Research Article

Effect of red ginseng NaturalGEL on skin aging

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A B S T R A C T

Background: In aged skin, degradation of collagen fibers, which occupy the majority of the extracellular matrix in the dermis, and changes of aquaporin 3 (AQP3) and skin constituents, such as hyaluronic acid and ceramide, cause wrinkles and decrease skin moisturization to contribute to dryness and lower elasticity skin. Red ginseng (RG) is used as a cosmetic and food material and is known to protect from UVB-induced cell death, increase skin hydration, prevent wrinkles, and have an antioxidative effect. But, in general, RG used as a material is the soluble liquid portion in the solvent, and the part that is not soluble in the solvent is discarded. Thus, we made the whole RG into microgranulation and dispersed in water to produce gel form for using entire RG, and it was named red ginseng NaturalGEL (RG NGEL).

Methods: RG NGEL was investigated for matrix metalloproteinases inhibitory activity, induction of Type I collagen, AQP3, hyaluronan synthetase 2, serine palmitoyl transferase, ceramide synthase 3, and filaggrin expression and compared with RG water extract.

Results: RG NGEL reduced the levels of UV-induced matrix metalloproteinases and increased Type I collagen in human fibroblast cells and upregulated AQP3, hyaluronan synthetase 2, serine palmitoyl transferase, ceramide synthase 3, and filaggrin expressions in human keratinocytes compared with RG water extract.

Conclusion: RG NGEL has the potential as an effective reagent for antiaging cosmetics to improve wrinkle formation and skin hydration.

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1. Introduction

The skin is composed the epidermis, dermis, and subcutaneous tissues. The epidermis, which is located at the outermost area of the skin, plays a role in protecting the skin from external stimuli or various pathogens and in maintaining moisture and lipid composition. The dermis is a deeper layer of the skin underlying the epidermis and is composed of collagen fibers, elastic fibers, and substrates. Collagen comprises about 90% in the extracellular matrix (ECM) of connective tissue in the dermis and directly affects the strength and tension of the skin [1]. Skin aging is a complex process that is caused by several factors. There are two main processes of cutaneous aging: intrinsic (or innate) and extrinsic aging. Intrinsic aging occurs by random cell damage during metabolic processes and depends on time. Extrinsic aging is caused by environmental stimuli, UV, air pollution, temperature, smoking, etc. [2]. In the aged skin, the number of dermis and epidermis cells may reduce, and the cells become less functional, resulting in wrinkle formation, pigmentation, dryness, thick skin, and decreased elasticity [3].

The exposure to UV light induces the production of matrix metalloproteinases (MMPs) in human skin. MMPs are responsible for the degradation of the ECM proteins such as collagen, elastin, and proteoglycans [3,4]. MMPs are divided into about five major subgroups according to their substrate characteristics, structure, and localization. Among them, MMP-1, as a collagenase, degrades Type I collagen (Col-I), the main form in human skin, and Col-III, and MMP-2 and MMP-9 are gelatinases that digest Col-I and Col-IV [4,5]. It was reported that collagen synthesis is reduced in naturally aged skin and photoaged skin. Also, this reduction in collagen synthesis with increasing age was caused by the increased MMPs level [6–8].

As mentioned earlier, the skin wrinkles as well as dries when it ages, and the stratum corneum (SC) plays a role in maintaining skin hydration...
moisture. In the SC, lipids, such as ceramide (CER), cholesterol, and free fatty acids, are responsible for the formation of the skin barrier and the prevention of transepidermal water loss (TEWL). Especially, CER constitutes about 50% of intercellular SC lipids and is generally produced by the de novo pathway, one of which is initiated by serine palmitoyl transferase (SPT) [9,10]. And, it is known that amino acids and derivatives produced by flaggrin (FLG) processing are the contributors to the natural moisturizing factors (NMFs). NMFs maintain skin hydration and retain water [11,12]. Also, decreased aquaporin 3 (AQP3) levels in the aged skin cause dry skin, reduced skin elasticity, and barrier function decline [13,14].

