

Effects of Yukmigeehwang-hwan Pharmacopuncture Extracts on the Collagenase Activity and Procollagen Synthesis in HS68 Human Fibroblasts and Tyrosinase Activity

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六味地黃丸 藥鍼液의 사람 피부아세포의 콜라게나제 활성 및 프로콜라겐 합성과 티로시나제 활성에 미치는 영향

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Abstract

목적 : 본 연구는 六味地黃丸 藥鍼液가 사람 피부 섬유아세포의 콜라게나제 활성 및 프로 콜라겐 합성에 미치는 영향과 티로시나제 활성에 미치는 효과를 측정하고자 실시하였다.

방법 : HS68 사람 정상 섬유아세포에 UVB 조사 후 六味地黃丸 藥鍼液가 type I procollagen 생성과 콜라게나제 효소활성에 미치는 효과와 티로시나제 효소활성에 미치는 효능을 평가하였다.

결과 : 六味地黃丸 藥鍼液은 UVB 조사된 세포의 콜라게나제 효소활성을 통계적으로 유의하게 억제하였고, 티로시나제 활성과 L-DOPA oxidation 활성 또한 통계적으로 유의하게 억제하였다. 그러나 티로시나제와 L-DOPA oxidation 억제활성의 정도는 미약하였다.

결론 : 六味地黃丸 藥鍼液의 콜라게나제 억제효능은 주름개선 약침 치료에 활용이 가능할 것으로 생각된다.

Key words : Yukmigeehwang-hwan, Liuweidihuang-wan, Type I procollagen, Collagenase, Tyrosinase

I. Introduction

Yukmigeehwang-hwan pharmacopuncture extracts (Liuweidihuang-wan in chinese, YPE) are composed of six herbal medicines¹⁾. YPE is classic decoction for the treatment of Kidney and Liver yin deficiencies

such as a general weakness of the lower back and legs, a light-headedness, vertigo, tinnitus and diminished hearing^{1,2)}.

In these days, aging seems to be treated as not a nature to accept but a disease or a disorder to overcome. Theories of aging fall into two categories, (1) programmatic theory and (2) stochastic theory. The programmed theory proposes a clock in our bodies that controls not only our process of development but also triggers our self-destruction.

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The stochastic theory proposes that the cross-linking of proteins and other cellular macromolecules leads to age-dependent diseases and disorders. Processes that are associated with cellular damage and aging are the production of free radicals (a process much enhanced after ultraviolet irradiation) and an increasing number of errors during DNA replication. Cellular manifestations of intrinsic aging include decreased life span of cells, decreased responsiveness of cells to growth signals, which may reflect loss of cellular receptors to growth factors, and increased responsiveness to growth inhibitors. All these findings are more pronounced in cells derived from photo-damaged skin³⁾. Aging process in skin (extrinsic aging) is generally referred to as photo-aging due to chronic exposure to short wavelength UV light (UVB) and is characterized by severe wrinkling and pigmentary changes, such as solar lentigo and mottled pigmentation on exposed areas such as the face, neck, and forearm⁴⁾. 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 matrix metalloproteinases (MMPs) in human skin *in vivo*^{5,6)}. MMPs including collagenase are considered key factors in the photo-aging process. Melanogenesis was induced after UV irradiation as well. The key regulator in melanogenesis is well known as a type of enzyme, tyrosinase. Tyrosinase is a copper-containing enzyme present in animal tissues that catalyzes the production of melanin⁷⁾.

In the present study, the effect of YPE on type I procollagen production and collagenase activity in human normal fibroblasts HS68 after UVB (312 nm) irradiation were investigated. The tyrosinase activity after treatment of YPE was measured as

well.

II. Materials and methods

1. Sample preparation

The *Rehmanniae Radix Preparata*, *Corni Fructus*, *Dioscoreae Rhizoma*, *Alismatis Rhizoma*, *Hoelen*, and *Moutan Cortex Radicis* were purchased from Omniherb (Korea). YPE was prepared as follow. The *Rehmanniae Radix Preparata* (24 g), *Corni Fructus* (12 g), *Dioscoreae Rhizoma* (12 g), *Alismatis Rhizoma* (9 g), *Hoelen* (9 g), and *Moutan Cortex Radicis* (9 g) 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 extracts were lyophilized by using freeze dryer (13.7 g). The lyophilized extract was dissolved in water and filtered three times through microfilter paper (Whatman no. 2, 0.45–0.2 μm). It was placed in a disinfected vial and sealed for further study.

