大韓本草學會誌 제29권 제1호(2014년 1월)
Kor. J. Herbology 2014 ; 29(1) : 7-12

ISSN 1229-1765(Print), ISSN 2288-7199(Online)
http://dx.doi.org/10.6116/kjh.2014.29.1.7.

Effects of Polygoni Multiflori Radix on the Elastase, and Collagenase Activities and the Procollagen Synthesis in Hs68 Human Fibroblasts

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ABSTRACT

Objectives : Polygoni Multiflori Radix (PMR), the roots of *Polygonum multiflorum* Thunberg, is used to nourish the blood and yin and used for preventing premature greying of the hair. There are some articles on its preventing effects on the melanogenesis. However, there is no report about its effects on the collagen and elastin. The present study was designed to investigate its effects on collagen metabolism and elastase activity.

Methods : The effects of PMR on type I procollagen production and collagenase activity in human normal fibroblasts Hs68 after UVB (312 nm) irradiation were measured by ELISA method. Cells were pretreated with the PMR for 24 hours prior to UVB irradiation. After UVB irradiation, cells were retreated with the sample and incubated for additional 24 hours. The amount of collagen type I was measured with a procollagen type I C-peptide assay kit. The activity of collagenase was measured with a MMP-1 human biotrak ELISA system. The elastase activities after treatment of PMR were measured as well.

Results : In the present study, the collagen production was not increased. However, the increased collagenase activity after UVB damage was significantly recovered to 50.2 ± 14.5%, 8.2 ± 3.1%, and 10.0 ± 3.3% (10, 30, and 100 μg/ml). The elastase activities (10, 100, and 1000 μg/ml) significantly reduced to 75.2 ± 5.2%, 40.3 ± 1.2%, and 27.0 ± 1.9%, respectively.

Conclusion : PMR showed the inhibitory effects on collagenase and elastase activity. These results suggest that PMR may have potential as an anti-aging ingredient in cosmetic herbal treatment.

Key words : Polygoni Multiflori Radix, Elastase, Collagenase, Collagen, Hs68, Fibroblasts

Introduction

Polygoni Multiflori Radix (PMR), the roots of *Polygonum multiflorum* Thunberg, is used to tonify the liver and kidney, nourish the blood, and augment the essence and used for the treatment of the patterns of yin or blood deficiency with such signs as dizziness, blurred vision, premature greying of the hair, weakness in lower back and knees, soreness in the exremities, nocturenal emissions, and insomnia[1,2]. According to recent studies, PMR has many pharmacological actions, such as anti-oxidation[3], neuroprotection[4], anti-cancer[5], hair growing effect of resting hair follicles[6], anti-inflammation[7], reducing the blood lipids[8], and anti-osteoporosis[9].

Solar ultraviolet (UV) radiation accelerates premature aging (photoaging), the symptoms of which include leathery texture, wrinkles, mottled pigmentation, laxity and sallowness[10]. Seo et al. reported that PMR has an inhibitory effect on the melanogenesis induced by UV radiation[11]. However, there are no studies of its effects on the collagen and elastin metabolism. The UV exposure would lead to the activation of receptors for epidermal growth factor, IL-1, and TNF-α which then activates AP-1 and matrix metalloproteinase-1 (MMP-1, a major metalloproteinase for collagen degradation) are expressed and activated[12].

Elastin is an aging related protein in connective tissue that is elastic and allows many tissues in the body to resume their shape after stretching or contracting. In the present study, the effects of PMR

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· 접수 : 2013년 12월 13일 · 수정 : 2014년 1월 20일 · 채택 : 2014년 1월 20일

on type I procollagen production and collagenase activity in human normal fibroblasts Hs68 using UVB irradiation was examined to find out collagen-related mechanism. The effect of PMR on elastase activity was measured as well to find out the potential cosmetic effects of PMR.

Materials and Methods

1. Materials

Polygoni Multiflori Radix was purchased from Omniherb (Daegu, Korea), and it had been authenticated by Prof. Bu (the department of herbal pharmacology, Kyung-Hee University). Polygoni Multiflori Radix extract (PMR) was prepared as follow. 100 g of PMR in 2,000 ml distilled water was extracted in a heating extractor at 100℃ for 3 hours. The extract was filtered through filter paper, and concentrated by using the rotary evaporator. The extract was lyophilized by using freeze dryer (21.1 g). All reagents without mentioning were purchased from Sigma-Aldrich (USA).

