30 min at 37°C, they were washed twice with 1 mL FBS-free DMEM. The cells were resuspended in 1 mL FBS-free DMEM and irradiated with UV-B radiation. The ROS levels were determined by monitoring fluorescence using multi-mode microplate reader (Synergy™ Mx, BioTek Instruments, Winooki, VT, USA).

Cellular viability assay

Cell viability was determined using an MTT assay which is used to assess metabolic activity.[14] Briefly, the cells, after treatment and/or irradiation, were lysed with dimethyl sulfoxide and a purple formazan product, generated from the reduction of MTT by the mitochondria of metabolically active cells, was determined by the absorbance at 540 nm.

Gelatin zymography

The gelatinolytic activity of proMMP-2 in conditioned medium was determined using zymographic analysis as previously described.[15] After SDS-10% PAGE gel was stained with 0.1% Coomassie Brilliant Blue R-250, proMMP-2 activities were convinced as clear zones against a blue background. The proMMP-2 band was confirmed in accordance with its molecular mass, 72 kDa, which was identified by protein molecular weight markers.

Western blot analysis

proMMP-2 proteins in cellular lysates were detected using Western blot analysis using anti-MMP-2 antibody (ALX-210-753, Enzo Life Sciences, Farmingdale, NY, USA) as a primary antibody. GAPDH, used as an internal standard, in cellular lysates was detected using anti-GAPDH antibody (LF-PA0212, AbFrontier, Seoul, Korea). In brief, total cellular proteins were separated on SDS-10% PAGE and electrotransferred to PVDF membranes. The blotted membrane was blocked with blocking buffer (2% BSA in 1X TBS-Tween 20), probed with primary antibodies overnight at 4°C, incubated with secondary antibody (goat anti-rabbit IgG-pAb-HRP-conjugate; ADI-SAB-300, Enzo Life Sciences, Farmingdale, NY, USA) for 1 h at the room temperature and developed using an enhanced West-save up™ (AbFrontier, Seoul, Korea).

Measurement of total glutathione

As previously described,[16] total GSH contents in cellular lysates were determined using an enzymatic recycling assay based on GR. The reaction mixture (200 μL), which contained 175 mM KH_2PO_4, 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM DTNB, 0.5 units/mL GR, and cellular lysate, was incubated at 25°C. Absorbance at 412 nm was monitored using a microplate reader. Total GSH content was represented as μg/mg protein.

Determination of superoxide dismutase activity

As previously described,[17] total superoxide dismutase (SOD) activity in cellular lysates was spectrophotometrically determined based upon the reduction of cytochrome c with xanthine/xanthine oxidase system. The reaction mixture (200 μL) contained 50 mM phosphate buffer (pH 7.4), 0.01 units/mL xanthine oxidase, 0.1 mM EDTA, 1 μM catalase, 0.05 mM xanthine, 20 μM cytochrome c and cellular lysate. A change in absorbance was monitored at 550 nm. The SOD activity was expressed as Δ_{550}/min/mg protein.

Statistical analysis

The results were represented as mean ± standard deviation differences between experimental groups were analyzed using the one-way analysis of variance followed by *post hoc* Tukey HSD test for multiple comparisons. A $P < 0.05$ was considered statistically significant.

RESULTS

Non-cytotoxicity on HaCaT keratinocytes

Prior to starting the main experiments, it was examined whether Rd had a toxic effect on the proliferation of HaCat keratinocytes. As shown in Figure 2a, Rd had no cytotoxic effects on the cellular viabilities of HaCaT keratinocytes. The viable cell numbers of the UV-B-irradiated cells were the same as those of the non-irridiated control cells, implying that the UV-B radiation had no damaging effects on the cellular viabilities [Figure 2b]. As shown in Figure 2b, Rd had no modulating effect on the cellular viabilities of HaCaT keratinocytes under UV-B irradiation. In brief, the UV-B radiation at 70 mJ/cm^2 is noncytotoxic and Rd, at the used concentrations, is noncytotoxic irrespective of UV-B irradiation.

