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Vegetable Peptones의 세포증식 및 콜라겐생성 촉진효과

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Effects of Vegetable Peptones on Promotion of Cell Proliferation and Collagen Production

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요 약: 피부노화의 주요 원인으로는 타입 I 콜라겐 생합성 저하 및 피부 진피세포의 증식 활성 감소를 들 수 있다. 효과적으로 피부노화를 관리하기 위해서는 안전하면서도 효능이 우수한 소재를 찾는 것이 필요하다. 이를 위해 본 연구진은 항노화소재를 스크리닝하였고, 완두콩과 밀 펩톤이 성체줄기세포의 세포증식을 증가시키는 효능을 관찰하였다. 완두콩과 밀 펩톤을 포함하는 식물성 펩톤은 다양한 크기의 펩타이드와 아미노산을 함유하고 있어 세포배양 시 첨가해 주면 영양공급원이나 growth factor로 세포 성장과 활성을 증가시키는 것으로 알려져 있다. 본 연구에서는 인간 진피섬유아세포를 이용하여 완두콩과 밀 펩톤이 세포증식 및 콜라겐합성에 미치는 영향과 이들의 작용기전을 규명하고자 하였다. 세포증식 실험에서 완두콩과 밀 펩톤은 유의성 있게 농도 의존적으로 세포증식을 유도하였다. 또한 인간 COL1A2 프로모터 루시퍼라아제와 타입 I 프로콜라겐 생합성 실험에서 완두콩 및 밀 펩톤이 COL1A2 프로모터의 활성화를 통해 타입I 프로콜라겐 생합성을 촉진시키고 있음을 확인하였다. TGF-β1 루시퍼라아제 리포터 실험과 TGF-β1 ELISA 실험에서는 완두콩 및 밀 펩톤이 TGF-β1 유전자의 발현을 촉진한다는 사실을 관찰하였고, 이러한 결과를 통해 완두콩 및 밀 펩톤의 콜라겐 생합성 촉진 기전이 TGF-β신호와 관련이 있음을 제시하였다. 즉, 완두콩 및 밀 펩톤은 TGF-β1의 발현촉진을 통해 콜라겐 생합성을 유도함을 유추할 수 있다. 완두콩과 밀 펩톤을 포함한 로션을 인체피부에 4주 동안 도포하여 피부자극을 관찰한 결과, 어떠한 부작용도 관찰되지 않았다. 이러한 연구결과를 종합해 볼 때 완두콩 및 밀 펩톤이 피부자극이 없으며 피부주름을 개선시킬 수 있는 소재로 사용가능할 것으로 기대된다.

Abstract: Skin aging appears to be principally attributed to a decrease in both levels of Type I collagen and regeneration ability of dermal fibroblasts. It is important to introduce an efficient and safe agent for effective management of skin aging. To this end, we performed screening for anti-ageing agents and then found that vegetable peptones (pea and wheat) promoted cell proliferation of adult stem cells. Vegetable peptones may be considered as useful medium additives because it can supply nutrients, peptides, amino acids or growth factor analogues. This study was designed to investigate effects of vegetable peptones on cell proliferation/collagen production and their possible mechanisms in human dermal fibroblasts. In cell proliferation assay, vegetable peptones significantly promoted cell proliferation in a concentration-dependent manner. In addition, human COL1A2 promoter luciferase and type I procollagen synthesis assays showed that vegetable peptones induce type I procollagen production through the activation of COL1A2 promoter. In both TGF-β1 luciferase reporter and ELISA assays, vegetable peptones was found to induce TGF-β1 production, suggesting that vegetable peptones induce type I procollagen production through the activation of TGF-β1. When applied topically in a human skin twice a day for an 4-week period of time, vegetable peptones

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did not induce any adverse reactions. Therefore, based on these results, we suggest the possibility that vegetable peptones may be considered as an attractive, wrinkle-reducing candidate for topical application.

