Department of Biological Sciences[1] and Department of Biochemistry[2], College of Natural Sciences, Kangwon National University, Chuncheon, Korea

Protective properties of ginsenoside Rb1 against UV-B radiation-induced oxidative stress in human dermal keratinocytes

Sun-Joo Oh[1], Kyunghoon Kim[1], Chang-Jin Lim[2]

Received December 6, 2014, accepted January 9, 2015

Prof. Chang-Jin Lim, Ph.D., Department of Biochemistry, College of Natural Sciences, Kangwon National University, 192-1 Hyoja-2-dong, Chuncheon 200-701, Korea
cjlim@kangwon.ac.kr

Pharmazie 70: 381–387 (2015) *doi: 10.1691/ph.2015.4884*

Ginsenosides, also known as ginseng saponins, are responsible for most pharmacological effect of ginseng. Ginsenoside Rb1 (Rb1) exerts a variety of pharmacological properties, including anti-inflammatory, antistress, anti-aging and anti-neurodegenerative activities. The aim of the present work was to assess the skin anti-photoaging properties of Rb1in human dermal keratinocyte HaCaT cells. The anti-photoaging activity was evaluated by analyzing the levels of reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) as well as cell viability for HaCaT cells under UV-B irradiation. Rb1 was able to suppress the ROS levels which were elevated under UV-B irradiation, and unable to influence cellular survival in UV-B-irradiated HaCaT cells. Rb1 diminished the enhancement of MMP-2 gelatinolytic activity in conditioned medium, which corresponded with the decreased MMP-2 protein levels in both conditioned medium and cellular lysate prepared from UV-B-irradiated HaCaT cultures. Rb1 could restore the total glutathione (GSH) and superoxide dismutase (SOD) activity diminished in UV-B-irradiated HaCaT cells. Ginsenoside Rb1 possesses skin anti-photoaging properties through scavenging ROS and decreasing MMP-2 levels possibly by enhancing antioxidant activity in keratinocytes under UV-B irradiation.

1. Introduction

Ginseng, the dried root of *Panax ginseng* C.A. Meyer (family Araliaceae), is a popular herbal medicine which is used to treat a wide variety of disorders, especially in Korea, China and Japan. Since its supplementary use as a general tonic has been spread all over the world, an interest in its pharmacological efficacies has been broadened. Ginsenosides are a class of steroidal glycosides which are categorized into three groups, such as protopanaxadiol (PPD)-, protopanaxatriol (PPT)- and oleanolic acid-type ginsenosides, based upon the chemical structures of their aglycones. Ginsenoside Rb1 (Rb1, Fig. 1) is one of main PPD-type ginsenosides that are most abundantly present in ginseng.

Although various kinds of pharmacological activities of Rb1 have been documented, most of them are originally based upon its antioxidative activity. Rb1 is protecting chondrocytes against hydrogen peroxide-induced apoptosis in part *via* the inhibition of mitochondrial permeability transition and capase-3 activity (Na et al. 2012). Rb1 exhibits a protective effect against hydrogen peroxide-induced oxidative stress in rat articular chondrocytes (Kim et al. 2012). Rb1 has potent effects on postoperative fatigue syndrome through improvement of energy metabolism and suppression of skeletal muscle oxidative stress (Tan et al. 2013). Rb1 exerts a cardioprotective effect on hydrogen peroxide-induced oxidative injury in cardiomycetes, which is associated with attenuated intracellular ROS generation and suppressed ROS-induced JNK activation (Li et al. 2012). Rb1 effectively protects human umbilical endothelial cells from hydrogen peroxide-induced senescence through the modulation of the hydrogen peroxide-altered caveolin-1 and

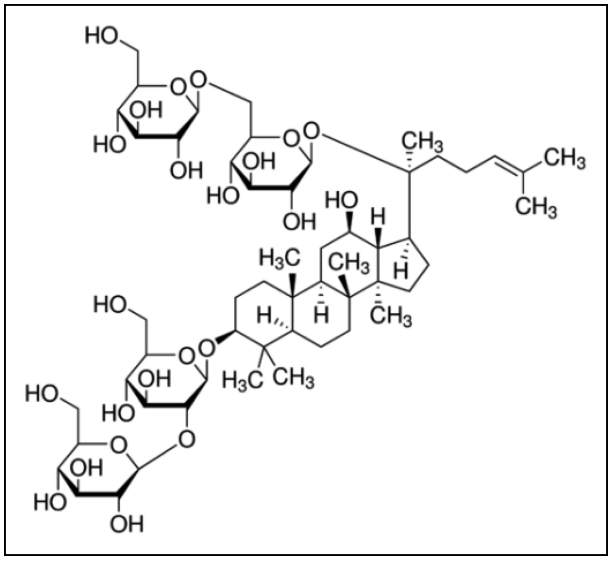

Fig. 1: Ginsenoside Rb1 (Rb1).

pAkt, two important regulators of endothelial nitric oxide synthase expression and activity (Liu et al. 2012). Rb1 inhibits β-amyloid-induced ROS overproduction and lipid peroxidation, and enhances the Bcl-2/Bax ratio and attenuates caspase-3 activation, which plays a protective role against β-amyloid-induced cell injury (Xie et al. 2010). Rb1 augments the cellular antioxidant defenses through endoplasmic reticulum-dependent heme oxygenase-1 induction *via* an estrogen receptor-related

PI3K/Akt-Nrf2-dependent signaling pathway, thereby protecting cells from 6-hydroxydopamine-induced oxidative stress in human dopaminergic cells (Hwang and Jeong 2010). Rb1 exhibits antioxidant effects and antagonizes hydrogen peroxide-induced cellular senescence by increasing intracellular superoxide dismutase activity and decreasing the malondialdehyde level and the production of intracellular ROS in hydrogen peroxide-treated human umbilical vein endothelial cells (Liu et al. 2011). Rb1 displays potent neuroprotective effects against oxidative injury induced by t-butylhydroperoxide in rat neural progenitor cells, implying its antioxidative effect through the activation of Nrf2 (Ni et al. 2014). Rb1 prevents retinal ganglion cells from apoptosis against hypoxia and oxidative stress through the mitochondrial pathway (Liu et al. 2013).