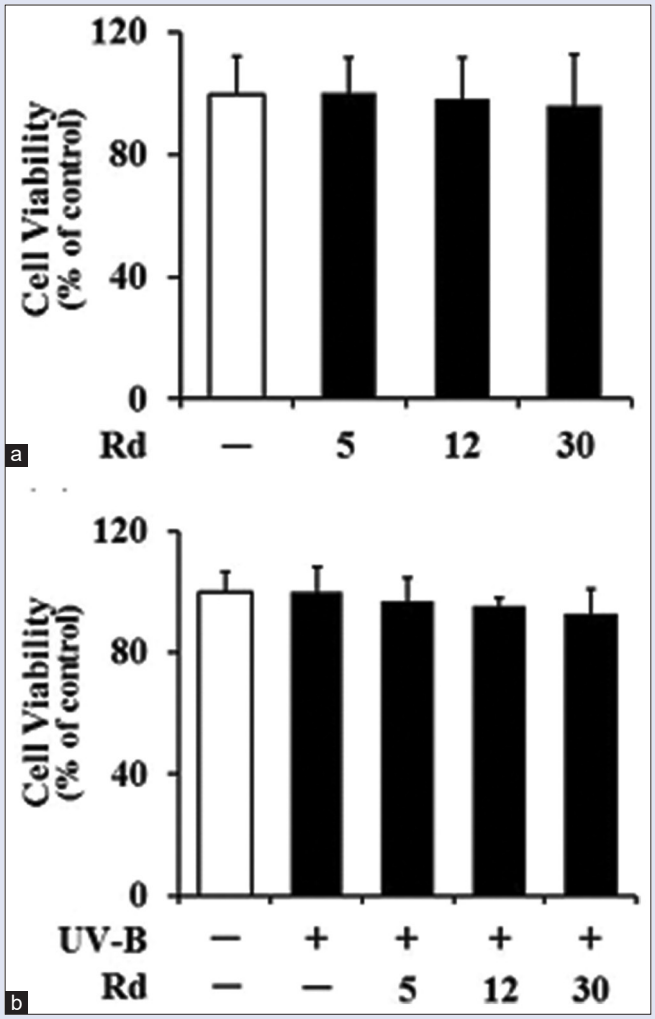

Figure 2: Non-toxic effect of Rd on the cellular viabilities in HaCaT keratinocytes in the absence (a) or presence (b) of ultraviolet-B irradiation. In (a), HaCaT cells were treated with Rd (0, 5, 12, and 30 μM) for 30 min and in (b) they were irradiated with ultravilet-B radiation after the Rd treatment. Cellular viabilities were expressed as % of non-treated control (at column width)

Downregulation of reactive oxygen species

When HaCaT keratinocytes were irradiated with UV-B radiation, their intracellular ROS levels were increased to 4.3 ± 0.5 fold over those of the non-irridiated control cells [Figure 3]. This confirms that 70 mJ/cm^2 UV-B radiation used in the present work with HaCaT keratinocytes induces photooxidative stress which can possibly lead to skin photoaging. When HaCaT cells were subjected to Rd at 5, 12, and 30 μM, their ROS levels were dropped to 82.5% ± 8.2%, 52.6% ± 6.2%, and 50.5% ± 7.2%, respectively, of the UV-B-induced ROS levels [Figure 3]. Its IC_{50} value was estimated to be 30.2 μM. Taken

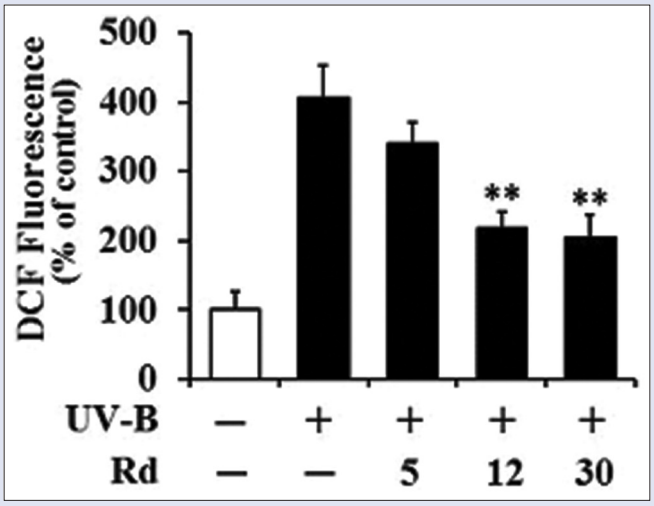

Figure 3: Attenuating effect of Rd on the reactive oxygen species levels in HaCaT keratinocytes under ultraviolet-B irradiation. HaCaT cells were pretreated with Rd (0, 5, 12 and 30 μM) for 30 min prior to the irradiation. Reactive oxygen species levels were represented as 2′,7′-dichlorodihydrofluorescein fluorescence arbitrary unit expressed as % of the non-treated control and each bar shows the mean ± standard deviation of the three independent experiments repeated in triplicate. $^{**}P < 0.01$ versus ultraviolet-B irradiation alone (at column width)