2. Reagents

All reagents were purchased from Sigma-Aldrich except as mentioned below (St. Louis, MO, USA).

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°C in a humidified atmosphere of 5% CO₂. When cells

reached above confluency, subculture was conducted at a split ratio of 1:3.

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 μ l/well)⁸⁾. Immediately after irradiation, fresh serum-free medium was added to the cells. After 24 hours incubation period, responses were measured. 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°C in 5% CO₂. Cells were pretreated with the sample at a concentration of 100, 10, 1 μ g/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°C for additional 4 hours. The medium containing MTT was discarded, and MTT formazan that had been produced was extracted with 200 μ l of DMSO. The absorbance was read at 595 nm with

a reference wavelength of 690 nm. The cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{[(\text{OD}_{595} \text{ of sample}) / (\text{OD}_{595} \text{ of control})] \times 100}{1}$$

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°C in 5% CO₂. Cells were pretreated with the sample at a concentration of 10, 30, and 100 μ g/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 pro-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 described⁹⁾. The reaction mixtures were prepared by adding 40 U of mushroom tyrosinase to 20 μ l of YPE dissolved in 0.1 M sodium phosphate buffer (pH 6.5), and then adding 40 μ l of 1.5 mM L-tyrosine and 220 μ l of 0.1 M sodium phosphate buffer. The resulting mixture (300 μ l) was incubated for 10 min at 37°C and then absorbance at 490 nm was measured. The same mixture, but without YPE extract, was used as a control.

8. Inhibition of L-DOPA oxidation

The inhibitory effect of YPE on L-DOPA oxidation was determined according to the method of Joshi with a slight modification¹⁰. 50 μ l of YPE dissolved in 0.1 M sodium phosphate buffer was added to 40 U of mushroom tyrosinase in 900 μ l of 0.1 M sodium phosphate buffer (pH 6.5). After 6 min of incubation at 37°C, 3 mM of L-DOPA was added. Then the mixture was incubated at 37°C for 15 min. Activities were quantified by measuring absorbance at 475 nm. The same mixture, but without YPE extract, was used as a control.

9. Statistical analysis

The results were expressed as means \pm standard error of the mean (SEM). Significances of changes were determined using the one-way ANOVA with a Dunnett's post hoc test. Values of $p < 0.05$ were considered significant.

III. Results

1. Cytotoxicity on HS68 human fibroblasts

In order to evaluate the cytotoxicity of YPE, samples were prepared at various concentrations and used to treat human fibroblasts (HS68). The results of this evaluation are shown in Fig. 1 at concentrations of 10, 30, 100 μ g/ml. The cell viability was recalculated into 100% of control group. The cell viabilities of YPE 10 μ g/ml treated, YPE 30 μ g/ml treated, YPE 100 μ g/ml treated are $101.8 \pm 1.4\%$, $101.1 \pm 0.0\%$, and $99.1 \pm 1.0\%$, respectively. YPE showed no cytotoxicity up to the effective concentration for anti-wrinkle

activity (less than 100 μ g/ml).

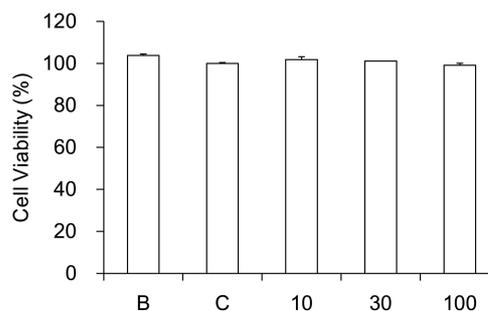


Fig. 1. Cell viability of Yukmigeehwang-hwan pharmacopuncture extracts (YPE) on HS68 human fibroblasts.

B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: YPE 10, 30, and 100 μ g/ml treated group. Data are expressed as the mean \pm SEM of three experiments.

2. Assay of collagen type I synthesis

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 synthesized¹¹. The amounts of type I collagen synthesis of YPE were shown in Fig. 2. YPE did not increase the expression of type I collagen at all concentrations of 10, 30, and 100 μ g/ml (13.3 ± 2.6 ng/ml, 15.9

± 3.6 ng/ml, and 16.2 ± 0.0 ng/ml) compared with control group (15.3 ± 1.6 ng/ml, Fig. 2).