2. Assay of elastase activity

The elastase activity was evaluated by using a modification of a previously reported method of Kraunsoe et al[13]. 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[14]. 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 10, 100, and 1000 μg/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:

$$\text{Elastase activity (\%)} = [(OD_{410} \text{ of control}) - (OD_{410} \text{ of sample}) / (OD_{410} \text{ of control})] \times 100$$

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. The cells were sub-cultivated with a split ratio of 1:3 every other day.

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. Cytotoxicity

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 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, 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:

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

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

7. Statistical analysis

The results were expressed as means ± standard error of the mean (SEM)(n = 3 experiments). 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. Effect of PMR on inhibition of elastase activity

The inhibitory effect of PMR on elastase activity was determined according to the method described previously[13,14]. The elastase activities of PMR-treated group at 10, 100, and 1000 μg/ml were 75.2 ± 5.2%, 40.3 ± 1.2%, and 27.0 ± 1.9%, respectively. The results showed the statistical significances between all concentrations of PMR-treatment groups and control group (Fig. 1).

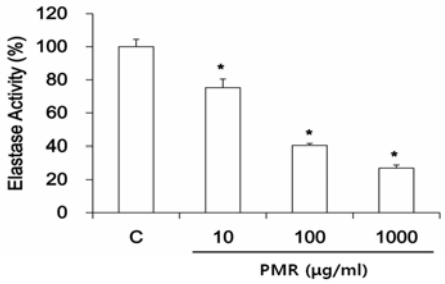

Figure 1. Effect of PMR on inhibition of elastase activity. C: control, distilled water treated group. 0.1, 1, and 10: Polygoni Multiflori Radix extract treated group (10, 100, and 1000 μg/ml). Data are expressed as the mean ± SEM of three experiments. *: significantly different from the control, $p < 0.05$.

2. Cytotoxicity of PMR in human fibroblasts

The cytotoxicity of PMR was evaluated with MTT method on human fibroblasts. 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 PMR-treated group at 10, 30 and 100 μg/ml are 106.5 ± 0.5%, 113.6 ± 0.4%, and 118.3 ± 6.4%, respectively. The cell viabilities of PMR treated groups were increased dose-dependently, and the results showed that PMR has no cytotoxicity up to the effective concentration in human fibroblasts (less than 100 μg/ml).

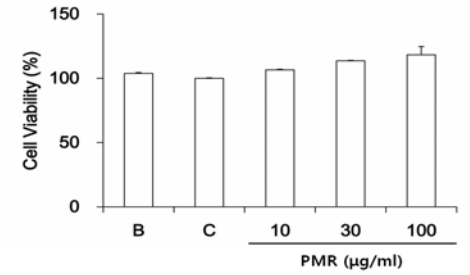

Figure 2. Cell viability of PMR on Hs68 human fibroblasts. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Polygoni Multiflori Radix extract (PMR 10, 30, and 100 μg/ml) treated group. Data are expressed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

3. Effect of PMR on collagen type I synthesis

The amounts of type I collagen synthesis of PMR were shown in Figure 3. The collagen amounts in PMR-treated group at 10, 30 and 100 μg/ml did not show the statistical significance (13.2 ± 2.3, 13.9 ± 2.3, and 14.0 ± 1.0 ng/ml, respectively).