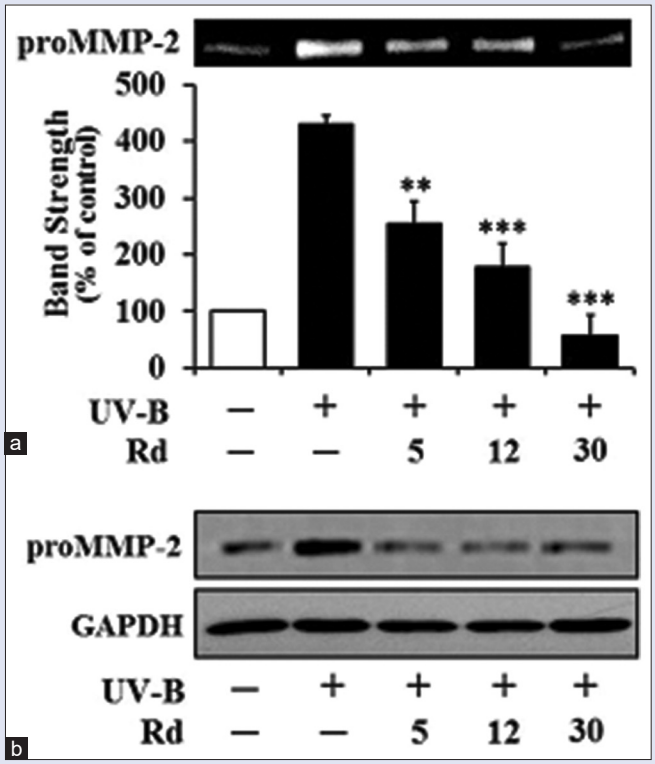

Figure 4: Suppressive effects of Rd on Promatrix metalloproteinase-2 at activity (a) and protein (b) levels in HaCaT keratinocytes under ultraviolet-B irradiation. HaCaT cells were pretreated with Rd (0, 5, 12, and 30 μM) for 30 min prior to the irradiation. In (a), the gelatinolytic activities of Promatrix metalloproteinase-2 in conditioned medium were expressed as % of non-treated control in the lower panel, after the band strengths were determined with densitometry. In (b), the Promatrix metalloproteinase-2 proteins were determined by the Western blot analysis using anti-metalloproteinase-2 antibody. Representatives of the three independent experiments are shown. $^{**}P < 0.01$; $^{***}P < 0.001$ versus ultraviolet-B irradiation alone (at column width)

together, Rd exhibits an attenuating activity against the UV-B-induced ROS in HaCaT keratinocytes, reasonably suggesting its skin antioxidant activity.

Downregulation of promatrix metalloproteinase-2

Like MMP-9, known as gelatinase B, MMP-2 (known as gelatinase A) degrades collagen Type IV, an important component of the basement membrane in skin and other substrates such as collagen Type V, VII and X, fibronectin and elastin.[18] UV-B irradiation was previously shown to augment proMMP-2 mRNAs and gelatinolytic activity, corresponding to proMMP-2, in HaCaT keratinocytes.[19] The proMMP-2 gelatinolytic activity was increased 4.3 ± 0.2-fold in the UV-B-irradiated cells than in the non-irridiated control cells [Figure 4a]. Rd at 5, 12 and 30 μM attenuated the intracellular ROS levels to 59.3% ± 9.5%, 41.3% ± 9.5% and 13.1% ± 8.9%, respectively, of those of the non-treated control cells [Figure 4a]. Its IC_{50} value was determined to be 9.7 μM.

As shown in Figure 4b, the western blot analysis showed that the UV-B irradiation significantly enhanced the proMMP-2 protein levels in the irradiated cells. Rd was able to markedly attenuate the UV-B-induced proMMP-2 protein levels [Figure 4b]. Collectively, Rd is capable of attenuating the proMMP-2 activity and production enhanced by UV-B irradiation in HaCaT keratinocytes, proposing that Rd downregulates proMMP-2 in UV-B-irradiated HaCaT keratinocytes.

