Anti-wrinkle effect of fermented black ginseng on human fibroblasts

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Abstract. Fermented black ginseng (FBG) is processed by the repeated steaming and drying of fresh ginseng followed by fermentation with *Saccharomyces cerevisiae*. It is known to possess anti-oxidative effects. Skin wrinkle formation is associated with oxidative stress and inflammatory reactions. The aim of this study was to determine whether FBG possesses anti-wrinkle activity using human fibroblasts (HS68). According to the Korea Ministry of Food and Drug Safety (MFDS) guidelines for the evaluation of the efficacy of functional anti-wrinkle cosmetics, we attempted to elucidate the effects of FBG on type I procollagen, matrix metalloproteinase (MMP)-1, MMP-2, MMP-9 and tissue inhibitor of metalloproteinase-2 (TIMP-2). In addition, the eye irritation potential of FBG was examined using the EpiOcular-EIT kit. Our results revealed that FBG was not cytotoxic at concentrations <10 μg/ml. It was considered as safe for the eyes at concentrations of up to 100 μg/ml. Treatment with FBG at concentrations from 0.3 to 10 μg/ml significantly (P<0.05) increased the type I procollagen expression levels from 117.61±1.51 to 129.95±4.47% in the human fibroblasts. By contrast, FBG significantly (P<0.05) decreased the MMP-1 expression level from 18.41±4.95 to 27.41±3.96%. FBG at 3 μg/ml also increased the expression of TIMP-2 up to 154.55%. However, FBG at 10 μg/ml decreased the expression levels of MMP-2 and MMP-9 to 45.15 and 66.65%, respectively. These results suggest that FBG has potential anti-wrinkle effects as a potential ingredient in cosmetics.

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Introduction

Skin functions as a barrier to protect the internal organs from environmental toxins. Skin consists of the epidermis, dermis and subcutaneous tissue. The epidermis is the outermost layer composed mainly of keratinocytes that secrete keratin protein and lipids to form the extracellular matrix (ECM), melanocytes to produce pigment, and Langerhans cells to present antigen (1). The dermis is the layer of skin beneath the epidermis. It is composed of connective tissues to provide tensile force and elasticity to skin through the ECM composed of collagen fibrils, microfibrils and elastic fibers (2). The subcutaneous tissue below the dermis is composed of fibroblasts to produce ECM proteins, macrophages to eliminate pathogens and adipocytes to conserve body fat (3-8).

The aging of the skin is induced by complex processes, including intrinsic (e.g., genetic mutation, cellular metabolism and hormonal changes) and extrinsic factors [e.g., chemicals, toxins, pollutants and ultraviolet (UV) radiation] (5,9-11). Aging skin is mainly associated with the general atrophy of ECM components with a decrease in the number of fibroblasts, reduced levels of collagen and elastin, and the disorganization of collagen fibrils and elastin fibers (12-14). Alterations in the levels of collagen and elastin primarily cause clinical symptoms of aging skin, such as wrinkles, sagging and laxity (15). The degradation of collagen and elastin in aged or photodamaged (UV-irradiation) skin is associated with matrix metalloproteinases (MMPs) released from epidermal keratinocytes and dermal fibroblasts (10,14-17).

The root of the ginseng plant (*Panax ginseng* Meyer, Araliaceae) is traditionally used as an herbal medicine in East Asian countries, including Korea, Japan and China. It is known to possess the ability to enhance physical performance, as well as to exert neuroprotective effects, enhance sexual function, and to exert anti-cancer effects (18-22). In addition, the antioxidant and/or anti-inflammatory effects of ginseng are considered to be relevant to its anti-aging effects on skin (23,24). Pharmacologically active components in ginseng include polysaccharides, polyacetylenes and gisenosides, with ginsenosides being considered as the most important component (25). To date, about 50 types of ginsenosides have been identified from the ginseng root. These natural ginsenosides from raw ginseng are converted to more stable, bioavailable and bioactive forms through the processes of drying and/or steaming (26).