Panax ginseng is a perennial herb that belongs to the Araliaceae family and the genus Panax and has been widely used as a medicinal herb as it has many benefits. Red ginseng (RG) is a red brown ginseng made by steaming and drying for a long time [15]. There are several studies related to skin. It was reported that RG ethanol extracts increased collagen synthesis and inhibited MMP-1 activity [16]. Ginsenoside F1 protects human keratinocytes from UVB-induced apoptosis, compound K increases the level of hyaluronic synthetase 2 (HAS2) in human keratinocytes, and 0.5% RG extract improves skin hydration by accumulating CER and increasing the expression of SPT in UV-irradiated hairless mice [17–19]. Also, RG residue extracts showed antioxidative effect and inhibited elastase activity [15].

When manufactured as a product, RG is extracted or concentrated using a solvent such as water or ethanol and is used as a powder or beverage according to the type of product required [20]. In this way, only the soluble portion of RG is used, and the non-soluble fiber part is discarded which then becomes an environmental pollution source [21]. However, these discarded RG also had antioxidant and antiwrinkle effects and increased immunity when used in chicken feed [15,21]. Therefore, we made the entire RG into gel form to use whole RG, including both the soluble and non-soluble RG portions. In the present study, we investigated RG NGEL and RG WE for UV-induced MMP-1, MMP-2, and MMP-9 expression; MMP-2 and MMP-9 gelatinase activity; and Coll-I expression in human fibroblast cells. In addition, we examined the expression of AQP3, HAS2, SPT, ceramide synthase 3 (CERS3), and FLG related to skin moisturization in human keratinocytes.

2. Materials and methods

2.1. RG WE

RG was extracted in distilled water for 2 h at 60 °C, and then the water extract was filtered using a 0.45-μm filter (Millipore, USA).

2.2. RG NGEL

RG powder (5 g) was stirred in purified water (100 ml) containing 0.2 mmol/g of (2,2,6,6-tetramethylpiperidin-1-yl)oxyxyl (TEPPO; Alfa Aesar, USA), 2 mmol/g of NaBr (Ducksan, Korea), and 10 mmol/g of NaClO (Daegung, Korea) at pH 10 and room temperature for 24 h. The mixture was maintained at pH 10–11 with 0.5 M NaOH (Ducksan, Korea). After oxidation, 0.175 ml of ethanol (Ducksan, Korea) was added to quench the reaction, and the pH of the mixture was adjusted to 6 with 1 M HCl (Ducksan, Korea). The mixture was washed with ethanol and purified water, then it is fibrillated using a microfluidizer (Microfluidics corporation, USA) and concentrated, followed by dispersing it in water and vacuum evaporating (Buchi, Switzerland) at 70 °C to form the gel.

2.3. Cell culture

Fibroblast cells were purchased from American Type Culture Collection (USA) and cultured in Iscove's modified Dulbecco's medium (Welgene, Korea) supplemented with antibiotics (Gibco, USA) and 10% fetal bovine serum (FBS; Gibco, USA). Immortalized human keratinocytes (HaCaT) were cultured in Dulbecco's modified Eagle's medium (Welgene, Korea) supplemented with antibiotics (Gibco, USA) and 10% FBS (Gibco, USA). Human epidermal keratinocyte- neonatal (HEKn) was purchased from Gibco (USA) and cultured in EpiliE medium (Gibco, USA). All conditions are maintained at 37 °C with 5% CO2 atmosphere.

2.4. High-performance liquid chromatography analysis

Chromatographic analysis was performed by HPLC (Shimadzu, prominence, Japan) equipped with a C18 column (250 × 4.6 mm id). The flow rate was 1 ml/min, the column temperature was 35 °C, and the peak was detected using an ultraviolet absorption spectrophotometer (UV 203 nm). Water (A) and acetonitrile (B) were used in the mobile phase. The ratio of (A) was 80% at 10 min, 60% at 35 min, 20% at 60 min, and 80% at 75 min.