Up-regulation of glutathione

The UV-B irradiation diminished the total GSH contents to 83.6% ± 8.2% of those of the non-irridiated control cells [Figure 5]. Rd at 5, 12 and 30 μM was able to recover the UV-B-reduced total GSH contents to 2.0 ± 0.2-, 2.3 ± 0.1-, and 2.6 ± 0.1-fold of those of the non-treated control cells (UV-B irradiation alone), respectively [Figure 5]. In brief, Rd is capable of up-regulating the total GSH levels in HaCaT keratinocytes under UV-B irradiation.

Up-regulation of superoxide dismutase

The UV-B irradiation attenuated the total SOD activity to 75.0% ± 6.8% of those of the non-irridiated control cells [Figure 6]. As shown in Figure 6, Rd at 5, 12, and 30 μM could also give rise to 1.6 ± 0.2-, 2.4 ± 0.3-, and 3.2 ± 0.2-fold enhancements in the total SOD activities, respectively, compared to those of the UV-B-irradiated cells, implying its upregulating capacity on total SOD in UV-B-irradiated HaCaT keratinocytes.

Collectively, Rd has up-regulating activities on at least some antioxidant components, including total GSH content and SOD activity, in HaCaT keratinocytes under UV-B irradiation.

DISCUSSION

A number of adverse biological effects, including photoaging and possibly photo-carcinogenesis, due to the chronic exposure to UV-B radiation in skin cells are triggered by the photochemical generation of ROS.[20] UV-B radiation induces photoaging through the up-regulation of MMPs and subsequent breakdown of extracellular matrix proteins, such as collagen, in skin cells, which is mediated by UV-induced ROS.[21] ROS and epidermal growth factor receptor activation are currently known to play central roles in UV-induced MMP expression through extracellular signal-regulated kinase-mediated AP-1 signaling.[21] This work

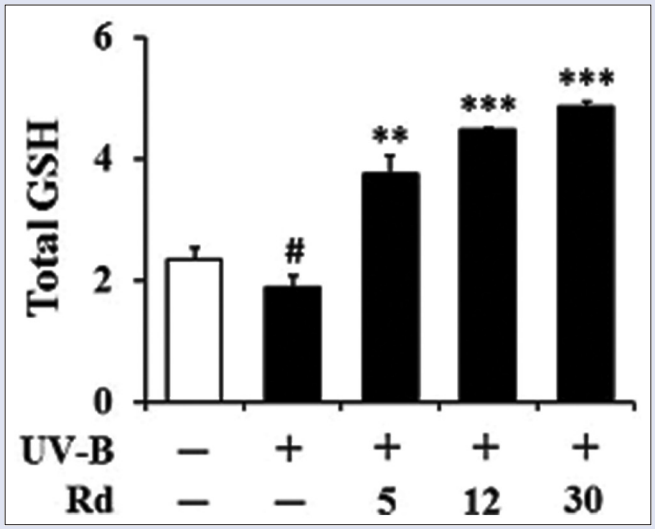

Figure 5: Augmenting effects of Rd on the total glutathione levels of HaCaT keratinocytes under ultraviolet-B irradiation. HaCaT cells were pretreated with Rd (0, 5, 12 and 30 μM) for 30 min prior to the irradiation. Total glutathione content in cellular lysates was expressed as μg/mg protein. $^{\#}P < 0.05$ versus the non-irridiated control. $^{**}P < 0.01$; $^{***}P < 0.001$ versus ultraviolet-B irradiation alone (at column width)