Among UV-A, -B and -C radiations, UV-B (280-315 nm) is primarily involved in skin photoaging, the symptoms of which are wrinkles, laxity, coarseness, mottled pigmentation, epidermal thickening, degradation of matrix macromolecules, vascularization and immunosuppression (Rabe et al. 2006). It acts chiefly on the basal cell layer of the skin, leading the initiation of a photooxidation reaction which damages the antioxidant status of the skin and enhances the cellular ROS levels (Ikehata and Ono 2011). UV-B-induced ROS sequentially brings about oxidative damages to both epidermal and dermal cells, and may ultimately cause apoptotic or necrotic dermal cell death (Rabe et al. 2006). Matrix metalloproteinases (MMPs) are a complex family of zinc-dependent endopeptidases, which are capable of degrading essentially all components of extracellular matrix (Curran and Murray 1999). Skin photoaging is associated with deterioration of the dermal extracellular matrix due to increased MMP expression and decreased collagen synthesis under oxidative stress (Chae et al. 2011; Kim et al. 2013). Collagen degradation, considered as a cause of aging in both naturally aged and photoaged skin, is related to the induction of MMPs which are secreted from epidermal keratinocytes and dermal fibroblasts (Lee et al. 2009).

In the present work, we demonstrate that Rb1 possesses suppressive properties on ROS and MMP-2 enhanced by UV-B irradiation in human dermal keratinocytes, but enhances the total GSH content and SOD activity diminished under UV-B irradiation, implying its potential skin anti-photoaging properties.

2. Investigations and results

2.1. Suppression of UV-B-induced ROS elevation by Rb1

Cultured HaCaT cells were pretreated with varying concentrations (0, 5.0, 12.0 or 30.0 μM) of Rb1 prior to the irradiation with 70 mJ/cm^{2}UV-B. As shown in Fig. 2A, UV-B irradiation, in the absence of Rb1 treatment, could cause approximately 3.2-fold enhancement in the ROS levels over that in the non-irradiated control cells. Rb1 attenuated the UV-B-induced ROS enhancement in a concentration-dependent manner (Fig. 2A). Pretreatment with Rb1 at 30.0 μM reduced the UV-B-induced ROS elevation to 29.9% of that of the cells treated with UV-B alone (Fig. 2A). As shown in Fig. 2B, the suppressive effect of Rb1 was also confirmed by confocal microscopic analysis. Treatment of Rb1 at 5.0, 12.0 and 30.0 μM could decrease the ROS elevation to 83.0%, 70.6% and 56.9% of that of the cells treated with UV-B alone, respectively (Fig. 2B). Collectively, Rb1 suppressed ROS levels enhanced by UV-B irradiation in dermal keratinocytes.

2.2. Cellular survival

To examine whether UV-B radiation at the used intensity and Rb1 at the used concentrations display cytotoxicities on keratinocytes or not, their effects on the cellular viabilities of keratinocytes were determined in a MTT assay. As shown in Fig. 3, UV-B irradiation alone could not give rise to a change in the cellular viabilities of keratinocytes, and their cellular viabilities remained to be similar to that of the non-irradiated keratinocytes. Rb1 was also unable to alter the cellular viabilities of keratinocytes under UV-B irradiation (Fig. 3). Taken together, neither UV-B irradiation nor Rb2, under conditions used in this work, displays cytotoxicities on keratinocytes.

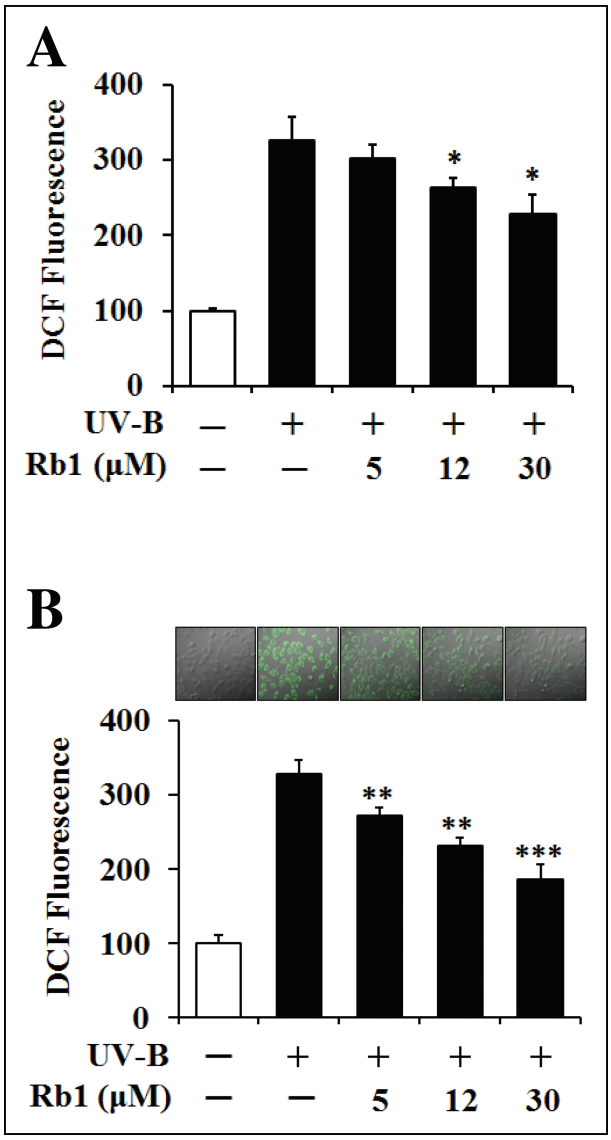

Fig. 2: Effect of Rb1 on reactive oxygen species (ROS) elevation in human HaCaT keratinocytes under irradiation with 70 mJ/cm^2 UV-B radiation. The intracellular ROS levels were determined using DCFH-DA in a microplate fluorometer (**A**) and dihydrorhodamine 123 in confocal microscopic analysis (**B**). In **A**, ROS levels are represented as DCF fluorescence arbitrary units expressed as percentage of control. In the lower panel of **B**, the ROS-associated fluorescent signals were quantified using Adobe Photoshop software. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ versus non-treated control (UV-B irradiation alone).

2.3. Down-regulation of matrix metalloprotenase-2 (MMP-2) by Rb1

Since irradiation with UV, including UV-B, is capable of enhancing expression of certain MMP family members, such as MMP-1, -2 and -9, which degrade collagen and other extracellular matrix proteins that compose the dermal connective tissue, chronic exposure to UV radiation impairs normal architecture of the skin, leading to skin photoaging (Quan et al.