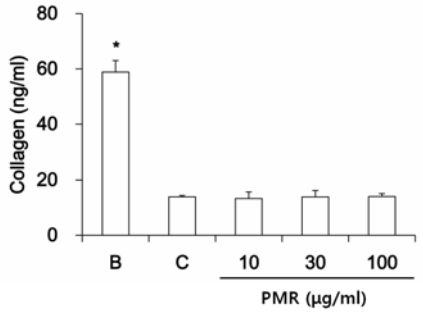

Figure 3. Effect of PMR on collagen type I synthesis in human fibroblast cells. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Polygoni Multiflori Radix extract (PMR 10, 30, and 100 μg/ml) treated group.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

4. Inhibitory effect of PMR on collagenase activity

To evaluate the collagenase activity, MMP-1 activity was quantitatively measured by using the previously described MMP-1 assay kit. The activities of MMP-1 in PMR-treated group were recalculated into 100% of control group (Fig. 4). PMR-treated group at 10, 30, and 100 μg/ml significantly reduced the MMP-1

activity (50.2 ± 14.5%, 8.2 ± 3.1%, and 10.0 ± 3.3%, $p < 0.05$).

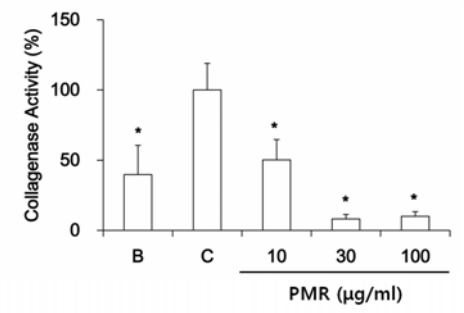

Figure 4. Effect of PMR on collagenase activity in human fibroblast cells. B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: Polygoni Multiflori Radix extract (PMR 10, 30, and 100 μg/ml) treated group. Data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

Discussions

PMR has been clinically used to prevent the aging process in the human body[1,2]. Its name can be translated into crow-black-haired man. Accordingly, it has been used to prevention and treatment from becoming grey hair. Contradictively, Seo *et al.* recently reported its whitening effects[11]. In the present study, we tried to find out its anti-aging effects especially in the aging skin via the mechanisms on the elastin and the collagen.

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[15]. In the present study, inhibitory effect of PMR on elastase activity was determined with elastase enzyme assay. PMR significantly and dose-dependently inhibited the elastase activity. The results provide that PMR has preventive effects on the aging process by decreasing elastase activity.

We examined the cytotoxicity of PMR with MTT method on human fibroblasts. The cell viabilities of PMR treated groups were increased dose-dependently, and the results showed that PMR has no cytotoxicity up to the effective concentration in human fibroblasts. This result means that PMR has protective effect of UV-B radiation-induced damage on human fibroblasts.

Collagen is one of the main components of body and plays an important agent for skin volume by itself. In nature, it is found exclusively in animals, especially in the flesh and connective tissues of mammals[16]. 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[17]. The amount of collagen is mainly due to the balance of synthesis and degradation. In this study, the amount of collagen type I was not increased after treatment of PMR.

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[18,19]. 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[20]. To evaluate the effect of PMR on the collagenase activity, MMP-1 activity was quantitatively measured. PMR significantly reduced the MMP-1 activity in all concentrations. Therefore, the result of this study provides that PMR has the anti-aging effects by reducing the MMP-1 activity.

In conclusion, PMR showed that the protective effect of UV-B radiation-induced damage and the inhibitory effects on collagenase and elastase. The preventive effects on the aging process in the human body might be thought to be related with the inhibitory effects on collagenase and elastase related mechanism considering the present data. These results suggest that PMR may have potential as an anti-aging ingredient in cosmetic herbal products. Further studies would need to unravel the exact molecular mechanisms of PMR's anti-wrinkle effects.

Conclusions

In this study, we determined the activities of elastase in PMR-treated groups. And the amounts of

type I collagen synthesis and collagenase activity were measured in PMR-treated human fibroblasts.

1. PMR significantly and dose-dependently inhibited the elastase activity.
2. The cell viabilities of PMR treated groups were increased dose-dependently, and the treatment of PMR has no cytotoxicity up to the effective concentration in human fibroblasts.
3. The collagen amounts in PMR-treated group did not show the statistical significance.
4. PMR significantly reduced the MMP-1 activity in all concentration on human fibroblasts.

We conclude that PMR has the protective effect of UV-B radiation-induced damage and the inhibitory effects on collagenase and elastase. These results suggest that PMR may have potential as an anti-aging ingredient in cosmetic herbal products.

Acknowledgement

This paper was supported by the Semyung University Research Grant of 2013.

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