Original Article

Anti-wrinkle Effects of *Cervi Pantotrichum Cornu* Pharmacopuncture Solution

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국문초록

녹용 약침액의 주름 개선 효과에 관한 연구

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목적 : 본 연구는 녹용약침액의 주름 개선 효과에 대하여 여러 가지 *in vitro* 실험을 통해 확인해 보고자 계획되었다.

방법 : 녹용약침액의 항산화효과를 DPPH(1,1-diphenyl-2-picrylhydrazyl) 자유라디칼 소거법을 이용하여 측정하였고, 또, elastase 억제 효과를 측정하였다. 그리고 녹용약침액이 사람 정상 섬유아세포 HS 68에서 UVB 조사 후 type I procollagen의 생산량 회복에 미치는 효과를 ELISA법을 이용하여 측정하였다.

결과 : 녹용약침액에서 우수한 DPPH 자유라디칼 소거 효과가 관찰되었다. 또한 통계적으로 유의한 elastase 활성 억제 효과를 관찰할 수 있었다. 사람 섬유아세포 HS 68을 이용한 실험에서는 녹용약침액 처치군에서 UVB 조사로 감소된 type I procollagen이 유의하게 회복되는 것이 관찰되었다.

결론 : 본 연구 결과에 의하면, 녹용약침액에는 유효한 주름개선 효과가 있어, 미용약침 소재로 개발할 수 있을 것으로 사료된다.

핵심 단어 : 녹용약침액, 주름 개선, elastase, DPPH, type I procollagen

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Ⅰ. Introduction

It is the latest trend to be looked younger than the actual age. In these days, aging seems to be treated not as a nature to accept but as a disease or a disorder to overcome.

There are two major theories of aging: the programmatic theorystates that aging is an inherent genetic process, and the stochastic theory states that aging represents random environmental damage. Processes that are associated with cellular damage and aging are the production of free radicals(a process much enhancedafter ultraviolet(UV) 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 photodamaged skin[1].

UV irradiation is responsible for the cutaneous damage after both acute and chronic exposure, and is believed to be an important etiology in human skin cancer and premature skin aging[2]. Ultraviolet-B(UVB)(312㎚) has a low level of skin penetration, but it can readily affect macromolecules in the epidermal layer, thus altering cellular functions via DNA damage, generation of reactive oxygen species (ROS), decreased in skin content of antioxidant compounds[3]. Recent studies have shown that ROS such as superoxide anion, hydroxyl radical, and hydrogen peroxide are responsible for UV-induced oxidative damage[4-6]. Nowadays, various natural compounds from both nutritive and non-nutritive sources were reported to protect against UV-induced skin damage[4,7,8].

In Oriental medicine, skin aging problem is thought to be due to the failure of nourishing the essence of the kidneys and insufficient nutrition supply for body organs[9]. *Cervi Pantotrichum Cornu* is considered asa core tonic ingredient which fortifies the primal yang and the kidneys, generates essence and nourishes the qi and blood of the body[10]. Therefore, it is likely to use *Cervi Pantotrichum Cornu* in skin aging such as premature wrinkle.

In the present study, I investigated the anti-wrinkle effects of *Cervi Pantotrichum Cornu* including anti-oxidative activities, elastase inhibitory activities and protective effects against the UVB-induced photodamage in human skin fibroblasts HS 68.

Ⅱ. Materials and methods

1. Sample preparation

Cervi Pantotrichum Cornu(Nokyong) was purchased from Omniherb(Korea). *Cervi Pantotrichum Cornu* Pharmacopuncture solution(CPC-HAS) was prepared according to the following steps. 2.0g of dried mixture of *Cervi Pantotrichum Cornu* was distilled in 100㎖ of saline, and 50㎖ of resulting distillates was filtered three times through micro-filter paper and syringe filter(Whatman # 2, 0.45㎛ to 0.2㎛). Filtered material was placed in the disinfected vial and was sealed for further study.