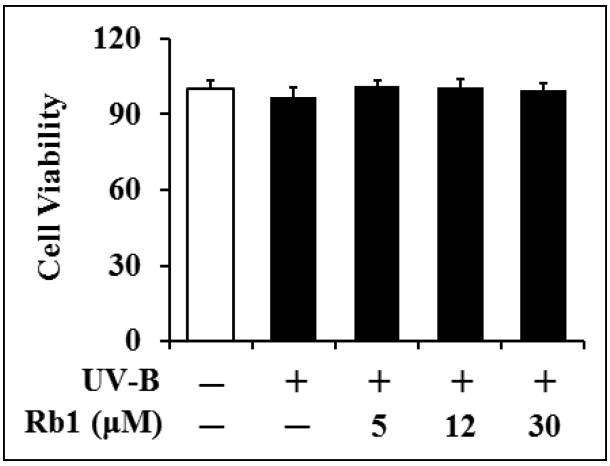

Fig. 3: Effect of Rb1 on cellular viability in human HaCaT keratinocytes under irradiation with 70 mJ/cm^2 UV-B radiation. The viable cell numbers, represented as the relative percentages, were determined using MTT assay.

2009). Rb1 attenuated the UV-B-enhanced MMP-2 gelatinolytic activity in a concentration-dependent fashion in keratinocytes (Fig. 4). Since Rb1 appeared to decrease the UV-B-enhanced MMP-2 gelatinolytic activity, MMP-2 protein levels in both conditioned medium and cellular lysate of keratinocytes were determined using western blotting analysis. The UV-B radiation alone significantly enhanced the MMP-2 protein levels in both conditioned medium and cellular lysate (Fig. 5). Rb1, at the concentrations of 5.0, 12.0 and 30.0 μM, could diminish the UV-B-enhanced MMP-2 protein levels in conditioned medium in a concentration-dependent manner (Fig. 5A). As shown in Fig. 5B, Rb1, used at the same concentrations, was able to attenuate the UV-B-enhanced MMP-2 levels in cellular lysates. Collectively, Rb1 is able to down-regulate the UV-B-enhanced MMP-2 of keratinocytes *via* modulating production and subsequent secretion of MMP-2 protein.

2.4. Up-regulation of total GSH by Rb1

Consistent with a previous report (Zhu and Bowden 2004), total GSH levels were diminished in keratinocytes irradiated with 70 mJ/cm^2 UV-B (Fig. 6A). Treatment with 5.0, 12.0 and 30.0 μM Rb1 was able to increase total GSH levels by 2.5-, 3.3- and 3.5-fold, respectively, compared to that of the UV-B-irradiated keratinocytes that did not receive treatment (Fig. 6A). Taken together, the GSH-enhancing property of Rb1 would be able

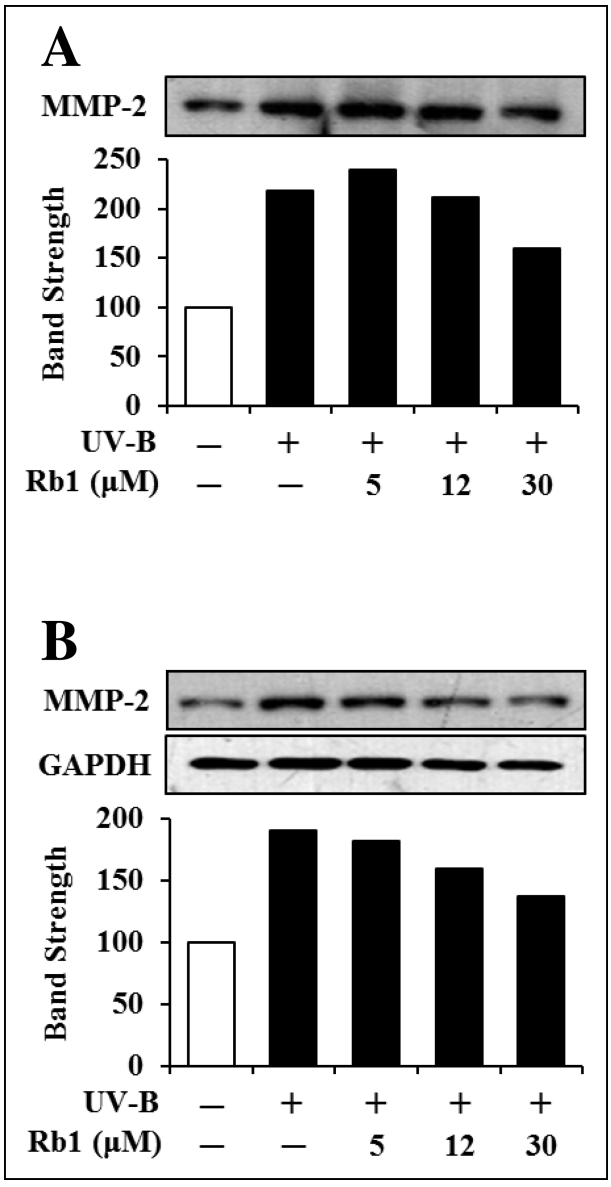

Fig. 5: Effect of Rb1 on matrix metalloproteinase-2 (MMP-2) protein levels in both conditioned media(**A**) and cellular lysates (**B**) obtained from human HaCaT keratinocyte cultures under irradiation with 70 mJ/cm^2 UV-B radiation. The MMP-2 proteins were determined using western blotting analysis with anti-MMP-2 antibodies. GAPDH was used as a protein loading control. The equal loading of conditioned media in **A** was shown in the right panel of Fig. 4. The relative band strength was determined with densitometry using the ImageJ software.