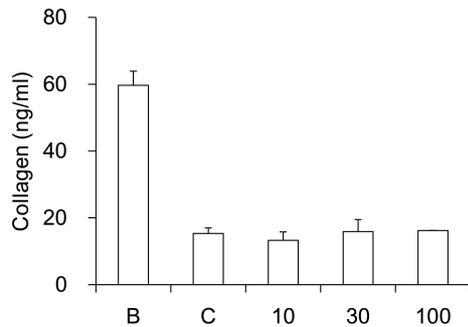


Fig. 2. Effect of Yukmigeehwang-hwan pharmacopuncture extracts (YPE) on collagen type I synthesis in human fibroblast cells.

B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: YPE 10, 30, and 100 μ g/ml treated group. Data are expressed as the mean \pm SEM of three experiments.

3. 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 YPE treatment were recalculated into 100% of control group (Fig. 3). YPE significantly reduced the MMP-1 activity at concentrations of 30 μ g/ml and 100 μ g/ml ($50.0 \pm 6.2\%$ and $37.0 \pm 3.4\%$ respectively, $p < 0.05$). 10 μ g/ml showed the tendency of reducing patterns. However, there was no significance ($59.0 \pm 8.3\%$, Fig. 3).

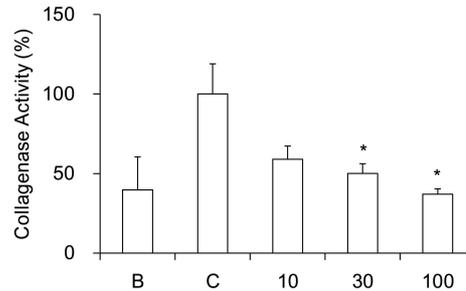


Fig. 3. Effect of Yukmigeehwang-hwan pharmacopuncture extracts (YPE) on collagenase activity in human fibroblast cells.

B: blank, distilled water treated group without UVB irradiation. C: control, distilled water treated group with UVB irradiation. 10, 30, and 100: YPE 10, 30, and 100 μ g/ml treated group. Data are expressed as the mean \pm SEM of three experiments. *: significantly different from the control, $p < 0.05$.

4. Tyrosinase activity assay

The activities of YPE on tyrosinase activity were recalculated into 100% of control group (Fig. 4). YPE significantly reduced the tyrosinase activity at concentrations of 10 mg/ml ($37.5 \pm 15.0\%$, $p < 0.05$). The tyrosinase activity of YPE 0.1 and 1 mg/ml treated group did not show any significance ($109.3 \pm 6.3\%$ and $99.3 \pm 2.9\%$, respectively).

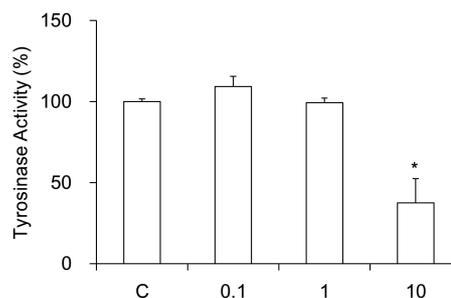


Fig. 4. Effect of Yukmigeehwang-hwan pharmacopuncture extracts (YPE) on tyrosinase activity.

C: control, distilled water treated group. 0.1, 1, and 10: YPE 0.1, 1, and 10 mg/ml treated group. Data are expressed as the mean \pm SEM of three experiments. *: significantly different from the control, $p < 0.05$.

5. L-DOPA oxidation

The activities of YPE on L-DOPA oxidation were recalculated into 100% of control group (Fig. 5). YPE significantly reduced the L-DOPA oxidation activity at concentrations of 10 mg/ml ($87.5 \pm 2.2\%$, $p < 0.05$). YPE 0.1 and 1 mg/ml treated groups did not show any activity ($101.8 \pm 0.0\%$ and $104.0 \pm 2.0\%$, respectively).