2.5. Zymography on gelatinase (MMP-2 and MMP-9) activity

Dermal fibroblasts were seeded on 60-mm plates and incubated for 1 day. And, cells were irradiated with UVA 6 J/cm² and then incubated overnight in Iscove's modified Dulbecco's medium containing 10% FBS and RG NG or RG WE (0, 100, 250, or 500 μg/ml). The conditioned media were collected using an Amicon Ultra-4 centrifugal filter (Millipore, USA). The proteins in the media were concentrated by centrifugation (13,000 g for 3 h at 4 °C). The proteins were separated on 10% zymogram gel (Invitrogen, USA), and the gels were renatured in 2.5% Triton X-100 and then were developed in buffer with 50 mM Tris-HCl, pH 7.6, 1 mM CaCl₂, 0.2 M NaCl overnight at 37 °C. The gels were stained with Coomassie blue (Brilliant blue G solution; Sigma, USA) and destained in buffer with 10% acetic acid and MeOH. Gelatinolysis activity, i.e., unstained areas on gel, was quantified by using Luminograph II and CSAnalyzer4 program (Atto, Japan). Zymography was performed in triplicates for each sample.

2.6. Cell viability assay

Cell viabilities were determined by 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay. Briefly, 2.5 mg/ml of MTT in phosphate buffered saline was added to wells of 24-well plates and incubated at 37 °C for 4 h, followed by the addition of dimethyl sulfoxide (Duksan, Korea). The absorbance was measured at 570 nm. Absorbance readings were subtracted from the value of blank wells. Cell viabilities were calculated as a percentage of control absorbance.

2.7. Extraction of total RNA and cDNA synthesis

Total RNA was extracted by using TriZol (Invitrogen, USA) following the manufacturer’s protocol. RNA concentrations were determined using a Qubit RNA BR Assay kit (Invitrogen, USA). A high-capacity RNA-to-cDNA kit (Applied Biosystems, USA) was then used for cDNA synthesis following the manufacturer’s protocol. The cDNA was stored at −20 °C until use.

2.8. Quantitative real-time polymerase chain reaction

Using Power SYBR Green PCR Mix (Applied Biosystems, USA) following the manufacturer’s protocol, quantitative real-time polymerase chain reaction (qRT-PCR) reactions were performed in triplicates. Primers for the amplification of MMP-1 (F: 5'-GAT GCG GTA TAC GAG GCC C-3', R: 5'-TCT AGG GAA GCC AAA GGA GCT-3'), MMP-2 (F: 5'-GAT GCC GTA TAC GAG GCC C-3', R: 5'-GAT...
Fig. 1. Cytotoxicity of RG NGEL and RG WE against human skin cells. (A) Fibroblast and (B) HaCaT cells were treated with various concentrations (0–500 μg/ml) of RG NGEL or RG WE for 24 h. Data are expressed as the mean value (±SD) from three separate experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

RG NGEL, red ginseng NaturalGEL; RG WE, red ginseng water extract.
showed no cytotoxicity at concentrations between 0 and 500 μg/ml in fibroblast cells. In keratinocytes, both RG NGEL and RG WE showed toxicity from 100 μg/ml (Fig. 1).

3.2. **RG NGEL induces the production of Col-I**

Collagen is a major constituent of connective tissue, and Col-I is the main form in human skin [1]. And, RG ethanol extract induced the synthesis of collagen and suppressed MMP-1 activity, but not WE [16]. To investigate whether RG NGEL and RG WE increase the production of Col-I, we performed qRT-PCR, and the results are shown in Fig. 2. RG WE did not increase Col-I mRNA expression, but the group treated with RG NGEL at 250 and 500 μg/ml induced Col-I expression at 128.9% and 131.2%, respectively (p < 0.01), as compared with the control group. These results suggested that RG NGEL has an effect on improvement of skin elasticity.

