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Effects of Bletillae Rhizoma on the Elastase, Collagenase, and Tyrosinase Activities and the Procollagen Synthesis in Hs68 Human Fibroblasts

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ABSTRACT

Objectives : Bletillae Rhizoma, the roots of *Bletilla striata*, is used to restrain the leakage of blood and stop bleeding. It can cure the sores, ulcers, and chapped skin. This study was designed to investigate the collagen metabolism, elastase and tyrosinase activity of Bletillae Rhizoma extract (BR).

Methods : The effects of BR on type I procollagen production and collagenase activity in human normal fibroblasts Hs68 after UVB (312 nm) irradiation were measured by ELISA method. The elastase activity, tyrosinase activity, and L-DOPA oxidation after treatment of BR were measured as well.

Results : In the present study, the collagen production (type I procollagen) was significantly increased to 15.7 ± 1.8 ng/ml at a concentration of BR 100 μg/ml in UVB damaged Hs68 cells. The increased collagenase activity after UVB damage was significantly recovered to 42.7 ± 0.7%, 54.5 ± 3.5%, and 38.4 ± 0.9% by BR 10, 30, and 100 μg/ml. The activities of BR 10 mg/ml on tyrosinase activity was significantly reduced to 45.1 ± 8.4% as well. However, there were no significant effects on the elastase activity and the L-DOPA oxidation.

Conclusion : BR showed the promoting effects of collagen synthesis and inhibitory effects of collagenase activity in Hs68, human normal fibroblast cells. And these could be thought to have the anti-wrinkle effects and whitening effects *in vitro*. These results suggest that BR may have potential as an anti-aging ingredient in cosmetic treatment.

Key words : Bletillae Rhizoma, Elastase, Collagenase, Tyrosinase, Collagen, Hs68, Fibroblasts

Introduction

Bletillae Rhizoma (BR), the roots of *Bletilla striata*, riches in phenanthrene derivates, blestiarenen A, B, C, batatasin III, polysaccharides, saponins, flavonoids, amino acids, and so on. It is used to restrain the leakage of blood and stop bleeding which includes vomiting blood, coughing of blood, and nosebleed. The traumatic injury could be used locally. It generates flesh and reduces swelling. If topically used, it can cure the sores, ulcers, and chapped skin. It can be used whether or not the sore has ulcerated, as it reduces the swelling of sores and helps accelerate the healing of ulcers, especially for chronic and nonhealing ulcers[1,2].

There are many causes inducing hemoptysis, coughing of blood. One of the main reasons is the collapse of small airway or pulmonary alveoli. Emphysema is a disease of the lung caused by destruction of pulmonary airway and alveoli. Elastase is an enzyme from the class of proteases (peptidases) that breaks down a fine tissue in the lung. It breaks down elastin, an elastic fiber that, together with collagen, determines the mechanical properties of connective tissue[3].

BR stops bleeding and cures the chapped skin as mentioned in medical literature[1,2]. The action of collagen plays an important role in wound healing process and collagen is well known antihemorrhagic (antihaemorrhagic) agent and now being used in surgery[4,5].

In the present study, the effects of BR on elastase

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activity and on collagen were investigated to find out whether the anti-hemoptysis effects were related with an elastase-regulating mechanism or a collagen-related mechanism. The effects of BR on type I procollagen production and collagenase activity in human normal fibroblasts Hs68 using UVB irradiation were examined to find out collagen-related mechanism. The effect of BR on tyrosinase activity was measured as well to find out the potential cosmetic effects of BR.

Materials and Methods

1. Materials

Bletillae Rhizoma was purchased from Omniherb (Korea). Bletillae Rhizoma extract (BR) was prepared as follow. 100 g of BR in 2,000 ml distilled water was heated in a heating extractor for 3 hours. The extract was filtered and concentrated by using the rotary evaporator. The extract was lyophilized by using freeze dryer (18.2 g). All reagents without mentioning were purchased from Sigma-Aldrich (USA).