Keywords: *cell proliferation, type I procollagen, COL1A2 promoter, vegetable peptones, irritation*

1. Introduction

Photoageing of skin is the combination of chronological ageing and the effects of cumulative exposure to ultraviolet (UV) radiation. Chronological skin ageing produces characteristic fine lines, whereas exposure to solar UV results in skin that is, in comparison, coarse, roughened and is deeply wrinkled, as also which is stiffer and less elastic[1-3]. Histologically, photoaged skin exhibits numerous alterations to the dermis. Destruction and loss of extracellular matrix (ECM) constituents at the dermal epidermal junction (DEJ) and in the dermis by matrix metalloproteinases (MMPs) are characteristic biochemical features. Changes include the deposition of dystrophic elastic fibres in the papillary dermis, termed solar elastosis[4], decreases in the major fibrillar collagens types I and III[5,6], reduction in the numbers of anchoring fibrils together with a reduced fibrillin-rich microfibrillar network proximal to the DEJ[7,8]. The expression of MMP is implicated in the proteolysis of these key proteins in both chronological but especially photoaging[9-11].

Plant-derived peptides (vegetable peptones), produced by enzymatic hydrolysis of selected vegetal raw materials rich in proteins, are used for both microbial broth and solid culture media[12]. More recently, vegetable peptones have been employed by many in cell culture medium formulation to fortify the amino acid content in small peptide form as substitutes for serum[13,14]. Our research group also found that vegetable peptones significantly promote adult stem cell proliferation, suggesting that vegetable peptones may be introduced as a serum-free culture medium for adult stem cells[15]. Physiologically, the uptake of peptones is linked to specialized transport systems, which are different from amino acid transporters. Inside the cells, the peptones are first clipped by proteases and the resulting free amino acids can then be used as nutrients via the TCA cycle or as precursors for other amino acids, nucleic acids or incorporated into proteins[16]. The nutritive supply of small peptides and free amino acids in the vegetable peptones could function as growth factors for animal cells. Heidemann *et al.*[17] also suggested a potential effect of higher-MW oligopeptides as growth or survival factors.

In this study, in order to assess the anti-wrinkle effects of vegetable peptones, we investigated the collagen production and cell proliferation-inducing effects of vegetable peptones *in vitro* experiments and the possible use of vegetable peptone as a topical agent for the management of skin wrinkling.

2. Materials and Methods

2.1. Cell Culture Treatment

Human dermal fibroblasts were obtained from AmorePacific (Korea) and were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, USA), penicillin (100 units/mL) (Invitrogen, USA), and streptomycin (100 mg/mL) (Invitrogen, USA). The cells were grown at 37 ℃ in a 95 % air/5 % CO_2 environment. Vegetable peptones (pea peptone, wheat peptone) were obtained from Fluka (France). Peptones used to prepare 1 % peptone medium in DMEM without serum by filtration through a 0.2 mm membrane filter. Human cord blood-derived mesenchymal stem cells (CB-MSCs) were obtained from Phochon Cha University (Korea). The cells were cultured in DMEM containing low glucose levels and supplemented with 15 % FBS, penicillin and streptomycin at 37 ℃. The medium was changed every 3 days until the cells were confluent, at which time they were passaged. Human adipose-derived stem cells (ADSCs)

were purchased from Invitrogen (USA). The cryopreserved cells were thawed at 37 ℃ and then immediately cultured in MesenPRO RSTM medium (Invitrogen, USA). The cells were then expanded using MesenPRO RSTM medium. In serum-free condition, cell culture medium was replaced with serum-free DMEM.