2. Reagents

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

3. Elastase activity

The elastase activity was evaluated by using a modification of a previously reported method of Kraunsoe et al[11]. 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 ㎚[12]. 2mM N-succinyl-Ala-Ala-Ala-p-nitroanilide was prepared in a 0.1M Tris - Cl buffer(pH 8.0), and this solution was added to the stock sample. Each sample solution was diluted to final concen-

trations of 1000, 100, 10㎍/㎖. The solutions were mixed thoroughly by tapping before an elastase (0.1360unit/㎖) stock solution was added. Solution was incubated for 10min at 37°C and the absorbance was measured at 410㎚.

4. DPPH free radical scavenging activity

The scavenging effect of sample on 1,1-diphenyl-2-picrylhydrazyl(DPPH) radicals was assayed according to the procedure described by Shimada et al[13]. The DPPH radical shows a deep violet color due to its unpaired electron, and radical scavenging capacity can be followed spectrophotometrically by the loss ofabsorbance at 540㎚[12]. Sample was added to 1㎖ of freshly prepared ethanolic solution containing a final DPPH radical concentration of 0.2mM. After it stood for 30min in the dark, the absorbance of the mixture was measured at 540㎚ against an ethanol control with a spectrophotometer. The percent scavenging capability was calculated according to the following equation :

DPPH free radical scavenging activity(%)

= [1-{(OD540 of sample) - (OD540 of sample blank)} / {(OD540 of control) - (OD540 of blank)}] × 100

5. Cell culture

HS 68 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% CO_2. When cells reached above confluency, subculture was conducted at a split ration 1:3.

6. UVB irradiation

A UVB lamp(Vilber Lourmat, France) was used as a UVB source. HS 68cells were rinsed twice with phosphate-buffered saline(PBS), and all irradiations were performed under a thin layer of PBS (200㎕/well). Immediately after irradiation, fresh serum-free medium was added to the cells. Responses were measured after an incubation period of 24hours. Mock-irradiated blanks followed the same schedule of medium changes without UVB irradiation.

7. 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[14]. The human fibroblast cells(HS 68) were seeded in 24-well plates at a density of 2×105/㎖ per well and cultured at 37°C in 5% CO_2 Cells were pretreated with the sample at a concentration of 100, 30, 10㎍/㎖ for 24hours 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㎎/㎖(final concentration) of MTT. The blank and control group was cultivated without sample treatment. The cells were then incubated at 37°C or additional 4h. The medium containing MTT was discarded, and MTT formazan that had been produced was extracted with 200㎕ of DMSO. The absorbance was read at 595㎚ with a reference wavelength of 690㎚. The cell viability was calculated as follows :

Cell viability(%) = [(OD595 of sample) / (OD595 of control)] × 100

8. Assay of collagen type I synthesis by an ELA kit

HS 68 human fibroblasts were inoculated into 24-well plate(2×105cells/well) and cultured at 37°C in 5% CO_2. Cells were pretreated with the sample at a concentration of 100, 30, 10㎍/㎖ for 24hours prior to UVB irradiation. After UVB irradiation, cells were retreated with the sample and incubated for additional 24hours. 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).

9. Statistical analysis

The results were expressed as means ± standard error of the mean(SEM). The data of differences between two groups was analyzed by ANOVA test with a Dunnett's post-hoc test. All differences(p value) less than 0.05 were considered significant.

Ⅲ. Results

1. Elastase activity

CPC-HAS was found to have the lowest elastase activity at a concentration of 1000㎍/㎖(25.4±1.7%, p<0.001). CPC-HAS 100㎍/㎖ and 10㎍/㎖ treated groups showed 60.1±5.8%(p<0.01) and 89.6±6.1% of elastase activity, respectively(Fig. 1).