2. Elastase activity inhibition

The elastase activity was evaluated by using a modification of a previously reported method of Kraunsoe et al[6]. In order to evaluate the inhibition of elastase activity, the amount of released p-nitroaniline, which was hydrolyzed from the substrate, N-succinyl-Ala-Ala-Ala-p-nitroanilide, by elastase, was read with a maximum absorbance at 410 nm[7]. In brief, 2 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide was prepared in a 0.1 M Tris-Cl buffer (pH 8.0), and this solution was added to the stock sample. Each sample solution was diluted to final concentrations of 0.1, 1, and 10 mg/ml. The solutions were mixed thoroughly by tapping before an elastase (0.136 unit/ml) stock solution was added. Solution was incubated for 10 min at 37℃, and the absorbance was measured at 410 nm. The percent activity of elastase was calculated according to the following equation:

Elastase activity (%)
= [(OD_{410} of control) - (OD_{410} of sample) / (OD_{410} of control)] × 100

3. Cell culture

Hs68 human fibroblasts (Health Protection Agency Culture Collections, UK) were cultured in Dulbecco's Modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum, 1% antibiotics at 37℃ in a humidified atmosphere of 5% CO_2. When cells reached confluency, subculture was conducted at a 1:3 split ratio.

4. UVB irradiation

A UVB lamp (Vilber Lourmat, France) was used as a UVB source. In brief, Hs68 cells were rinsed twice with phosphate-buffered saline (PBS), and all irradiations were performed under a thin layer of PBS (200 μl/well). Immediately after irradiation, fresh serum-free medium was added to the cells. Responses were measured after an incubation period of 24 hours. Mock-irradiated blanks followed the same schedule of medium changes without UVB irradiation.

5. Cell viability

General viability of cultured cells was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The human fibroblast cells (Hs68) were seeded in 24-well plates at a density of 2×10^5/ml per well and cultured at 37℃ in 5% CO_2. Cells were pretreated with the sample at a concentration of 100, 10, 1 μg/ml for 24 hours prior to UVB irradiation. After UVB irradiation, cells were retreated with the sample and incubated for additional 24 hours, before being treated with 0.05 mg/ml (final concentration) of MTT. The blank and control group was cultivated without sample treatment. The cells were then incubated at 37℃ for additional 4h. The medium containing MTT was discarded, and MTT formazan that had been produced was extracted with 200 μl of DMSO. The absorbance was read at 595 nm with a reference wavelength of 690 nm. The cell viability being calculated as follows:

Cell viability (%)
= [($OD_{595\text{-}690}$ of sample) / ($OD_{595\text{-}690}$ of control)] × 100

6. Assays of collagen type I synthesis and collagenase inhibition

Hs68 human fibroblasts were inoculated into 24-well plate (2×10^5 cells/well) and cultured at 37℃ in 5% CO_2. Cells were pretreated with the sample at a concentration of 10, 30, and 100 μg/ml for 24 hours prior to UVB irradiation. After UVB irradiation, cells were retreated with the sample and incubated for additional 24 hours. The blank and control group was cultivated without sample treatment. After culturing, the supernatant was collected from each well, and the amount of collagen type I was measured with a procollagen type I C-peptide assay kit (Takara Bio, Japan). The activity of collagenase was measured with

a matrix metalloproteinase-1 (MMP-1) human biotrak ELISA system (Amersham life science, USA).

7. Tyrosinase inhibition assay

Tyrosinase activity was determined essentially as previously described[8]. The reaction mixtures were prepared by adding 40U of mushroom tyrosinase to 20 μl of BR dissolved in distilled water (25 and 50 mg/ml), and then adding 40 μl of 1.5 mM L-tyrosine and 220 μl of 0.1 M sodium phosphate buffer (pH 6.5). The resulting mixture (300 μl) was incubated for 10 min at 37℃ and then absorbance at 490 nm was measured. The same mixture, but without BR extract, was used as a control.

8. Inhibition of L-DOPA oxidation

The inhibitory effect of BR on L-DOPA oxidation was determined according to the method of Joshi et al[9]. with a slight modification. 50 μl of BR dissolved in 0.1 M sodium phosphate buffer (25 and 50 mg/ml) was added to 40 U of mushroom tyrosinase in 900 μl of 0.1 M sodium phosphate buffer (pH 6.5). After 6 min of incubation at 37℃, 3 mM of L-DOPA was added. Then the mixture was incubated at 37℃ for 15 min. L-DOPA oxidation was quantified by measuring absorbance at 475 nm. The same mixture, but without BR extract, was used as a control.

9. Statistical analysis

The results were expressed as means ± standard error of the mean (SEM). Significances of changes were evaluated using one-way analysis of variance (ANOVA) with Dunnett's post hoc test using the SPSS ver. 10.1 (SPSS Inc., USA). Values of $p < 0.05$ were considered significant.

Results

1. Elastase activity

The inhibitory effect of BR on elastase activity was determined according to the method described previously. The elastase activity of BR 0.1 mg/ml, BR 1 mg/ml, and BR 10 mg/ml treated are 94.5 ± 5.1%, 109.1 ± 2.6%, and 89.8 ± 4.7%, respectively. BR did not show the elastase inhibitory effect (Fig. 1).