2.2. Cell Proliferation Assay

CB-MSCs, ADSCs and human dermal fibroblast cells were cultured in MesenPRO RSTM medium (Invitrogen, USA) or DMEM including 10 % FBS and 1x antibiotic solution, respectively. Cells were incubated at 37 ℃ in a 5 % CO_2 incubator. Cells were seeded on 24-well plates. After 24 h, cells were cultured in DMEM under serum-free conditions for 4 days in the presence of different concentrations of vegetable peptone. General viability of cultured cells was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) to formazan. MTT (1 mg/mL in PBS) was added to each well, 1/10 volume of media. Cells were incubated at 37 ℃ for 3 h, and harvested by centrifugation. After then, dimethyl sulfoxide (DMSO) (Sigma, USA) was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm with a spectrophotometer (PowerWave, Biotek Inc. USA).

2.3. Transfection and Luciferase Reporter Gene Assay

Human dermal fibroblast cells were transiently transfected with firefly luciferase reporter gene under the control of human collagen type I alpha 2 (COL1A2) responsible elements and Renilla luciferase expression vector driven by thymidine kinase promoter (Promega, USA) by superfect reagent (Invitrogen, USA)[18]. The transfected cells were transferred to 6-well plates and incubated for 24 h at a density of 8×10^5 cells/mL. After 24 h, the cells were further cultured in the presence of or absence of vegetable peptone for 10 h. Luciferase activity were determined using a Dual Luciferase Assay system (Promega, USA) and a LB953 luminometer (Berthold, Germany) and were expressed as a ratio of COL1A2-dependent firefly luciferase activity divided by control thymidine kinase Renilla luciferase activity (relative luciferase unit). Results were confirmed by three independent transfections.

2.4. Quantitative Detection of Type I Collagen

The quantity of type I collagen in the cells was determined using a commercially available kit (Takara Bio Inc., Japan). This kit is capable of detecting procollagen type I carboxy-terminal peptide using polyclonal antibodies, rather than directly measuring collagen. Human dermal fibroblast cells were then incubated in either the presence or the absence of vegetable peptone or fibroblast growth factor-2 (FGF-2) for 24 h, and then the culture supernatants were harvested and measured with a sandwich immunoassay kit, which was utilized in accordance with manufacturer's instructions (Takara Bio Inc., Japan). The measurement was performed with a microplate at 450 nm[19].

2.5. TGF-β1 Reporter Gene Assay

Human dermal fibroblasts were transfected with pMetLuc-TGF-β1 promoter[20] vector using superfect transfection reagent according to the manufacturer's instructions (QIAGEN, USA). After 24 h of transfection chemicals were treated to the culture media. Following stimulation, 50 μL of the culture media was used to measure reporter gene expression. Luciferase activity was assayed by the Ready-To-Glow™ secreted luciferase reporter assay (Clontech, USA). Fluorescence was measured using a luminometer.

2.6. TGF-β1 ELISA Assay

After incubating human dermal fibroblasts on the vegetable peptone treated dishes for 48 h, we harvested the conditioned medium and then measured the concentration of TGF-β1 by ELISA kit (Quantikine human TGF-β1 immunoassay), according to the manufacturer's instructions (R&D systems, USA).