Raw ginseng is processed into white ginseng by a simple drying process or into red ginseng by steaming and drying processes to preserve or improve the efficacy (27). Black ginseng is made from raw ginseng by repetitive steaming and drying processes. After being subjected to steaming and drying processes (9 times), raw ginseng will become black ginseng (28). The fermentaion of black ginseng using *Saccharomyces cerevisiae* can produce more active gisenosides (29,30). Fermented black ginseng (FBG) has different ratios of bioactive ingredients and contents of ginsenosides compared to white or red ginseng (31,32). However, the effects of FBG on skin remain unclear. Thus, the aim of this study was to determine the *in vitro* toxicity and anti-aging effect of FBG as a cosmetic ingredient on skin to provide safety and efficacy data to support the use of FBG as a comsmetic ingredient.

Materials and methods

Reagents. FBG was obtained from (Ginseng By Pharm Co., Ltd., Wonju, Korea). Its detailed composition information has been previously described (26). All media required for cell growth, such as Dulbecco's modified Eagle's medium (DMEM) high glucose, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA (0.25%) were purchased from (Gibco-BRL Inc., Franklin Lakes, NJ, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT), retinoic acid, Dulbeco's phosphate-buffered saline (D-PBS) were purchased from (Sigma-Aldrich, St. Louis, MO, USA). Cell lysis buffer, phenylmethylsulfonyl fluoride (PMSF), antibodies against MMP-9 (sc-3852S), β-actin (sc-1616), horseradish peroxidase-conjugated anti-rabbit IgG (7074) and anti-mouse IgG (7076) were purchased from (Cell Signaling Technology, Inc., Danvers, MA, USA). Polyvinylidene fluoride (PVDF) and antibody against tissue inhibitor of metalloproteinase (TIMP)-2 (MAB 3310) were purchased from Millipore (Billerica, MA, USA). All other reagents were of the highest quality available.

Cell culture. Human skin fibroblasts (HS68) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM containing 10% FBS and 1% of penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay. The HS68 human fibroblasts were seeded onto 96-well plates at a density of $5x10^4$ cells/well and incubated for 48 h, as previously described (10). After 48 h of incubation, various concentrations of FBG (10, 25, 50, 100, and 200 µg/ml in fresh medium) or distilled water (DW; control) were used to treat the cells. The cells were further cultured for 48 h. Subsequently, 200 µl of MTT (0.5 mg/ml MTT in fresh medium) was added to each well followed by incubation at 37°C for 3 h. The MTT medium was removed by aspiration and 200 µl of dimethyl sulfoxide was added to each well. After reacting for 10 min at room temperature, formazan production was detected by measuring the optical density at 570 nm on a PowerWave XS microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Data were then expressed as a percentage of viable cells compared to viable cells in the DW-treated control.

Eye irritation test. The ocular irritation potential of FBG was examined using the EpiOcular Eye Irritation Test (OCL-200-EIT; MatTek Corp., Ashland, MA, USA). Following incubation at 37°C in 5% CO_2 overnight, OCL-200-EIT was pre-wet with 20 µl Ca^{++}- and Mg^{++}-free D-PBS (Sigma-Aldrich) for 30±2 min. After pre-wetting, 50 µl of FBG (10 and 100 µg/ml) was topically applied to the pre-wet OCL-200-EIT and incubated for 30±2 min. Tissues included in OCL-200-EIT kit were then rinsed with 300 ml D-PBS, post-soaked with 5 ml fresh medium, and incubated for 2±0.4 h at 37°C with 5% CO_2. To measure cell viability, OCL-200-EIT was incubated with MTT solution (1 g/ml) for 3 h. After extracting isopropanol, the absorbance of formazan was measured at 570 nm on a PowerWave XS microplate reader (BioTek Instruments, Inc.). The mean value for each test substance was calculated from 2 wells. D-PBS was used as a control. Data were expressed as a percentage of the viability compared to that of the D-PBS-treated control.