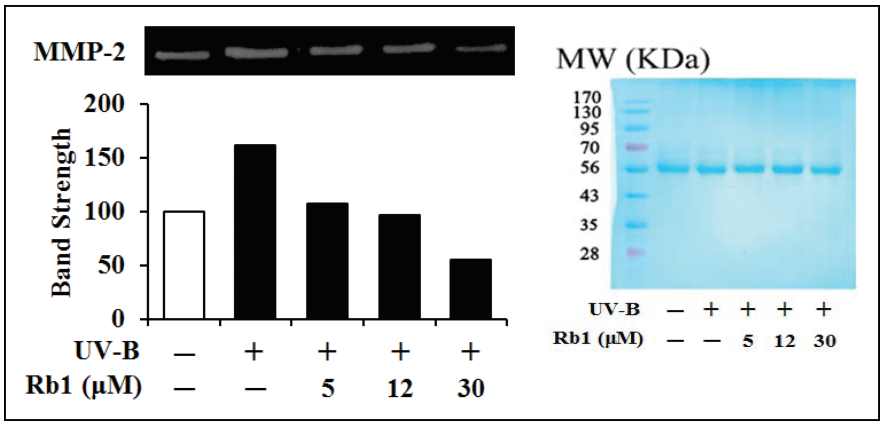

Fig. 4: Effect of Rb1 on matrix metalloproteinase-2 (MMP-2) gelatinolytic activity in the conditioned media obtained from human HaCaT keratinocyte cultures under irradiation with 70 mJ/cm^2 UV-B radiation. Mammalian cells were subjected to fresh media with the indicated concentrations (0, 5.0, 12.0 or 30.0 μM) of Rb1 for 30 min prior to the irradiation. The gelatinolytic activity of MMP-2 in conditioned medium was detected using gelatin zymography. In the right panel, the equal loading of conditioned media was shown by the use of Coomassie Blue staining of the identical gel. The relative band strength was determined with densitometry using the ImageJ software.

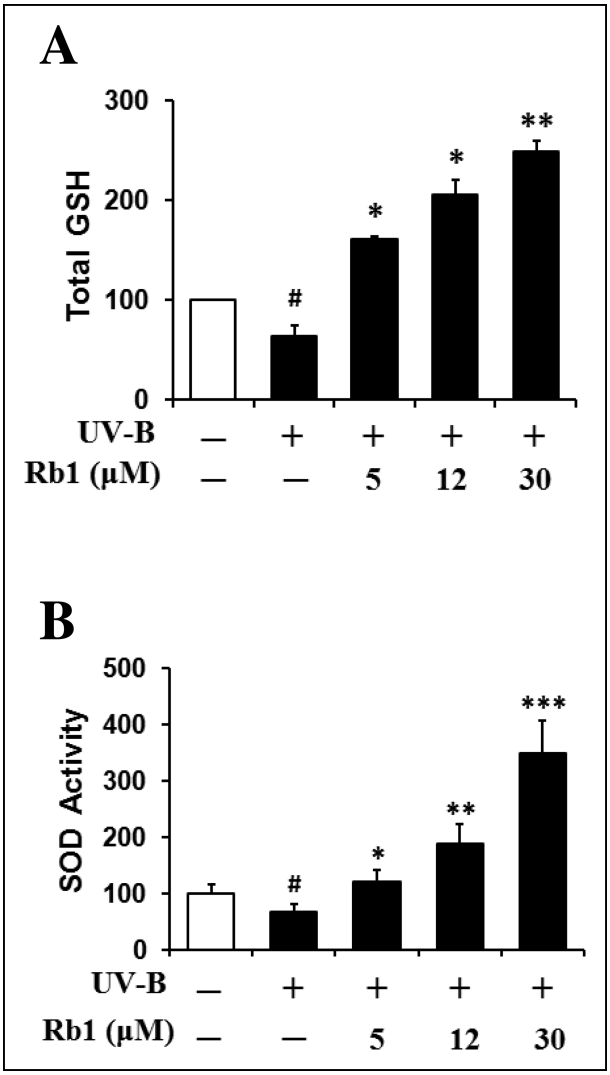

Fig. 6: Effect of Rb1 on total glutathione (GSH, **A**) and superoxide dismutase (SOD, **B**) activity levels in cellular lysates prepared from human HaCaT keratinocyte cultures under irradiation with 70 mJ/cm^2 UV-B radiation. In **A**, total GSH contents were expressed as μg/mg protein. In **B**, SOD activity, expressed as relative percentages, was measured using a spectrophotometric assay. $^{\#}$, $P < 0.05$ versus non-irradiated control: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus non-treated control (UV-B irradiation alone).

to combat the deleterious effects of UV-B irradiation in dermal keratinocytes.

2.5. Up-regulation of total superoxide dismutase (SOD) activity by Rb1

As shown in Fig. 6B, total SOD activity was decreased by irradiation with 70 mJ/cm^2 UV-B in keratinocytes. This diminishment could be restored by treatment with Rb1. Rb1 at 5.0, 12.0 and 30.0 μM could enhance SOD activities by 1.8-, 2.8- and 5.2-fold, respectively, compared to that of the UV-B-irradiated keratinocytes without the reception of Rb1 treatment (Fig. 6B). In brief, Rb1 is capable of restoring SOD activity diminished under UV-B irradiation in keratinocytes.

3. Discussion

In addition to the antioxidation-related activities of Rb1, its diverse activities, especially on neuroprotection, have been assessed using various experimental models. Rb1 possesses potent neuroprotective effects against ischemia, glutamate neurotoxicity, seizures, motor impairment, and cell loss in the striatum (Cheng et al. 2005). Rb1 exhibits a neuroprotective effect against glutamate-induced injury through the inhibition of autophage in cortical neurons, which is linked with the diminishment of autophage-related Beclin-1 by Rb1 (Chen et al. 2010). Rb1 is effectively able to control stress-related hippocampal dysfunction through the suppression of the stress-mediated decline of brain-derived neurotrophic factor and the enhancement of the stress-mediated elevation of heat shock protein 70 level (Kim et al. 2014). Rb1 protects neonatal rat cardiomyocytes from hypoxia/ischemia injury *in vitro via* inhibiting apoptosis based upon the inhibition of the mitochondrial apoptotic pathway (Yan et al. 2014). Rb1 modulates obesity-induced inflammation and improves central leptin sensitivity in high fat diet-induced obesity (Wu et al. 2014). ROS are implicated in various inflammatory diseases including cancer, diabetes, cardiovascular disease, autism, cataract, Alzheimer's disease and aging (Lû et al. 2012). Administration of Rb1 results in an amelioration of the clinical arthritis score in collagen-induced arthritis mice (Kim et al. 2007). Rb1 suppresses UV-induced apoptosis of keratinocytes, which results from a reduction in UV-specific DNA lesions due to the induction of DNA repair (Cai et al. 2009). Homocysteine, an independent risk factor for cardiovascular disease by its multiple effects on vascular cells and thrombosis factors, inhibits endothelial proliferation with increased generation of the superoxide anion, which is effectively blocked by Rb1 (Ohashi et al. 2006). Rb1 can reduce hydroxyl radical and hypochlorous acid, two strong ROS (Lû et al. 2012). According to various reports on the antioxidative properties of Rb1, Rb1 is assumed to be capable of scavenging diverse ROS, such as superoxide anion, hydrogen peroxide, hydroxyl radical and hypochlorous acid. The present work demonstrates that Rb1 exhibits an antioxidative activity also on cultured dermal keratinocytes exposed to UV-B radiation by decreasing the ROS elevation. This finding further supports that most pharmacological activities of Rb1 might be ascribed to its antioxidative properties.