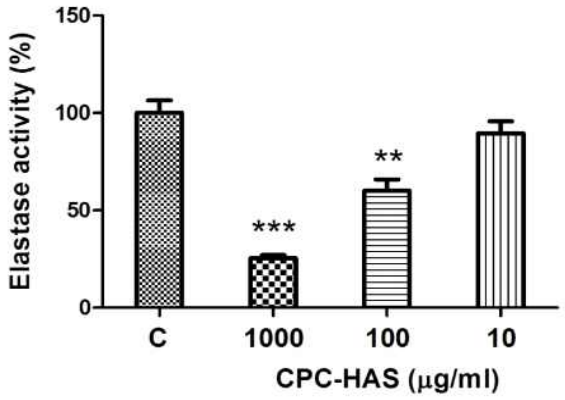

Fig. 1. Effect of CPC-HAS on inhibition of elastase activity
C : control, distilled water treated.
CPC-HAS : *Cervi Pantotrichum Cornu* herbal acupuncture solution treated group.
Data are expressed as the mean±SEM of three experiments.
** : significantly different from the control, $p<0.01$.
*** : significantly different from the control, $p<0.001$.

2. DPPH free radical scavenging capability

CPC-HAS 10㎎/㎖ treated groups had the highest scavenging capability of 63.9±6.2%, while CPC-HAS 2㎎/㎖ and CPC-HAS 0.4㎎/㎖ treated groups had 33.6±3.9% and 8.3±1.3%, respectively(Fig. 2).

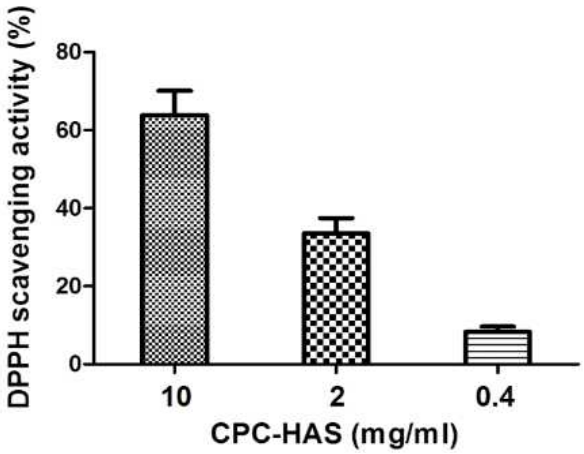

Fig. 2. DPPH free radical scavenging capability of CPC-HAS
CPC-HAS : *Cervi Pantotrichum Cornu* herbal acupuncture solution treated group.
Data are expressed as the mean±SEM of three experiments.

3. Cytotoxicity

The cell viability was recalculated into 100% of control group. The cell viabilities of CPC-HAS 100 ㎍/㎖ treated, CPC-HAS 30㎍/㎖ treated and CPC-

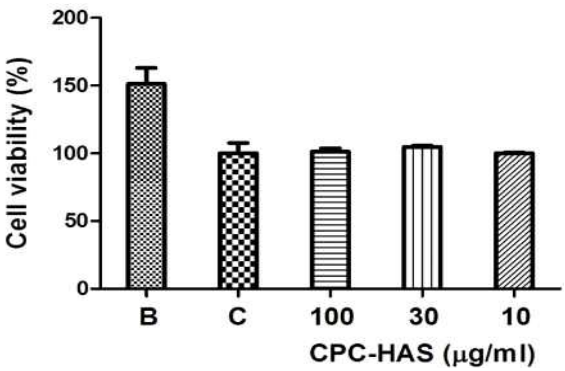

Fig. 3. Cell viability of CPC-HAS on HS 68 human fibroblasts
B : blank, distilled water treated group without UVB irradiation.
C : control, distilled water treated group with UVB irradiation.
CPC-HAS : *Cervi Pantotrichum Cornu* herbal acupuncture solution treated group.
Data are expressed as the mean±SEM of three experiments.