Quantification of type I procollagen and MMP-1 levels. The levels of type I procollagen and MMP-1 in the human fibroblasts were quantified using the procollagen Type I C-peptide (PIP) EIA kit (Takara Bio, Inc., Otsu, Japan) and the Human MMP-1 ELISA kit (Young In Frontier, Seoul, Korea), respectively. The human fibroblasts were seeded at a density of $5x10^4$ cells/well. After 48 h, the cells were treated with DW (control), various concentrations of FBG (0.3, 1, 3 and 10 µg/ml), or 0.03 µg/ml retinoic acid for 48 h. The levels of type I procollagen and MMP-1 from the cultured media were measured using the Type I PIP EIA kit and Human MMP-1 ELISA kit according to the manufacturer's instructions. Data were expressed as the percentage of expression compared to that of the DW-treated control.

Quantification of MMP-2, MMP-9 and TIMP-2 levels. The expresssion levels of MMP-2, MMP-9 and TIMP-2 were measured by western blot analysis. The human fibroblasts were treated with DW (control), various concentrations of FBG (0.3, 1, 3 and 10 µg/ml), or 0.03 µg/ml retinoic acid for 48 h. To extract total protein, the treated cells were incubated with cell lysis buffer (Cell Signaling Technology, Inc.) containing 1 mM PMSF (Cell Signaling Technology, Inc.) for 5 min on ice. Following incubation, whole cell lysates were briefly sonicated and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was stored at -80°C until use. Before running on a gel, the protein concentration was determined by Bradford assay. Total protein (25 µg) was separated by 10% SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, Darmstadt, Germany) using a transfer apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. The membranes were incubated in blocking buffer (5% w/v skim milk in TBST) for 3 h. Primary antibody (1:1,000 for MMP-2, 1:500 for MMP-9, and 1:500 for TIMP-2) was incubated with the transferred membranes at 4°C overnight. After washing the membranes with TBST, the membranes were incubated with the secondary antibody (anti-rabbit for MMP-2 and MMP-9, anti-mouse IgG for TIMP-2 at 1:1,000 dilution) for 2 h at room temperature for MMP-2 or overnight at 4°C for MMP-9 and TIMP-2. After washing the membranes with TBST, protein signal was detected by horseradish peroxidase detection
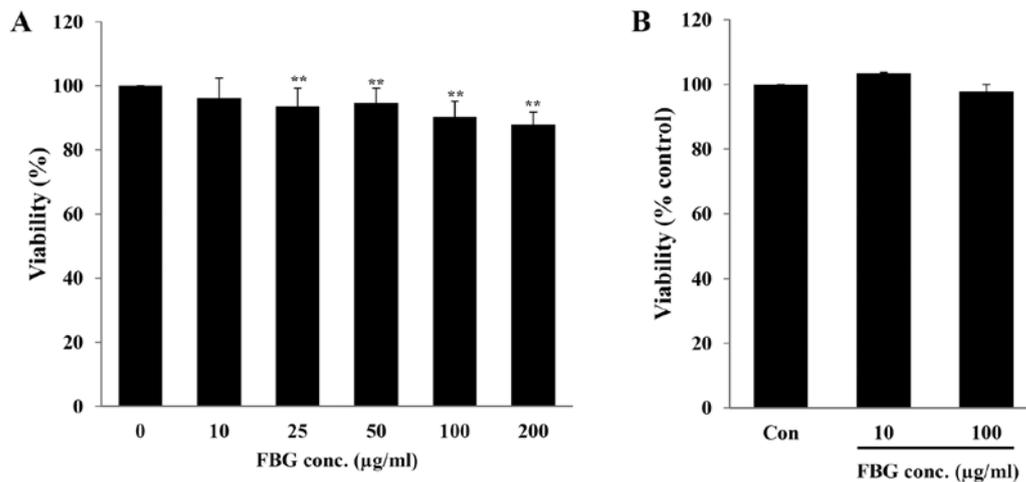
Figure 1. (A) Cytotoxicity of FBG. Human skin fibroblasts (HS68) were treated with DW or FBG (10-200 µg/ml) for 48 h and cell viability was determined by MTT assay. The data are the means ± standard deviation values of 3 individual experiments. Each value was compared with the control using analysis of variance, followed by Bonferroni's test (**P<0.01). (B) *In vitro* eye irritation was tested for FBG (10 µg/ml and 100 µg/ml) using the OCL-200-EIT. D-PBS was used as a control. FBG, fermented black ginseng; DW, distilled water; OCL-200-EIT, EpiOcular Eye Irritation Test; D-PBS, Dulbeco's phosphate-buffered saline.

system (Sigma-Aldrich). Data were expressed as a percentage of expression compared to that of the DW-treated control.