Oxidative stress, which is caused by an imbalance between pro-oxidant processes and antioxidant defense systems, disturbs redox homeostasis in living cells. Chronic exposure of the skin cells to UV light from the sun or artificial sources gives rise to an oxidative stress, resulting in skin photoaging. UV-A and UV-B radiation from sunlight is currently believed to be responsible for approximately 90% of skin photoaging of humans (Jenkins 2002; Ritté and Fisher 2002). In the present work, when cultured dermal keratinocytes were subjected to UV-B radiation, MMP-2 activity and production were significantly enhanced. MMP-2 (64 kD), secreted as proMMP-2 (72 kD), is located on the cell surface and requires activation to exert its catalytic activity (Ellenrieder et al. 2000). After proMMP-2 is recruited to the cell surface by interacting with tissue inhibitor of metalloproteinase-2 (TIMP-2) bound to membrane type 1 MMP (MT1-MMP) by forming a ternary complex, it is activated on the cell surface by free MT1-MMP closely located to the ternary complex (Nagase 1998). MT1-MMP is currently known to be the most common physiological activator of proMMP-2 (Sato and Takino 2010). Type 1 collagen stimulates proteolytic activation of constitutively secreted proMMP-2 on the cell surface (Morley et al. 2007). The induction of TIMP-2 by cholesterol causes the conversion of proMMP-2 into active MMP-2 through the mediation of JNK and ERK in human dermal fibroblasts (Kim et al. 2010). Independent of the MT1-MMP/TIMP-2 pathway, activation of proMMP-2 by snake venom proteinases is known to be another mechanism in human fibroblasts (Saravia-Otten et al. 2004). Although the precise mechanism(s) on the down-regulating properties of Rb1 on MMP-2 elevation in UV-

B-irradiated keratinocytes remain unknown, scavenging of ROS by Rb1 would be estimated to act as a crucial step.

As an intracellular small-molecular weight antioxidant, GSH plays central roles in maintaining cellular redox homeostasis and protecting living cells against oxidative stress and injury. UV-B irradiation-induced GSH depletion in cultured human keratinocytes mimics the pathogenesis of several cutaneous disorders (Zhu and Bowden 2004). Decreased activity of γ-glutamylcysteine synthetase and diminished cysteine uptake through the functional inhibition of the system Xc(-), a cysteine transporter on cell membrane (Zhu and Bowden 2004), are implicated in pathogenesis. GSH depletion has also been shown in a commercially available reconstituted human epidermis model of UV-B radiation (Meloni and Nicolay 2003). When mouse lenses are exposed to UV-B radiation, simultaneous diminishment in both ATP and GSH, which can be prevented by caffeine, were observed (Varma et al. 2008). In the aquatic organism Tubifex, UV-B radiation was found to induce production of singlet oxygen, superoxide anions, and hydroxyl radicals, and to decrease levels of GSH, DNA, RNA, and protein (Misra et al. 2002).

Several pure ginsenosides were reported to restore GSH levels after depletion caused by several kinds of stresses. Ginsenoside Rg1 decreases malondialdehyde levels and intracellular ROS and enhanced superoxide dismutase activity and total GSH in colistin-treated rat pheochromocytoma cells, implying that neuroprotective effects may be mediated by inhibition of oxidative stress (Jiang and Li 2014). Rg1 also enhances total GSH levels in Schwann cells exposed to hydrogen peroxide (Ma et al. 2013), and Rb2, a PPD-type ginsenoside, decreases blood malondialdehyde and elevates total GSH in ovariectomized mice. Re also restores GSH levels in serum of diabetic rats and attenuates diabetes-associated cognitive decline (Liu et al. 2012). Ginsenoside Rd was shown to increase total GSH and promote antioxidant activities of catalase, superoxide dismutase, and glutathione peroxidase in cultured hippocampal neurons exposed to oxygen-glucose deprivation (Ye et al. 2009). Rd also enhances total GSH and levels of γ-glutamylcysteine synthetase heavy chain in a rat hepatocyte cell line (Kim et al 2007). Although GSH-restoring effects were not detected in the experiments using UV-B irradiation, some ginsenosides were shown to enhance GSH levels depleted by stresses.

Eukaryotic cells produce three different SOD isozymes which regulate intracellular superoxide anion ($O_2^{-\bullet}$) levels by catalyzing the dismuation of $O_2^{-\bullet}$ to H_2O_2; Cu/ZnSOD in cytosol, MnSOD within mitochondrial matrix, and an extracellular form similar to Cu/ZnSOD (Frank et al. 2000). Based upon immunohistochemical studies, it was revealed that Cu/ZnSOD is chiefly expressed in keratinocytes (Kobayashi et al. 1993), suggesting that the SOD activity in cellular lysates, determined in the present work, might arise from Cu/ZnSOD. Cu/ZnSOD exhibits a crucial defensive role against UV-B-induced injury of HaCaT cells (Sasaki et al. 2000). *Ginkgo biloba* extracts has a restoring capability on SOD activity diminished in normal human epidermal keratinocytes under trichloroethylene-induced stress (Zhu et al. 2005). Likewise, Rb1 could up-regulate SOD activity diminished under UV-B-induced oxidative stress in keratinocytes, which might partly support the skin anti-photoaging properties of Rb1.

The results presented herein imply that Rb1 restores GSH and SOD depletion caused by exposure to UV-B radiation. Although the mechanisms of these restoring activities remain unknown, restoration of antioxidant components, such as GSH and SOD, might be an initiating event that leads to lowering of ROS in dermal keratinocytes. In conclusion, Rb1 has skin photoprotective properties that may occur through restoration of GSH and SOD levels, reduction of ROS elevation, and down-regulation of MMP-2. Together, the results propose that Rb1 should be considered as one of natural resources for the manufacture of anti-photoaging cosmetics which may have similar or enhanced activities with fewer side effects.