3.3. **RG NGEL regulates the expression and activity of MMPs**

MMP-1 is a typical collagenase-degrading collagen, and MMP-2 and MMP-9 are gelatinases that digest Col-I and Col-IV. These MMPs contribute for wrinkle formation and reduction of skin elasticity [4]. To examine RG NGEL and RG WE inhibiting MMPs, we measured MMP-1, MMP-2, and MMP-9 levels and MMP-2 and MMP-9 activity. As shown in Fig. 3, after treatment with RG NGEL, MMP-1, MMP-2, and MMP-9 mRNA levels were decreased by approximately 43.7%, 30.3%, and 25.1%, respectively (p < 0.001). RG WE reduced MMP-1 and MMP-2 levels by about 16.6% and 29.5%, respectively (p < 0.001) and decreased MMP-9 only at 500 μg/ml. And, RG NGEL significantly suppressed the activity of MMP-2 and MMP-9 by 72.5% (inactive form, p < 0.001), 79.2% (active form, p < 0.001), and 61.3% (p < 0.01) (Fig. 4). After treatment with RG WE, MMP-2 activity was inhibited only at 500 μg/ml, and the activity of MMP-9 was not inhibited. These findings indicated that RG NGEL can prevent wrinkle formation by regulating MMPs induced during the aging process compared with RG WE.

3.4. **RG NGEL upregulates the levels of skin hydration–related factors**

Changes in skin water content and barrier function occur as the skin ages. AQP3, as a membrane channel, is responsible for allowing
water, glycerol, and others to pass through the membrane. It was known that decrease of AQP3 causes skin dryness and reduction of skin elasticity [13,14]. HA, a component of the ECM, is important for skin hydration and is synthesized by a specific enzyme called HAS. Among three HASs, HAS2 is a major isoform producing HA in skin [22]. In the SC, CER, cholesterol, and free fatty acids are responsible for the prevention of water loss. CER is generally produced through the de novo pathway, one of which is initiated by SPT [10]. And, pro-FLG is processed to FLG repeat units, and FLG is degraded into free amino acids, which contribute to formation of the NMFs that retain water content on the skin [11].

We determined whether RG NGEL and RG WE affected mRNA expression of these moisturization-related factors. RG NGEL significantly increased the levels of AQP3 and FLG by 145.4% (\(p < 0.001\)) and 134% (\(p < 0.001\)), respectively, in a dose-dependent manner, compared with RG WE. And, the mRNA levels of SPT and HAS2 were upregulated by RG NGEL compared with RG WE (Fig. 5A). We also performed immunoblotting to examine the protein expression of CERS3 and FLG. Results are shown in panel B and C in Fig. 5. After treatment with RG NGEL, CERS3 was effectively increased by more than two times than after treating with control (\(p < 0.01\)). RG WE induced CERS3 by 154.2% (\(p < 0.01\)), and FLG
protein level rose by 169.4% \((p < 0.001)\) on treating with RG WE compared with treating with RG WE. These results suggested that RG NGEL might induce the levels of AQP3, HAS2, SPT, FLG, and CERS3 to contribute to skin moisturization and elasticity.

3.5. **HPLC analysis of RG NGEL and RG WE**

To investigate ginsenosides, the main components of RG, contained in RG WE and RG NGEL, we performed HPLC analysis.
Accurate analysis of these two substances was performed by comparing the HPLC peak with standard materials of Rgl1, Re, Rb1, Rh1-S, and Rh1-R (Sigma-Aldrich, USA). In the RG NGEL analysis, no peak was found. RG WE contained 1.13% of Rgl1, 1.28% of Re, 2.89% of Rb1, 0.94% of Rh1-S, and 0.46% of Rh1-R.