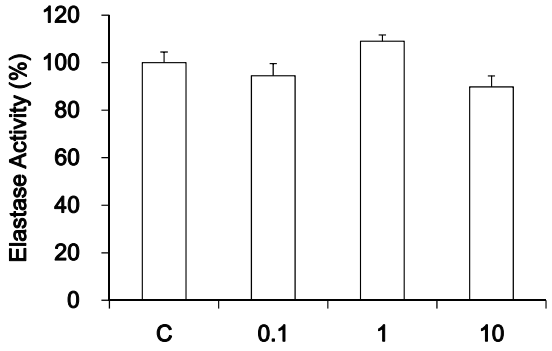

Figure 1. Effect of BR on inhibition of elastase activity. C: control, distilled water treated group. 0.1, 1, and 10: Bletillae Rhizoma extract treated group (0.1, 1, and 10 mg/ml). Data are expressed as the mean ± SEM of three experiments.

2. Cytotoxicity on Hs68 human fibroblasts

In order to evaluate the cytotoxicity of BR, samples were prepared at various concentrations and used to treat human fibroblasts (Hs68). The results of this evaluation are shown in Figure 2 at concentrations of 10, 30, and 100 μg/ml. The cell viability was recalculated into 100% of control group. The cell viabilities of BR 10 μg/ml treated, BR 30 μg/ml treated, BR 100 μg/ml treated are 102.5 ± 1.4%, 104.5 ± 0.5%, and 99.1 ± 0.0%, respectively. BR showed no cytotoxicity up to the effective concentration (less than 100 μg/ml).

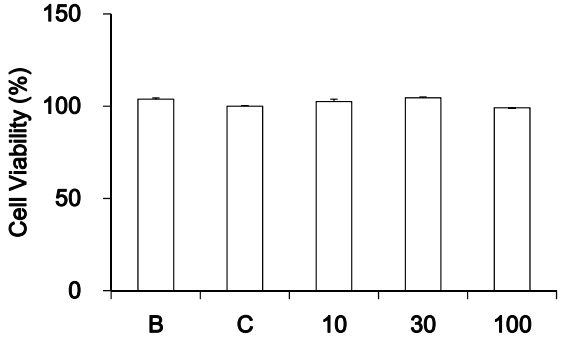

Figure 2. Cell viability of BR on Hs68 human fibroblasts. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Bletillae Rhizoma extract (BR 10, 30, and 100 μg/ml) treated group. Data are expressed as the mean ± SEM of three experiments.

3. Assay of collagen type I synthesis

The amounts of type I collagen synthesis of BR were shown in Figure 3. BR significantly increased the expression of type I collagen at a concentration of 100 μg/ml (15.7 ± 1.8 ng/ml) compared with control group. The collagen amounts of BR 30 and 10 μg/ml treated group did not show the statistical significance

(13.3 ± 0.8 and 10.3 ± 0.6 ng/ml, respectively).

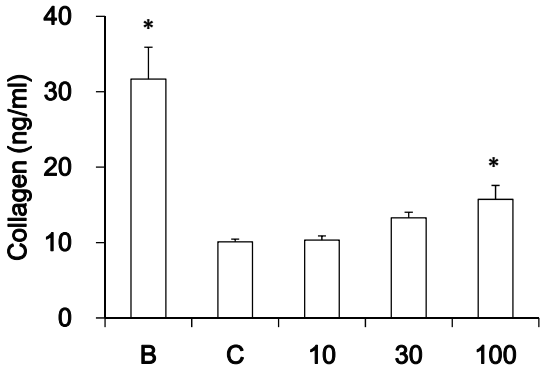

Figure 3. Effect of BR on collagen type I synthesis in human fibroblast cells. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Bletillae Rhizoma extract (BR 10, 30, and 100 μg/ml) treated group. Data are expressed as the mean ± SEM of three experiments. * significantly different from the control, $p < 0.05$.

4. Assay of collagenase activity

To evaluate the collagenase activity, matrix metalloproteinase-1 (MMP-1) activity was quantitatively measured by using the previously described matrix metalloproteinase-1 assay kit. The activities of MMP-1 of BR treatment were recalculated into 100% of control group (Fig. 4). BR significantly reduced the MMP-1 activity at concentrations of 10, 30, and 100 μg/ml (42.7 ± 0.7%, 54.5 ± 3.5%, and 38.4 ± 0.9%, $p < 0.05$).