2.7. Assessment of the Cutaneous Acceptability of the Investigational Product

To evaluate the irritation effect of vegetable pep-

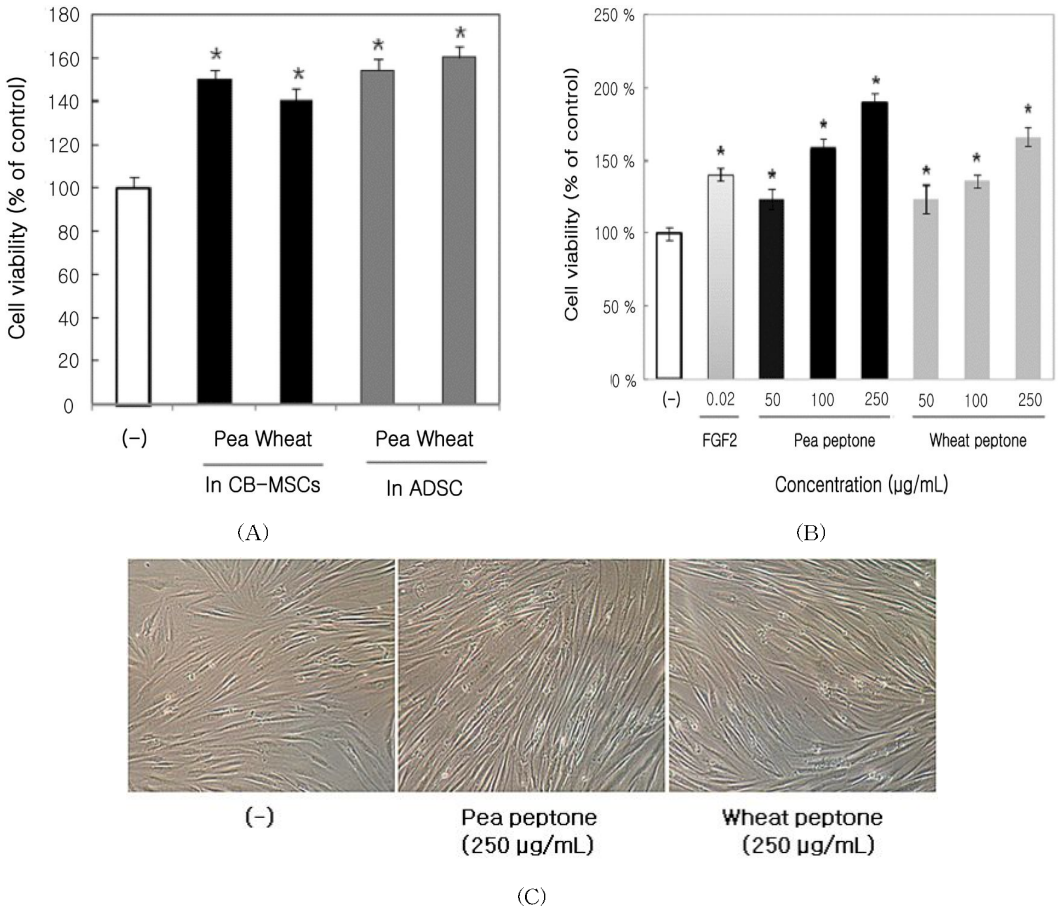


Figure 1. Effects of vegetable peptones on cell proliferation. Human cord blood-derived stem cells (CB-MSCs), human adipose-derived stem cells (ADSCs) and human dermal fibroblasts were seeded on 24-well plates. After 24 h, cells were cultured in DMEM under serum-free conditions for 4 days in the presence of different concentrations of vegetable peptones, respectively. (A and B) The cell viability was then determined by MTT assay, as described in the Materials and Methods section. Graph is a presentation of three different experiments carried out in triplicate each time. $p < 0.05$ compared with vehicle-treated control, Student's t-test, (-): untreated serum-free control. (C) Photographs of human dermal fibroblasts which were treated with vegetable peptones for 4 days, (-): untreated serum-free control.

tones for clinical applications to human skin, test product (vegetable peptone lotion 2 %) were each applied to separate target areas on the face of twenty participants twice a day (in the morning and evening) for an 4 weeks period of time. The dermatologist observed the initial state of the test area as well as after 4 weeks of treatment with the investigational product.