HAS 10㎍/㎖ treated are 101.2±2.5%, 104.7± 0.8%, and 100.0±0.8%, respectively. CPC-HAS showed no cytotoxicity up to the effective concentration for anti-wrinkle activity(less than 100㎍/㎖)(Fig. 3).

4. Assay of collagen type I synthesis

The amounts of type I collagen synthesis of CPC-HAS were recalculated into 100% of control group(Fig. 4). CPC-HAS significantly($p<0.05$) increased the expression of type I collagen at a concentration of 100㎍/㎖(144.4±16.6). CPC-HAS 30㎍/㎖(115.6±7.6) and 10㎍/㎖(126.6±15.1) treated group showed the increase of type I collagen synthesis, but there was no significance.

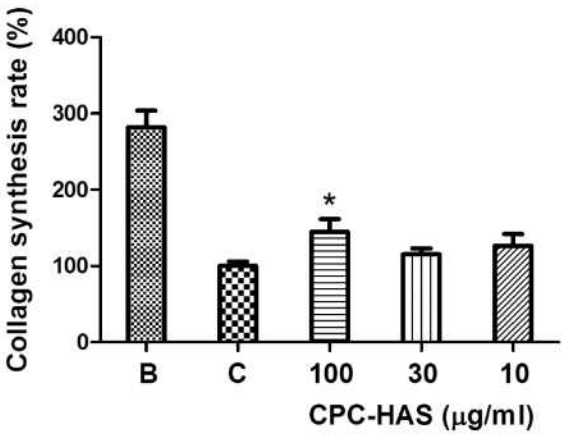

Fig. 4. Effect of CPC-HAS on collagen type I synthesis in human firboblast cells
B : blank, distilled water treated group without UVB irradiation.
C : control, distilled water treated group with UVB irradiation.
CPC-HAS : *Cervi Pantotrichum Cornu* herbal acupuncture solution treated group.
Data are expressed as the mean±SEM of three experiments.
* : significantly different from the control, $p<0.05$.

Ⅳ. Discussion and Conclusion

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. The unifying pathogenic agents for these changes are UV-generated ROS which deplete and damage non-enzymatic and enzymatic antioxidant defense systems of the skin. As well as causing permanent genetic changes, ROS activate cytoplasmic signal transduction pathways in resident fibroblasts that are related to growth, differentiation, senescence and connective tissue degradation[15].

Elastase is an enzyme from the class of proteases or peptidases, that break down proteins. It, specifically, breaks down elastin, an elastic fiber that, together with collagen, determines the mechanical properties of connective tissue[16]. 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[17]. Elastin is the main component of the elastic fibers of the connective tissue and tendons. The elastic fibers in the skin, together with the collagenous fibers, form a network under the epidermis[18]. Elastase also plays a critical role in inflammatory processes[19]. The enzyme has drawn much attention, primarily because of its reactivity and non-specificity. It is able to attack all major connective tissue matrix proteins, including elastin, collagen, proteoglycans, and keratins. Since this elastic fiber is easily decomposed by elastase secretion and activation caused by exposure to UV light or ROS, an approach that inhibits the elastase activity could also be applied as a useful method to protect against skin aging[19].

Cervi Pantotrichum Cornu(Nokyong), processed young antlers of Cervus nippon Temminck, Cervus elaphus Linne and Cervus canadensis Erxleben[10], considered as a nobel ingredient in Korean medicine.

It is one of the major restoratives which fortifies the primal yang, generates essence and augments the bonemarrow. Furthermore, it tonifies the kidneys, strengthens the sinews and bones and nourishes the qi and blood. Clinically, it is used to treat fatigues, impotences, cold extremities, sorenesses and lacks of strengths in the lower backs and knees, mental retardations, insufficient growths and so on[20].