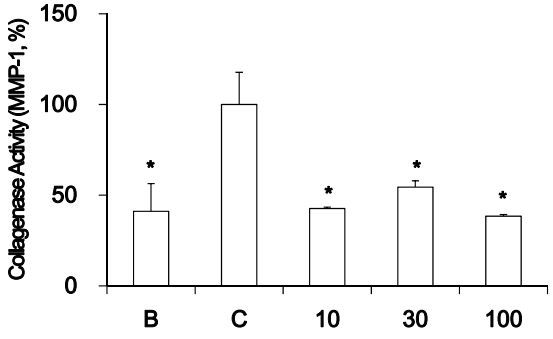

Figure 4. Effect of BR on collagenase activity in human fibroblast cells. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Bletillae Rhizoma extract (BR 10, 30, and 100 μg/ml) treated group. Data are expressed as the mean ± SEM of three experiments. * significantly different from the control, $p < 0.05$.

5. Tyrosinase activity assay

The activities of BR on tyrosinase activity were recalculated into 100% of control group (Fig. 5). BR significantly reduced the tyrosinase activity at concentrations of 10 mg/ml (45.1 ± 8.4%, $p < 0.05$). The tyrosinase activity of BR 0.1 and 1 mg/ml treated groups did not show any significance (116.4 ± 2.3% and 83.8 ± 3.5%).

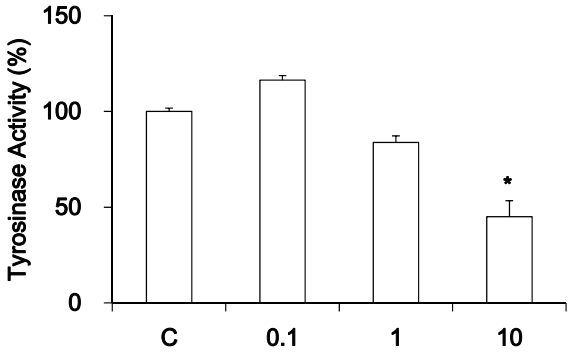

Figure 5. Effect of BR on tyrosinase activity. C: control, distilled water treated group. 0.1, 1, and 10: Bletillae Rhizoma extract (BR 0.1, 1, and 10 mg/ml) treated group. Data are expressed as the mean ± SEM of three experiments. * significantly different from the control, $p < 0.05$.

6. L-DOPA oxidation

The activities of BR on L-DOPA oxidation were recalculated into 100% of control group. There was no significant difference (Fig. 6).

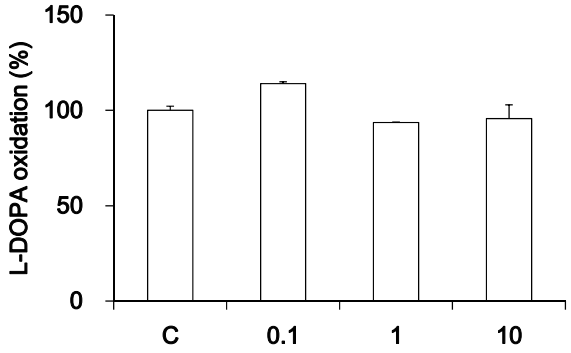

Figure 6. Effect of BR on L-DOPA oxidation. C: control, distilled water treated group. 0.1, 1, and 10: Bletillae Rhizoma extract (BR 0.1, 1, and 10 mg/ml) treated group. Data are expressed as the mean ± SEM of three experiments.

Discussion

BR has been clinically used to treat coughing of blood-streaked sputum with Eriobotryae Folium. BR tonifies the lung, breaks up stasis, and stops bleeding. Combined, the two herbs stop coughs, transform phlegm, and stop bleeding, and are used in the treatment of heat from deficiency in the lung leading to stasis and coughing of blood. BR powder has been clinically used to accelerate the healing of ulcers, especially for chronic and nonhealing ulcers[1,2].

Elastase breaks down elastin, an elastic fiber that, together with collagen, determines the mechanical properties of connective tissue. Actually, elastase is the only enzyme that is capable of degrading elastin, an insoluble elastic fibrous protein in animal connective tissues. It is capable of hydrolyzing nearly all proteins, including supporting and structural proteins of the connective tissue such as collagen and elastin[10]. In the present study, inhibitory effect of BR on elastase activity was determined with elastase enzyme assay. However, BR did not have any effect on elastase.