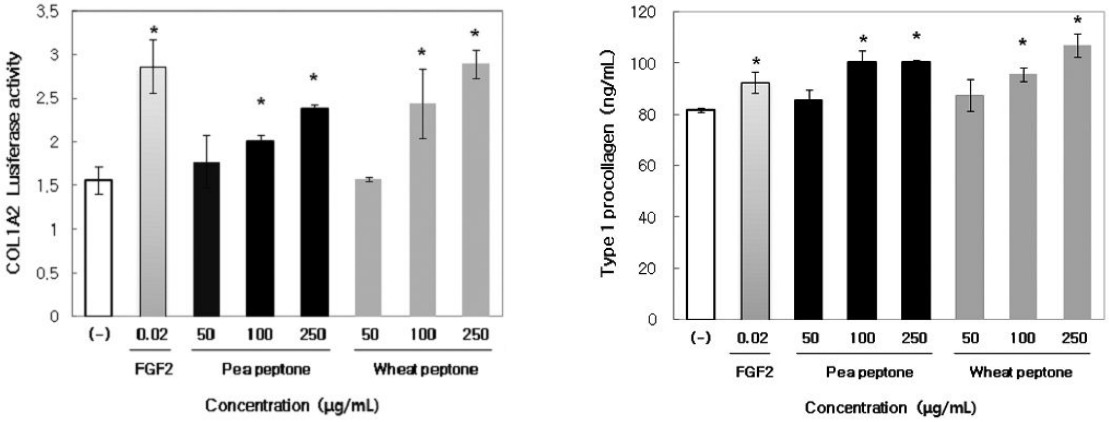
2.8. Statistics

The statistical significance of the data was determined by Student's t-test. $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Vegetable Peptones Promote Cell Proliferation of Human Dermal Fibroblast Cells and Human Adult Mesenchymal Stem Cells

In our previous study, we found that among 7 vegetable peptones (soy, wheat, broadbean, potato, pea, papaic soy, lupin), vegetable peptones derived from pea and wheat only promoted the proliferation of human CB-MSCs and ADSCs[15]. In this study, we confirmed this cell-proliferating effects of vegetable peptones on adult stem cells and then evaluated the in-



(A) (B)

Figure 2. Effects of vegetable peptones on collagen synthesis in human dermal fibroblasts. (A) Activity of COL1A2 promoter in response to vegetable peptones in transfectant human dermal fibroblasts. Transfectant human dermal fibroblasts were treated with indicated concentrations of vegetable peptones for 16 h. Cell lysate was assayed for luciferase activity. All values were significant $p < 0.05$ compared with values for untreated control, (-): untreated serum-free control. (B) Effects of vegetable peptones on type 1 procollagen production in human dermal fibroblasts. human dermal fibroblasts were treated with vegetable peptones. vegetable peptones increased type I procollagen production. Graph is a presentation of three different experiments carried out in triplicate each time. $p < 0.05$ compared with vehicle-treated control, Student's t-test, (-): untreated serum-free control.

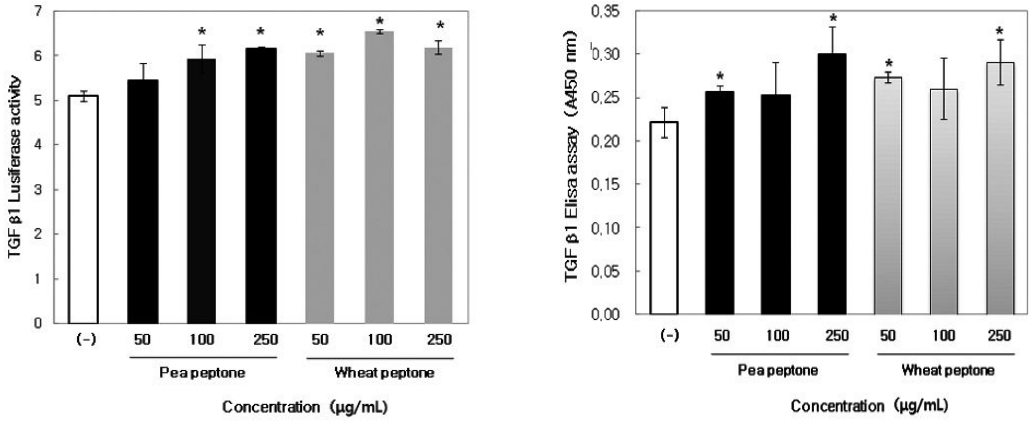


Figure 3. Effects of vegetable peptones on TGF-β pathway. (A) Activity of TGF-β1 promoter in response to vegetable peptones in transfectant human dermal fibroblasts. Transfectant human dermal fibroblasts were treated with indicated concentrations of vegetable peptones for 16 h. Cultured media was assayed for luciferase activity. All values were significant $p < 0.05$ compared with values for untreated control, (-): untreated serum-free control. (B) Effect of vegetable peptone on TGF- β1 production in human dermal fibroblasts. Vegetable peptones increased TGF-β1 production. Graph is a presentation of three different experiments carried out in triplicate each time. $p < 0.05$ compared with vehicle-treated control, Student's t-test, (-): untreated serum-free control.