4. Experimental

4.1. Reagents

Ginsenoside Rb1 (Rb1, purity ≥ 98%) was obtained from Ambo Institute (Seoul, Korea). Bovine serum albumin (BSA), gelatin, Bradford reagent, sodium dodecyl sulfate (SDS), GSH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), dihydrorhodamine 123, cytochrome c, xanthine, and xanthine oxidase were from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were from HyClone Laboratories Inc. (Logan, UT, USA). Cell lysis buffer was from Promega Korea (Seoul, Korea). All other chemicals used in this work were of the highest grade commercially available.

4.2. Cell culture

An immortalized human keratinocyte cell line, HaCaT (ATCC, Manassas, VA, USA) was grown in DMEM containing 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. Prior to the treatments, the 1 x 10^5 cells were typically seeded on 24-well plates and cultured overnight, washed twice with 1 ml phosphate-buffered saline (PBS), and replaced with 1 ml FBS-free medium. After UV-B irradiation and/or Rb2 treatment, the mammalian cells were grown under the same culture conditions described above.

4.3. UV-B irradiation

As a UV-B source, an ultraviolet lamp (peak, 312 nm; model VL-6 M, Vilber Lourmat, Marine, France) was used. A radiometer (model VLX-3W, Vilber Lourmat, Marine, France) with a sensor (bandwidth, 280 to 320 nm; model CX-312, Vilber Lourmat, Marine, France) was used to monitor the radiation intensity. The mammalian cells used in this work were irradiated with 70 mJ/cm^2 UV-B radiation at 25 °C.

4.4. Preparation of cellular lysates

For preparation of cellular lysates, adherent cells were washed twice with PBS and stored on ice for 5 min. The cells were harvested by scraping off the bottom of the dish with a cell scraper and centrifuged at 15,000 rpm for 10 min. The cell pellets were resuspended in cell lysis buffer [25 mM tris-phophate (pH 7.8), 2 mM CDTA, 2 mM DTT, 10% glycerol, 1% Triton X-100] and stored for 30 min on ice. Cellular lysates were taken by centrifugation at 15,000 rpm for 15 min.

Protein contents in cellular lysates were measured according to the procedure of Bradford (1976) using BSA as a standard

4.5. Determination of intracellular ROS

To fluorometrically determine intracellular ROS in cultured keratinocytes, a redox-sensitive fluorescent probe DCFH-DA, which produces the fluorescent 2',7'-dichlorofluorescein (DCF; $\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 530 nm) upon enzymatic reduction and subsequent oxidation by ROS, was used as previously described (Royall and Ischiropoulos 1993). After the treatment with Rb1 and/or 20 μM DCFH-DA for 30 min at 37°C, the cells were washed twice with 1 ml FBS-free medium. The cells are resuspended in 1 ml FBS-free medium and irradiated with 70 mJ/cm^2 UV-B. The intracellular ROS levels were immediately quantitated by Multi-Mode Microplate Reader (Synergy^{TM} Mx, BioTek Instruments, Winooki, VT, USA).

In confocal microscopic analysis, the cells were treated with Rb1 and/or 20 μM dihydrorhodamine 123 for 30 min at 37°C, irradiated with 70 mJ/cm^2 UV-B, and immediately analyzed using Confocal Laser Scanning Microscope (Fluoview-FV300, Olympus, Tokyo, Japan). The assays were repeated at least three times.

4.6. Cell viability assay

To determine the cellular survivals of the keratinocytes in the presence of Rb1, the cell viability was determined using MTT assay which is used to assess metabolic activity (Freshney 1993).The cells were treated with Rb1 for 30 min. After removing the medium by suction, the cells were treated with 5 μg/ml MTT in medium for 4 h. The cells were then lysed with dimethyl sulfoxide, and the amount of formazan, generated from the

reduction of MTT by the mitochondria of living cells, was quantitated by the absorbance at a wavelength of 540 nm.

4.7. Gelatin zymography

The gelatinolytic activity of MMP-2 in culture supernatants was determined using zymographic analysis as previously described (Kleiner and Stetler-Stevenson 1994). The cells were further incubated for 24 h at 37°C, and twice washed with 1 ml PBS. The cells in 1 ml FBS-free medium were treated with Rb1 for 30 min and irradiated with 70 mJ/cm^2 UV-B. The culture supernatants, taken from the irradiated culture incubated for 24 h at 37°C, were fractionated on 10% (w/v) SDS-PAGE gel impregnated with 1 mg/ml gelatin under a non-reducing condition. The proteins in the gel were renatured by shaking with 2.5% triton X-100 at room temperature for 30 min, which was repeated two times, and incubated in incubation buffer (50 mM tris buffer, pH 7.8, 5 mM $CaCl_2$, 0.15 M NaCl, 1% Triton X-100) for 24 h. After the gel was stained with 0.1% Coomassie Brilliant Blue R-250, gelatin-degrading enzyme activities were convinced as clear zones against a blue background. MMP-2 activity band was identified in accordance with its molecular mass, which was estimated by molecular mass markers.

4.8. Western blotting analysis

In order to detect MMP-2 in conditioned medium and cellular lysate, western blotting analysis was performed using anti-MMP-2 (ALX-210-753, Enzo Life Sciences, Farmingdale, NY, USA) and anti-GAPDH (LF-PA0212, Young In Frontier, Seoul, Korea) antibody as primary antibodies. Both culture supernatants and cellular lysates were run on 10% (w/v) SDS-PAGE and electrotransferred to PVDF membranes. The membranes were blocked with blocking buffer (2% BSA in 1x TBS-Tween 20), probed with primary antibody 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 room temperature, and developed with the use of an enhanced West-save up ™ (AbFrontier, Seoul, Korea)

4.9. Quantitation of total glutathione (GSH)

As previously described (Nakagawa et al. 1990), total GSH content was determined using an enzymatic recycling assay based on GR (Sigma-Aldrich, St Louis, MO, USA). The reaction mixture (200 µl), containing 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 was reported as µg/mg protein.

4.10. Determination of SOD activity

As previously described (Lee et al. 2002), total SOD activity in cellular lysates was spectrophotometrically determined as 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 the wavelength of 550 nm.