Statistical analysis. The means ± standard deviations of the expression values were calculated using Microsoft Excel. The statistical significance (P<0.05 or P<0.01) of apparent differences in protein expression among pre-dosing and treatments were assessed using analysis of variance followed by Bonferroni's test in Prism 5.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

In vitro toxicity of FBG. The *in vitro* toxicity of FBG on the skin and eyes, the major exposure routes of cosmetic ingredients, was evaluated using human skin fibroblasts (HS68) and the EpiOcular Eye Irritation test (OCL-200-EIT), respectively. When the HS68 cells were treated with 10, 25, 50, 100, and 200 µg/ml FBG for 48 h, cell viability was 96.17±6.36, 93.68±5.64, 94.64±4.66, 90.31±4.97 and 88.01±3.87%, respectively. Only FBG at 10 µg/ml did not exhibit any significant difference in cell viability (P>0.05) compared to the control (without FBG treatment) (Fig. 1A). The EpiOcular-EIT results revealed that FBG at 10 and 100 µg/ml caused no potential eye irritation compared to the control (PBS treatment) (Fig. 1B). These results suggest that FBG at a concentration of <10 µg/ml is not cytotoxic. In addition, FBG does not cause eye irritation at concentrations up to 100 µg/ml.

Effect of FBG on the production of collagen. To examine the effect of FBG on collagen synthesis in skin, the fibroblasts were treated with FBG at non-cytotoxic concentrations. Our results revealed that FBG at 0.3, 1, 3, and 10 µg/ml significantly (P<0.05) increased type I procollagen production to 117.61±1.51, 122.62±2.69, 128.07±5.76, and 129.95±4.47%, respectively, compared to the control (without FBG treatment). The positive control, retinoic acid at 0.03 µg/ml, significantly increased (P<0.05) type I procollagen production to 120.88±5.82% compared to the control (without FBG treatment) (Fig. 2).
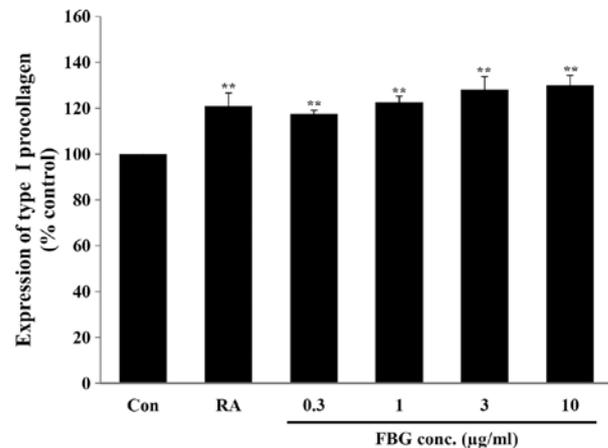

Figure 2. Effect of FBG on type I procollagen in human fibroblasts. Human fibroblasts were treated with DW (Con; control), RA (0.03 µg/ml retinoic acid) or FBG (10-200 µg/ml) for 48 h and the expression of type I procollagen was measured using the Procollagen Type I C-peptide (PIP) EIA kit. The data are the means ± standard deviation values of 3 individual experiments. Each value was compared with the control using analysis of variance, followed by Bonferroni's test (**P<0.01). FBG, fermented black ginseng; DW, distilled water.