Collagen is one of the main components of body and plays an important agent for hemostatic agent by itself. In nature, it is found exclusively in animals, especially in the flesh and connective tissues of mammals[11]. It is the main component of connective tissue, and is the most abundant protein in mammals, making up about 25% to 35% of the whole-body protein content[12]. In this study, the amount of collagen type I was significantly increased at a concentration of BR 100 μg/ml.

To evaluate the effect of BR on the collagenase activity, matrix metalloproteinase-1 (MMP-1) activity was quantitatively measured. BR significantly reduced the MMP-1 activity in all concentrations.

It has been shown that UV irradiation leads to the formation of reactive oxygen species (ROS) that activate the mitogen-activated protein (MAP) kinase pathway, which subsequently induces the expression and activation of MMPs in human skin in *vivo*[13,14]. MMPs including collagenase are considered key factors in the photoaging process. The skin aging is one of the most obvious evidence of aging. The skin is increasingly exposed to ambient UV-irradiation thus increasing risks for photooxidative damage with long-term detrimental effects like photoaging, characterized by wrinkles, loss of skin tone and resilience. Photoaged skin displays alterations in the cellular component and extracellular matrix with accumulation of disorganized elastin and its microfibrillar component fibrilin in the deep dermis and a severe loss of interstitial collagens, the major structural proteins of the dermal connective tissue. Therefore, agents with the ability to elevate extracellular matrix protein levels or inhibit the major collagen-degrading enzymes like MMPs would prove to be useful in the development of effective anti-aging agents[15].

In order to evaluate the cytotoxicity of BR, samples were prepared at various concentrations and used to treat human fibroblasts (Hs68). There was no cytotoxicity in all treated concentrations.

To evaluate the amount of collagen type I synthesis that occurred upon exposure to the sample, collagen type I was quantitatively detected by using the previously described procollagen type I C-peptide assay kit. Collagens are synthesized as precursor molecules, called procollagens. These molecules contain additional peptide sequences, usually referred to as 'propeptides', at both the amino-terminal end and the carboxy-terminal end. These propeptides are cleaved from the collagen triple-helix molecule during its secretion, after which the triple-helix collagens are polymerized into extracellular collagen fibrils. Thus, the amount of free propeptide stoichiometrically reflects the amount of collagen molecules synthesized[7]. In this study, the amount of collagen type I was significantly increased at a concentration of BR 100 μg/ml.

To evaluate the collagenase activity, matrix metalloproteinase-1 (MMP-1) activity was quantitatively measured. BR significantly reduced the MMP-1 activity. In contrast to the collagen synthesis results, all concentrations of BR showed the significant effect in MMP-1 activity. It is some hints, which might be interpreted as suggesting the possibility of the usage for MMP-1 enzyme inhibitor. However, further research should be performed.

BR has been used topically on skin. Accordingly, the cosmetic application was evoked and the whitening effects were measured using tyrosinase-enzyme assay in the present study.

Tyrosinase refers to an oxidase, which is the rate limiting enzyme for controlling the production of melanin. Melanin synthesis begins in the liver where phenylalanine is converted to tyrosine by the action of phenylalanine hydroxylase. The oxidation of L-Tyrosine to L-DOPA is then catalysed by the action of tyrosinase enzymes within the melanocyte's melanosome. In the next step L-DOPA is oxidized to DOPAquinone. From DOPAquinone, the melanin synthesis pathways diverge to produce either eumelanin or pheomelanin[8,9]. Tyrosinase, an oxidase, converts tyrosine and L-DOPA in order. And both tyrosine and L-DOPA were catalyzed by tyrosinase and related with melanin pigmentation. Accordingly, either inhibition from tyrosine to L-DOPA or inhibition from L-DOPA to DOPAquinone could inhibit a melanin synthesis.

The activities of BR on tyrosinase activity and were significantly effective at 10 mg/ml. There was no effect on L-DOPA oxidation. These results suggest that BR may have potential as an anti-aging ingredient in cosmetic herb.

In conclusion, BR showed the inhibitory effects on collagenase and slightly increased the synthesis of

collagen. The inhibitory effect of BR on elastase was not found out. The effects on vomiting blood, coughing of blood, nosebleed, a traumatic injury, sores, ulcers, and chapped skin might be thought to be related with collagen related mechanism considering the present data. Furthermore, those data could be thought that BR has anti-wrinkle effects. These results suggest that BR may have potential as an anti-aging ingredient in cosmetic herbal products. I think further studies will be needed to unravel exactly under the molecular mechanisms.

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