These days, there are many researches about effects of *Cervi Pantotrichum Cornu* on various kinds of disease. Chen et al reported anti-lipid peroxidation of *Cervi Pantotrichum Cornu*[21], while Kim et al studied effects of *Cervi Pantotrichum Cornu* on Osteoporosis[22]. Effects of *Cervi Pantotrichum Cornu* and fermented *Cervi Pantotrichum Cornu* on longitudinal bone growth were reported by Kim et al[23], and Lee et al[24], and hematopoietic action of *Cervi Pantotrichum Cornu* was studied by Kim et al[25]. For *Cervi Pantotrichum Cornu* Pharmacopuncture, protective and anti-arthritic effects[26] and analgesic effects[27] were reported. Also, it was studied in vivo that *Cervi Pantotrichum Cornu* Pharmacopuncture improved adrenal cortical insufficiency[28], diabetes mellitus[29], hypothyroidism[30], growth and the intellectual development[31]. However, there was few study for anti-wrinkle effects so far.

In order to investigate the potential of CPC-HAS as an active ingredient for wrinkle-care cosmetics, I measured its DPPH free radical scavenging activity, elastase inhibitory activity, and type I collagen synthesis in normal human fibroblasts HS 68.

The elastase acticvity of CPC-HAS was determined according to the method described previously. The elastase acticvity was recalculated into 100% of control group. CPC-HAS showed the elastase inhibitory effect in dose dependent manner. CPC-HAS was found to have the lowest elastase activity at a concentration of 1000㎍/㎖(25.4±1.7%, $p<0.001$). CPC-HAS 100㎍/㎖ and 10㎍/㎖ treated groups showed 60.1±5.8%($p<0.01$) and 89.6±6.1% of elastase activity, respectively(Fig. 1).

It has been reported that free radicals induced by ultraviolet light or oxidative stress accelerate skin aging[32]. Assays of the free radical scavenging capacity were carried out by the DPPH method. The free radical scavenging capacity of sample was measured at each concentration(10, 2 and 0.4㎎/㎖). A dose dependent free radical scavenging capability was observed in sample treated groups. CPC-HAS 10㎎/㎖ treated groups had the highest scavenging capability of 63.9±6.2%, while CPC-HAS 2㎎/㎖ and CPC-HAS 0.4㎎/㎖ treated groups had 33.6±3.9% and 8.3±1.3%, respectively(Fig. 2).

In order to evaluate the cytotoxicity of CPC-HAS, samples were prepared at various concentrations and used to treat human fibroblasts(HS 68). The results of this evaluation are shown in Fig. 3 at concentrations of 100, 30 and 10㎍/㎖. The cell viability was recalculated into 100% of control group. The cell viabilities of CPC-HAS 100㎍/㎖ treated, CPC-HAS 30㎍/㎖ treated and CPC-HAS 10㎍/㎖ treated are 101.2±2.5%, 104.7±0.8%, and 100.0±0.8%, respectively. CPC-HAS showed no cytotoxicity up to the effective concentration for anti-wrinkle activity(less than 100㎍/㎖).

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[12]. The amounts of type I collagen synthesis of CPC-HAS were recalculated into 100% of control group(Fig. 4). CPC-HAS significantly ($p<0.05$) increased the expression of type I collagen at a concentration of 100㎍/㎖(144.4±16.6). CPC-HAS 30㎍/㎖(115.6±7.6) and 10㎍/㎖(126.6±15.1) treated group showed the increase of type I collagen synthesis,

but there was no significance.

The significant DPPH free radical scavenging activity was observed in CPC-HAS. Also, elastase activity was significantly inhibited by CPC-HAS. Furthermore, type I procollagen production reduced by UVB irradiation was recovered by CPC-HAS in HS 68 cells.

In conclusion, CPC-HAS showed the anti-wrinkle effects in vitro. These results suggest that CPC-HAS may have potential as an anti-aging ingredient in cosmetic Pharmacopuncture. I think further studies will be needed to unravel exactly under the molecular mechanisms.

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