4. Discussion

Most RGs are extracted or concentrated using a solvent such as water or ethanol and then used as a material for the product [20]. Because RG is only used by dissolving in solvents, a portion that is not soluble in water is abandoned in large quantities, which causes environmental pollution. However, the discarded part is known to have antioxidant and antiwrinkle effects. To utilize the whole RG, we made the entire RG into microgranulation and dispersed in water to make gel form. It has been reported that ginsenosides contained in RG were effective in collagen production, inhibition of MMP-1 activity, protection against UVB-induced cell death, improvement of skin hydration, and antioxidation [15–19]. We focused on the effect of RG NGEL on skin aging in this study by using measurement of wrinkle formation–related factors, Col-I, and MMPs in human fibroblast cells and investigating to maintain skin moisture. UV irradiation increases MMPs, MMP-1, MMP-2, and MMP-9, followed by degradation of collagen [4,23]. RC NGEL increased the mRNA level of Col-I, but not RG WE (Fig. 2), and significantly inhibited UV-induced MMP-1, MMP-2, and MMP-9 expression compared with RG WE (Fig. 3). In addition, RG NGEL suppressed the activity of MMP-2 and MMP-9 compared with UV irradiation (Fig. 4). These results showed that RG NGEL has the improvement of skin elasticity and wrinkle formation. In our analysis of RG WE, Rb1 content was 2.89%, slightly higher than that of Rb1 5, 10 mg/ml, which showed an increase in procollagen and inhibition of MMP-1 activity in previous studies [16]. However, in our studies, RG WE inhibited MMPs expression but did not increase Col-I expression.

Our results showed that RG NGEL effectively increased transcription of AQP3, FLG, HAS2, and SPT (Fig. 5A) and protein expression of FLG and CERS3 compared with RG WE (Fig. 5B and C). These results suggested that RG NGEL has the effect of skin hydration and elasticity by inducing moisturization-related factors, AQP3, HAS2, SPT, FLG, and CERS3.

In previous studies, tissue inhibitor of matrix metalloproteinases (TIMPs) bind noncovalently to MMPs and inhibit them [24,25]. Alternatively, UV irradiation increases both MMPs and TIMPs, which are controlled by factors other than TIMP [26,27]. Another key factor is activator protein 1 (AP-1), which is activated by themitogen- activated protein kinase signaling pathway. UV-induced AP-1 increases MMPs, leading to destruction of collagen [28,29]. In addition, collagen fibers as well as elastin fibers control cutaneous elasticity. Elastin, comprising 3–4% of the dermis layer, affects skin elasticity and is important in maintaining the physiological functions of the skin [30]. Besides, destruction of elastic fibers is caused by MMP-12 or elastase in the photoaged skin [4,20,30]. Also, it was known that CER increases the expression of caspase-14 and that the degradation of FLG into amino acids contributes to NMFs maintaining water content [11,12]. Therefore, further studies are needed on TIMP, mitogen-activated protein kinase pathway, AP-1 regulatory mechanisms, skin elasticity, and the alteration of caspase-14, an FLG-processing enzyme.

As a result of HPLC analysis of ginsenosides contained in RG, it was found that RG NGEL did not contain any ginsenosides (Fig. S1). To further investigate this result, we conducted an additional LC-MS analysis (data not shown). Because the TEMPO reaction is an oxidative process, ginsenosides were expected to break off the sugar or split into smaller molecules after TEMPO reaction [31–33].

In the present study, the active compounds showing the effect of RG NGEL could not be identified. However, we found that RG NGEL showed wrinkling and elasticity improvement and moisturizing effect even without ginsenosides. Therefore, further investigations are required to clarify components structure and active ingredients contained in RG NGEL.

In conclusion, RG NGEL reduced wrinkle formation by increasing Col-I, a main form in human skin and a predominant constituent of ECM proteins, and suppression of MMP-1, MMP-2 and MMP-9 expression to cause collagen or elastin degradation. And, further RG NGEL increased the levels of AQP3 and HAS2 associated with skin hydration. In addition, RG NGEL promoted the expression of SPT, CERS3, and FLG, which are important for skin moisturization, thereby enhancing skin barrier and preventing water loss compared with RG WE. Therefore, we suggest that RG NGEL can be used as a cosmetic reagent to help prevent skin aging effectively.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2018.09.006.

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