volvement of peptones in the proliferation of dermal fibroblasts using an MTT assay. As shown in Figure 1A, pea peptone and wheat peptones significantly increased cell proliferation of human CB-MSCs and ADSCs. Like in adult stem cells, pea peptone and wheat peptones significantly increased the cell growth rate in

Table 1. The Result of Human Skin Irritation (n = 12)

No	Test material	4 Weeks					Reaction grade[b]	
		±	1+	2+	3+	4+	4 Weeks	Mean
1	Lotion	-	-	-	-	-	0	0
2	Lotion (Pea peptone 2 %)	-	-	-	-	-	0	0
3	Lotion (Wheat peptone 2 %)	-	-	-	-	-	0	0

a) No reaction.
b) Reaction grade = Σ[{Grade x No. of responders}/{4 (Maximum grade) × 30 (Total subjects)}] × 100 × (1/2)

dermal fibroblasts by 89 % and 66 %, respectively (Figure 1B and 1C).

3.2. Effects of Vegetable Peptones on Type I Procollagen Synthesis

Significant progress has been made in understanding the expression of COL1A2 gene and its transcriptional regulation by cytokines and growth factors. As a preliminary step to determine if vegetable peptones affect collagen production, we performed COL1A2 luciferase assay. As shown in Figure 2A, vegetable peptone increased COL1A2 reporter activity in a concentration-dependent manner. This result suggests the possibility that vegetable peptones may be involved in the production of human collogen type I alpha 2 We further studied the effect of vegetable peptones on the production of human collagen type I alpha 2 synthesis. As shown in Figure 2B, consistent with the finding in Figure 2A, vegetable peptones significantly increased the production of type I procollagen dose-dependently, confirming that vegetable peptones induce production of type I procollagen and also suggesting the collagen production-inducing function of vegetable peptones through activation of COL1A2 promoter. FGF2 was employed as a positive control.

3.3. Effects of Vegetable Peptones on TGF-β Pathway

We further studied the effect of vegetable peptones on the expression of TGF-β1. As shown in Figure 3A, vegetable peptones increased TGF-β1 reporter activity in a concentration-dependent manner. This result suggests the possibility that vegetable peptone may be involved in the production of human TGF-β1. As shown in Figure 3B, consistent with the finding in Figure 3A, vegetable peptones significantly increased the production of TGF-β1 dose-dependently, confirming that vegetable peptones induce production of TGF-β1.

3.4. Assessment of the Cutaneous Acceptability of the Investigational Product

To evaluate the irritation effect of vegetable peptones for clinical applications to human skin, test product (vegetable peptone lotion 2 %) were each applied to separate target areas on the face of twenty participants for a total treatment duration of 4 weeks. The dermatologist assessed physical and objective signs and any other symptom linked to the investigational product applications. There was no any advers events from finished subject of the study (Table 1). Globally, no abnormal clinical sign linked to the investigational product was noted, by the dermatologist evaluation, after the 4 weeks of treatment. This result suggests that vegetable peptones are safe to use and might be introduced as a possible anti-wrinkle agent for the management of skin ageing.

4. Conclusion

In conclusion, the data acquired in this study demonstrate that vegetable peptones can increase cell proliferation and synthesis of type I collagen in dermal fibroblasts, and is safe to use. These results suggest that vegetable peptones might be introduced as a possible anti-wrinkle agent for the management of skin ageing.

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