Effect of FBG on MMPs. Subsequently, we analyzed the levels of MMPs associated with collagen degradation in FBG-treated HS68 fibroblasts. Our results revealed that FBG at concentrations of 0.3, 1, 3 and 10 µg/ml significantly (P<0.05) decreased the MMP-1 levels by 18.41±4.96, 19.35±6.39, 21.53±7.81 and 27.41±3.96%, respectively compared to the levels of MMP-1 in the control HS68 cells (without FBG treatment). The positive control, retinoic acid at 0.03 µg/ml, also significantly (P<0.05) decreased the MMP-1 level by 37.78±7.71% compared to the level of MMP-1 in the control HS68 cells not treated with FBG (Fig. 3). In addition, in the cells treated with FBG at concentrations of 0.3, 1, 3, and 10 µg/ml, the expression levels of MMP-2 and MMP-9 were 76.32, 74.28, 52.71 and 45.15% and 106.66, 100.00, 90.53 and 66.65% compared to those of the control, respectively. The positive control, retinoic acid at
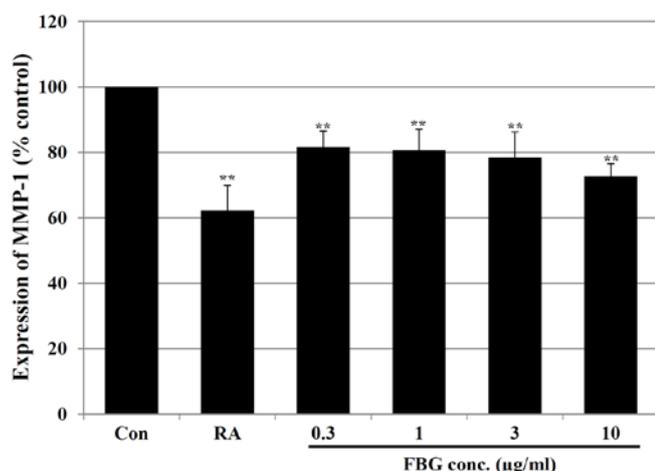
Figure 3. Effect of FBG on MMP-1 in human skin fibroblasts. Human skin fibroblasts were treated with DW (Con; control), RA (0.03 µg/ml retinoic acid) or FBG (0.3-10 µg/ml) for 48 h and The expression of MMP-1 was measured using the human MMP-1 ELISA kit. The data are the means ± standard deviation values of 3 individual experiments. Each value was compared with the control using analysis of variance, followed by Bonferroni's test ([**]P<0.01). FBG, fermented black ginseng; DW, distilled water; MMP-1, matrix metalloproteinase-1.

0.03 µg/ml, decreased the expression of MMP-2 and MMP-9 by 24.18% and 41.07% compared to control (Fig. 4). These results suggest that the levels of these MMPs were inhibited by FBG in a dose-dependent manner. The reduction in the levels of MMPs may be associated with the increased in type I collagen production caused by FBG treatment.

Effect of FBG on the expression of TIMP-2. TIMP-2 expression was examined in the FBG-treated HS68 cells. After the HS68 cells were treated with FBG at 0.3, 1, 3 and 10 µg/ml, the expression level of TIMP-2 was increased to 122.66, 137.45, 154.55 and 126.76% compared to that of the untreated control. The positive control, retinoic acid at 0.03 µg/ml, increased the level of TIPM-2 to 143.06 % compared to that of the control (Fig. 5). These results suggest that FBG increases the levels of TIMP-2, thus causing the inhibition of MMPs.