4.11. Statistical analysis

The results are presented as mean ± SD. Statistical comparisons between experimental groups were performed using Kruskal-Wallis test, followed by Dunn's *post hoc* test for pairwise individual comparison. A P value less than 0.05 was considered statistically significant.

Acknowledgments: This study was supported by a grant of the Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant No.: A103017).This study was also supported by 2014 Research Grant from Kangwon National University(No. 120140161). The authors are grateful to Ms. Suyeon Lee for technical assistance.

References

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Cai BX, Jin SL, Luo D, Lin XF, Gao J (2009) Ginsenoside Rb1 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair. Biol Pharm Bull 32: 837–841.

Chae S, Piao MJ, Kang KA, Zhang R, Kim KC, Youn UJ, Nam KW, Lee JH, Hyun JW (2011) Inhibition of matrix metalloproteinase-1 induced by oxidative stress in human keratinocytes by mangiferin isolated from *Anemarrhena asphodeloides*. Biosci Biotechnol Biochem 75: 2321–2325.

Chen Z, Lu T, Yue X, Wei N, Jiang Y, Chen M, Ni G, Liu X, Xu G (2010) Neuroprotective effect of ginsenoside Rb1 on glutamate-induced neurotoxicity: with emphasis on autophagy. Neurosci Lett 482: 264–268.

Cheng Y, Shen LH, Zhang JT (2005) Anti-amnestic and anti-aging effects of ginsenoside Rg1 and Rb1and its mechanism of action. Acta Pharmacol Sin 26: 143–149.

Curran S, Murray GI (1999) Matrix metalloproteinases in tumour invasion and metastasis. J Pathol 189: 300–308.

Ellenrieder V, Alber B, Lacher U, Hendler SF, Menke A, Boeck W, Wagner M, Wilda M, Friess H, Büchler M, Adler G, Gress TM (2000) Role of MT-MMPs and MMP-2 in pancreatic cancer progression. Int J Cancer 85: 14–20.

Frank S, Kämpfer H, Podda M, Kaufmann R, Pfeilschifter J (2000) Identification of copper/zinc superoxide dismutase as a nitric oxide-regulated gene in human (HaCaT) keratinocytes: implications for keratinocyte proliferation. Biochem J 346 Pt 3: 719–728.

Freshney RI (1994) Culture of animal cells: a manual of basic technique, 4th ed, New York: Wiley-Liss Press.

Hwang YP, Jeong HG (2010) Ginsenoside Rb1 protects against 6-hydroxydopamine-induced oxidative stress by increasing heme oxygenase-1 expression through an estrogen receptor-related PI3K/Akt/Nrf2-dependent pathway in human dopaminergic cells.Toxicol Appl Pharmacol 242: 18–28.

Ikehata H, Ono T (2011) The mechanisms of UV mutagenesis. J Radiat Res 52: 115–125.

Jenkins G (2002) Molecular mechanisms of skin ageing. Mech Ageing Develop 123: 801–810.

Jiang GZ, Li JC (2014) Protective effects of ginsenoside Rg1 against colistin sulfate-induced neurotoxicity in PC12 cells, Cell Mol Neurobiol 34: 167–172.

Kim HA, Kim S, Chang SH, Hwang HJ, Choi YN (2007) Anti-arthritic effect of ginsenoside Rb1 on collagen induced arthritis in mice. Int Immunopharmacol 7: 1286–1289.

Kim M, Kim SO, Lee M, Park Y, Kim D, Cho KH, Kim SY, Lee EH (2014) Effects of ginsenoside Rb1 on the stress-induced changes of BDNF and HSP70 expression in rat hippocampus. Environ Toxicol Pharmacol 38: 257–262.

Kim MS, Oh GH, Kim MJ, Hwang JK (2013) Fucosterol inhibits matrix metalloproteinase expression and promotes type-1 procollagen production in UVB-induced HaCaT cells. Photochem Photobiol 89: 911–918.

Kim ND, Pokharel YR, Kang KW (2007) Ginsenoside Rd enhances glutathione levels in H4IIE cells via NF-kappaB-dependent gamma-glutamylcysteine ligase induction. Pharmazie 62: 933–936.

Kim S, Oh JH, Lee Y, Lee J, Cho KH, Chung JH (2010) Induction of tissue inhibitor of matrix metalloproteinase-2 by cholesterol depletion leads to the conversion of proMMP-2 into active MMP-2 in human dermal fibroblasts. Exp Mol Med 42: 38–46.

Kim S, Na JY, Song KB, Choi DS, Kim JH, Kwon YB, Kwon J (2012) Protective effect of ginsenoside Rb1 on hydrogen peroxide-induced oxidative stress in rat articular chondrocytes. J Ginseng Res 36: 161–168.

Kleiner DE, Stetler-Stevenson WG (1994) Quantitative zymography: detection of picogram quantities of gelatinases. Anal Biochem 218: 325–329.

Kobayashi T, Saito N, Takemori N, Iizuka S, Suzuki K, Taniguchi N, Iizuka H (1993) Ultrastructural localization of superoxide dismutase in human skin. Acta Derm Venereol 73: 41–45.

Lee YM, Kim YK, Kim KH, Park SJ, Kim SJ, Chung JH (2009) A novel role for the TRPV1 channel in UV-induced matrix metalloproteinase (MMP)-1 expression in HaCaT cells. J Cell Physiol 219: 766–775.

LeeYY, Kim HG, Jung HI, Shin YH, Hong SM, Park EH, Sa JH, Lim CJ (2002) Activities of antioxidant and redox enzymes in human normal hepatic and hepatoma cell lines. Mol Cells 14: 305–311.

Li J, Shao ZH, Xie JT, Wang CZ, Ramachandran S, Yin JJ, Aung H, Li CQ, Qin G, Vanden Hoek T, Yuan CS (2012) The effects of ginsenoside Rb1 on JNK in oxidative injury in cardiomyocytes. Arch Pharm Res 35: 1259–1267.

Liu Z, Chen J, Huang W, Zeng Z, Yang Y, Zhu B (2013) Ginsenoside Rb1 protects rat retinal ganglion cells against hypoxia and oxidative stress. Mol Med Rep 8: 1397–1403.

Liu DH, Chen YM, Liu Y, Hao BS, Zhou B, Wu L, Wang M, Chen L, Wu WK, Qian XX (2012) Ginsenoside Rb1 reverses H_2O_2-induced senescence in human umbilical endothelial cells: involvement of eNOS pathway. J Cardiovasc Pharmacol 59: 222–230.