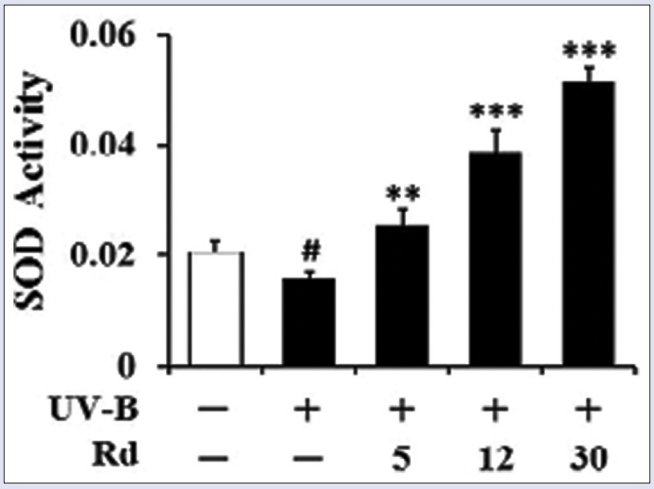

Figure 6: Augmenting effect of Rd on the total superoxide dismutase activity levels of HaCaT keratinocytes under ultraviolet-B irradiation. HaCaT cells were pretreated with Rd (0, 5, 12, and 30 μM) for 30 min prior to the irradiation. Total superoxide dismutase activity in cellular lysates was expressed as Δ_{550}/min/mg protein. $^{\#}P < 0.05$ versus the non-irridiated control. $^{**}P < 0.01$; $^{***}P < 0.001$ versus ultraviolet-B irradiation alone (at column width)

demonstrates that Rd is capable of attenuating the UV-B-induced ROS in HaCaT keratinocytes, which implies that Rd can block the triggering stage of the skin photoaging process.

ROS is considered as a major initiating factor to up-regulate MMPs in keratinocytes and fibroblasts via activation of receptor proteins on the cell membrane of those cells and to degrade fiber components in dermis, leading to wrinkle formation.[22] UV-B-induced ROS activates latent transforming growth factor-β complex by stimulating MMP-2 and-9 activities.[23] In this work, we could verify that Rd, in addition to its ROS-scavenging activity, diminish the UV-B-induced proMMP-2 at both activity and protein levels, directly implying its probable skin anti-photoaging potential. In other words, Rd has a down-regulating capability on the production and subsequent secretion of proMMP-2 in keratinocytes.

Downregulation of intracellular SOD and GSH can practically subject the living cells to oxidative stress. To the contrary, certain compounds, like some natural origin substances, are known to control the cellular redox homeostasis through up-regulating antioxidant components, such as SOD and GSH. For example, jellyfish collagen and its hydrolysate are able to alleviate the UV-induced abnormal changes of oxidative indicators, such as SOD, GSH peroxidase and catalase activities and GSH and malondialedehyde contents and protect skin lipid and collagen from the UV radiation damages.[24] A cell-permeable SOD, Tat-SOD, was shown to suppress tumor necrosis factor-α-induced MMP-9 expression via an ROS-NF-κB-dependent mechanism in HaCaT keratinocytes.[25]

Until the present, the diverse pharmacological properties of Rd have been proved to be mediated through the upregulation of antioxidant components in both cultured cells and animals. Rd plays a protective role on hydrogen peroxide-induced cytotoxicity in rat pheochromocytoma PC12 cells through enhancing the enzymatic activities of SOD and GSH peroxidase.[26] In a rat hepatocyte cell line H4IIE, Rd increases both cellular GSH contents and the levels of γ-glutamylcysteine sythetase heavy chain at the transcriptional level.[27] It attenuates the aging-related oxidative damages by elevating the GSH/GSSG ratio and the activities of GSH peroxidase and GR that are significantly lower in old senescence-accelerated mice than in young ones.[28] It was suggested to be used as a curing agent against induced ulcerative colitis through promoting antioxidant capacities, including SOD and GSH peroxidase and attenuating nitric oxide levels and inducible nitric oxide synthase activity.[9] Rd ameliorates Aβ(25-35)-induced oxidative stress by decreasing the ROS production and malondialdehyde level and increasing the levels of SOD and GSH peroxidase.[1] Similarly, this work demonstrates that Rd is able to enhance the total GSH and SOD activity levels diminished by UV-B irradiation in HaCaT keratinocytes. Although the correct underlying mechanism(s) remain to be uncovered, the upregulation by Rd of antioxidant components, such as GSH and SOD, might be an initiating step to display its antioxidant activity and subsequent attenuating activity on proMMP-2.

CONCLUSION

Rd, at non-toxic concentrations, exerted counteracting properties against elevated ROS and proMMP-2 and attenuated GSH and SOD in HaCaT keratinocytes under 70 mJ/cm^2 UV-B irradiation. These findings imply that Rd possesses a protective potential against UV-B-induced photooxidative stress presumably through upregulating antioxidant components, such as GSH and SOD. Accordingly, it is primarily concluded that Rd, one of the major ginsenosides of ginseng, possesses an anti-photoaging ability on UV-B-irradiated skin, which can be applied as a desirable natural resource with fewer side effects for the manufacture of anti-photoaging functional cosmetics.

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Conflicts of interest

There are no conflicts of interest.

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