Discussion

Ginsenosides are the major active components responsible for the pharmacological properties of ginseng (25). Ginsenosides can be grouped into protopanaxadiol, protopanaxatriol and oleanolic saponins based on their chemical structures (33). It has been previously reported that 20-*O*-b-d-glucopyranosyl-20(*S*)-protopanaxadiol (compound K) of ginsenosides has anti-metastatic, anti-angiogenic and anti-allergic activities *in vivo* (34,35). In particular, skin wrinkles and xerosis can be ameliorated by the topical application of compound K that induces hyaluronan synthase 2 in human keratinocytes and increases hyaluronan content in aged hairless mouse skin (36). In addition, skin wrinkles in humans can be improved by extracts from red ginseng roots with increased levels of type I procollagen (37). Collectively, these data suggest that the topical application of ginseng extracts can enhance its anti-wrinkle effects on human skin.
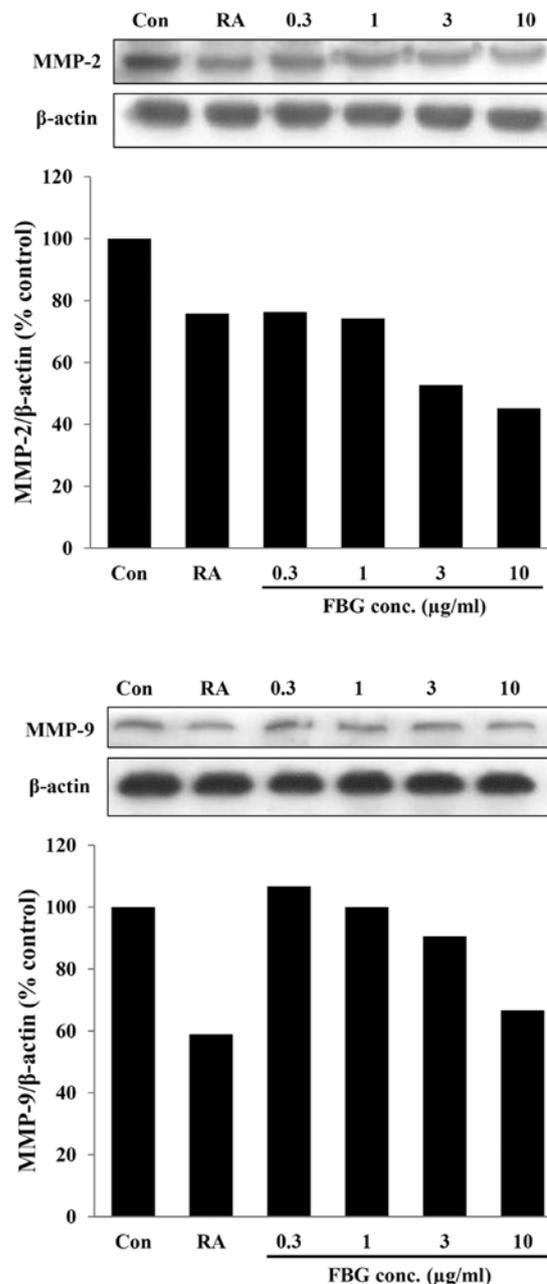

Figure 4. Effects of FBG on MMP-2 and MMP-9 in human skin fibroblasts. Human skin fibroblasts were treated with DW (Con; control), RA (0.03 µg/ml retinoic acid) or FBG (0.3-10 µg/ml) for 48 h and the expression of MMP-2 and MMP-9 was measured by western blot analysis and normalized to the expression of β-actin. FBG, fermented black ginseng; DW, distilled water; MMP, matrix metalloproteinase.

FBG extracts display different compositions of ginsenosides by more complex processes, such as repetitive steaming and drying with fermentation using *Saccharomyces cerevisiae* compared to fresh, white, or red ginseng (38,39). Such differences have been considered to be able to enhance their antioxidant and free radical scavenging activities (29,40-42). However, the anti-wrinkle effects of FBG extracts have not been studied on human skin. In the present study, FBG extracts significantly increased the expression of type I procollagen in human fibroblasts. This result indicates that FBG extracts have anti-wrinkle effects by increasing type I procollagen level in human skin.
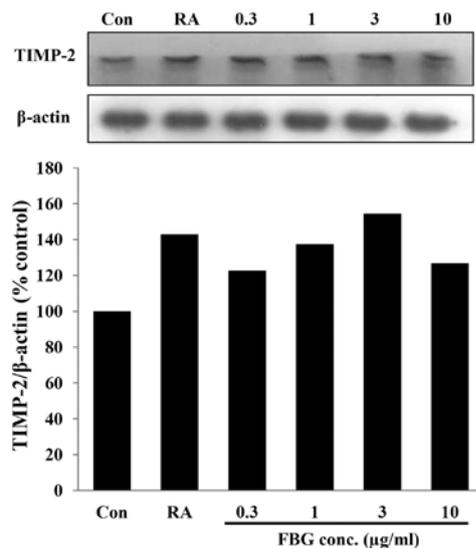
Figure 5. Effects of FBG on TIMP-2 in human skin fibroblasts. Human skin fibroblasts were treated with DW (Con; control), RA (0.03 µg/ml retinoic acid) or FBG (0.3-0 µg/ml) for 48 h and the expression of TIMP-2 was measured by western blot analysis and normalized to the expression of β-actin. FBG, fermented black ginseng; DW, distilled water; TIMP-2, tissue inhibitors of metalloproteinase-2.