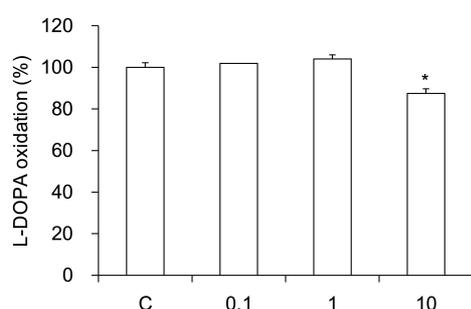


Fig. 5. Effect of Yukmigeehwang-hwan pharmacopuncture extracts (YPE) on L-DOPA oxidation.

C: control, distilled water treated group. 0.1, 1, and 10: YPE 0.1, 1, and 10 mg/ml treated group. Data are expressed as the mean \pm SEM of three experiments.

IV. Discussion and conclusion

YPE is composed of six herbal medicines. The *Rehmanniae Radix Preparata*, *Corni Fructus*, *Dioscoreae Rhizoma*, *Alismatis Rhizoma*, *Hoelen*, and *Moutan Cortex Radicis* are ground into powder and formed into small pills with honey¹⁾. YPE is a classic decoction for the treatment of Kidney and Liver yin deficiencies. When Kidneys are weakened, the marrow will become depleted and there will be a general weakness of the skeletal structure which is focused in the area of the body, most closely associated with the Kidneys, the lower back and legs. The eyes are nourished by the

Liver; lack of nourishment manifests itself as light-headedness and vertigo. The ears are nourished by the Kidneys; lack of nourishment manifests itself as tinnitus and diminished hearing^{1,2)}.

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 photo-oxidative damage with long-term detrimental effects like photo-aging, characterized by wrinkles, loss of skin tone and resilience. Photo-aged skin displays alterations in the cellular component and extracellular matrix with accumulation of disorganized elastin and its microfibrillar component fibrillin in the deep dermis and a severe loss of interstitial collagens, the major structural proteins of the dermal connective tissue. MMPs are known to be upexpressed in human fibroblasts within hours after exposure to UV irradiation and are, therefore, considered key factors in the photo-aging process. Therefore, agents with the ability to elevate ECM protein levels or inhibit the major collagen-degrading enzymes like MMPs would prove to be useful in the development of effective anti-aging agents. Collagen is a group of naturally occurring proteins. In nature, it is found exclusively in animals, especially in the flesh and connective tissues of mammals¹²⁾. 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 content¹³⁾. Collagen, in the form of elongated fibrils, is mostly found in fibrous tissues such as tendon, ligament and skin, and is also abundant in cornea, cartilage, bone, blood vessels, the gut, and intervertebral disc. In muscle tissue it serves as a major component of endomysium. Collagen constitutes 1% to 2% of muscle tissue, and accounts for 6% of the weight of strong,

tendinous muscles¹⁴). Collagen occurs in many places throughout the body. So far, only 29 types of collagen have been identified and described. Over 90% of the collagen in the body, however, is of type I, II, III, and IV. Among them, collagen type I is placed at skin, tendon, vascular, ligature, organs, and bone (main component of bone). Collagen-related diseases most commonly arise from genetic defects or nutritional deficiencies that affect the biosynthesis, assembly, postranslational modification, secretion, or other processes involved in normal collagen production.

In this study, we evaluate the cytotoxicity of YPE on human fibroblasts (HS68) at various concentrations. There was no cytotoxicity in all YPE-treated concentrations. However, the amount of collagen type I was not increased at all concentration of YPE treatments.

To evaluate the collagenase activity, matrix metalloproteinase-1 (MMP-1) activity was quantitatively measured. YPE significantly reduced the MMP-1 activity at a concentration of 30 µg/ml and 100 µg/ml in a dose-dependent manners.

Tyrosinase is one of the important enzymes that has a key role in pigmentation process¹⁵). L-DOPA oxidation was also undertaken by tyrosinase. The effects of YPE on tyrosinase activity and L-DOPA oxidation were significantly effective at 10 mg/ml treatments.

In conclusion, YPE showed the anti-wrinkle and whitening effects. However, the whitening effects are not enough to be used as whitening agents. These results suggest that YPE may be a potential pharmacopuncture as an anti-aging pharmacopuncture treatments such as wrinkle care therapies. I think further studies will be needed to unravel exactly under the effects in vivo and clinical experiments

and the molecular mechanisms of the effects

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