Liu DH, Chen YM, Liu Y, Hao BS, Zhou B, Wu L, Wang M, Chen L, Wu WK, Qian XX (2011) Rb1 protects endothelial cells from hydrogen peroxide-

induced cell senescence by modulating redox status. Biol Pharm Bull 34: 1072–1077.

Liu YW, Zhu X, Li W, Lu Q, Wang JY, Wei YQ, Yin XX (2012) Ginsenoside Re attenuates diabetes-associated cognitive deficits in rats. Pharmacol Biochem Behav 101: 93–98.

Lü JM, Weakley SM, Yang Z, Hu M, Yao Q, Chen C (2012) Ginsenoside Rb1 directly scavenges hydroxyl radical and hypochlorous acid. Curr Pharm Des18: 6339–6347.

Ma J, Liu J, Wang Q, Yu H, Chen Y, Xiang L (2013) The beneficial effect of ginsenoside Rg1 on Schwann cells subjected to hydrogen peroxide induced oxidative injury. Int J Biol Sci 9: 624–636.

Meloni M, Nicolay JF (2003) Dynamic monitoring of glutathione redox status in UV-B irradiated reconstituted epidermis: effect of antioxidant activity on skin homeostasis. Toxicol *In Vitro* 17: 609–613.

Misra RB, Babu GS, Ray RS, Hans RK (2002) Tubifex: a sensitive model for UV-B-induced phototoxicity. Ecotoxicol Environ Saf 52: 288–295.

Morley ME, Riches K, Peers C, Porter KE (2007) Hypoxic inhibition of human cardiac fibroblast invasion and MMP-2 activation may impair adaptive myocardial remodelling. Biochem Soc Trans 35: 905–957.

Na JY, Kim S, Song K, Lim KH, Shin GW, Kim JH, Kim B, Kwon YB, Kwon J (2012) Anti-apoptotic activity of ginsenoside Rb1 in hydrogen peroxide-treated chondrocytes: stabilization of mitochondria and the inhibition of caspase-3. J Ginseng Res 36: 242–247.

Nagase H (1998) Cell surface activation of progelatinase A (proMMP-2) and cell migration. Cell Res 8: 179–186.

Nakagawa K, Saijo N, Tsuchida S, Sakai M, Tsunokawa Y, Yokota J, Muramatsu M, Sato K, Terada M, Tew KD (1990) Glutathione-S-transferase pi as a determinant of drug resistance in transfectant cell lines. J Biol Chem 265: 4296–4301.

Ni N, Liu Q, Ren H, Wu D, Luo C, Li P, Wan JB, Su H (2014) Ginsenoside Rb1 protects rat neural progenitor cells against oxidative injury. Molecules 19: 3012–3024.

Ohashi R, Yan S, Mu H, Chai H, Yao Q, Lin PH, Chen C (2006) Effects of homocysteine and ginsenoside Rb1 on endothelial proliferation and superoxide anion production. J Surg Res 133: 89–94.

Quan T, Qin Z, Xia W, Shao Y, Voorhees JJ, Fisher GJ (2009) Matrix-degrading metalloproteinases in photoaging. J Invest Dermatol Symp Proc 14: 20–24.

Rabe JH, Mamelak AJ, McElgunn PJ, Morison WL, Sauder DN (2006) Photoaging: mechanisms and repair. J Am Acad Dermatol 55: 1–19.

Rittié L, Fisher GJ (2002) UV-light-induced signal cascades and skin aging. Ageing Res Rev 1: 705–720.

Royall JA, Ischiropoulos H (1993) Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 302: 348–355.

Royall JA, Ischiropoulos H (1993) Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 302: 348–355.

Saravia-Otten P, Frisan T, Thelestam M, Gutiérrez JM (2004) Membrane independent activation of fibroblast proMMP-2 by snake venom: novel roles for venom proteinases. Toxicon 44: 749–764.

Sasaki H, Akamatsu H, Horio T (2000) Protective role of copper, zinc superoxide dismutase against UVB-induced injury of the human keratinocyte cell line HaCaT. J Invest Dermatol 114: 502–507.

Sato H, Takino T (2010) Coordinate action of membrane-type matrix metalloproteinase-1 (MT1-MMP) and MMP-2 enhances pericellular proteolysis and invasion. Cancer Sci 101: 843–847.

Tan S, Zhou F, Li N, Dong Q, Zhang X, Ye X, Guo J, Chen B, Yu Z (2013) Anti-fatigue effect of ginsenoside Rb1 on postoperative fatigue syndrome induced by major small intestinal resection in rat. Biol Pharm Bull 36: 1634–1639.

Varma SD, Hegde KR, Kovtun S (2008) UV-B-induced damage to the lens *in vitro*: prevention by caffeine. J Ocul Pharmacol Ther 24: 439–444.

Wu Y, Yu Y, Szabo A, Han M, Huang XF (2014) Central inflammation and leptin resistance are attenuated by ginsenoside Rb1 treatment in obese mice fed a high-fat diet. PLoS One 9: e92618.

Xie X, Wang HT, Li CL, Gao XH, Ding JL, Zhao HH, Lu YL (2010) Ginsenoside Rb1 protects PC12 cells against β-amyloid-induced cell injury. Mol Med Rep 3: 635–639.

Yan X, Tian J, Wu H, Liu Y, Ren J, Zheng S, Zhang C, Yang C, Li Y, Wang S (2014) Ginsenoside rb1 protects neonatal rat cardiomyocytes from hypoxia/ischemia induced apoptosis and inhibits activation of the mitochondrial apoptotic pathway. Evid Based Complement Alternat Med 149195.

Ye R, Li N, Han J, Kong X, Cao R, Rao Z, Zhao G (2009) Neuroprotective effects of ginsenoside Rd against oxygen-glucose deprivation in cultured hippocampal neurons. Neurosci Res 64: 306–310.

Zhu M, Bowden GT (2004) Molecular mechanism(s) for UV-B irradiation-induced glutathione depletion in cultured human keratinocytes. Photochem Photobiol 80: 191–196.

Zhu QX, Shen T, Tu DY, Ding R, Liang ZZ, Zhang XJ (2005) Protective effects of *Ginkgo biloba* leaf extracts on trichloroethylene-induced human keratinocyte cytotoxicity and apoptosis. Skin Pharmacol Physiol 18: 160–169.