Type I collagen is the most abundant protein in skin connective tissue. It maintains skin structure with other types of collagen (III, V and VII), elastin, proteoglycans, fibronectin and other ECM proteins (43). Type I procollagen is synthesized in human dermal fibroblasts and secreted into the dermal extracellular space where it undergoes proteolytic processing. Finally, type I collagen forms collagen bundles (fibre bundles) that are responsible for the elasticity associated with other ECM proteins (5,13). Fibrillar (types I and III) collagen can characteristically reduce chronologically aged and photo-damaged skin (14,44,45). The degradation of type I collagen is closely associated with MMPs, a family of zinc-requiring endoprotease with the capacity to degrade all components of ECM (46). In particular, MMP-1 of the MMPs initiates the degradation of types I and III fibrillar collagens, while MMP-9 further degrades collagen fragments generated by collagenases (44). MMP-2 and MMP-9 together can cleave elastin, type IV collagen, and several other ECM molecules while MMP-2 can digest interstitial collagen types I, II, and III (47). It has been previously suggested that the function of MMP-1 is directly involved in the reduction of type I procollagen. MMP-2 and MMP-9 also regulate the expression of type I procollagen (47,48). All known MMPs are inhibited by 4 homologous TIMPs (49). TIMP-2 of TIMPs inhibits ECM proteolysis in several tissues by directly inhibiting metalloproteinases, including MMP-2 (50). TIMP-2 is also known to be required for the activation of MMP-2 through association with MMP-14 (50,51). In this study, FBG extracts significantly inhibited the expression of MMP-1 in human fibroblasts. In addition, the expression levels of MMP-2 and MMP-9 were dose-dependently decreased by FBG. Moreover, the expression of TIMP-2 exhibited a generally increased tendency by FBG treatment. Collectively, these results suggest that the increase in the level of type I procollagen caused by FBG may be induced by the inhibition of MMP-1, MMP-2 and MMP-9.

The inhibition of these MMPs may correlated with the upregulation of TIMP-2 due to FBG treatment.

Retinoic acid can effectively attenuate the clinical symptoms of photodamaged skin. It can reverse the adverse consequences of chronological aged skin (14,45,52). Treatment with retinoic acid can stimulate fibroblast proliferation, new collagen synthesis and the degradation of out-of-date or damaged collagen (53). The effects of retinoic acid are mainly associated with the inhibition of MMPs (16,17). Additionally, retinoic acid results in the selective downregulation of MMP-9 and the simultaneous upregulation of TIMP-1 in human bronchoalveolar lavage cells (54). It can also reduce the expression of MMP-2 in human breast cancer cells, suggesting that the inhibition of MMP-2 may be due to upregulated TIMP-2 (55). In this study, we demonstrated that the treatment of human fibroblasts with retinoic acid exerted similar effects with FBG treament as regards the expression patterns of type I procollagen, MMP-1, MMP-2, MMP-9 and TIMP-2. These results suggest that FBG and retinoic acid may share similar mechanisms as regards their anti-wrikle effects on human skin.

In conclusion, our *in vitro* dermal study demonstrated that FBG treatment increased type I procollagen correlating with associated regulators. In addition, we assessed the non-toxic concentration of FBG through cosmetic exposure routes using human fibroblasts and ocular tissues. Conclusively, we demonstrated that FBG may be used as a safe cosmetic ingredient with anti-wrinkle effects.

Acknowledgements

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