J. Soc. Cosmet. Scientists Korea
Vol. 30, No. 3, November 2004, 393-397

L-카르니틴의 사람피부에 대한 항노화 효과

이 범 천† · 최 태 부* · 심 관 섭 · 이 근 수 · 박 성 민 · 이 천 일 · 표 형 배

한불화장품(주) 기술연구소, *건국대학교 생물화학공학부

Anti-aging Effects of L-Carnitine on Human Skin

Bum-Chun Lee†, Tae-Boo Choe*, Gwan-Sub Sim, Geun-Soo Lee, Sung-Min Park, Chun-Il Lee, and Hyeong-Bae Pyo

R&D Center, Hanbul Cosmetics Co., 72-7, Yongsung-ri, Samsung-myun, Umsung-kun, Chungbuk 369-830, Korea
*Section of Chemical and Biological Engineering, Konkuk University

요 약: L-Carnitine (β-hydroxy-γ-trimethyl-ammoniumbutyric acid)은 분자량이 적은 수용성 분자로서 세포 내 지방 대사에서 중요한 역할을 수행한다. 지방산의 운반 분자인 아실-코에이(acyl-CoA)가 미토콘드리아의 세포막을 투과하지 못하기 때문에 지방산은 CoA로부터 카르니틴으로 운반되어 미토콘드리아에서 작용한다. 노화와 연관된 L-carnitine의 기능을 확인하기 위하여 MMP inhibition assay와 자외선 조사에 의해 유도된 MMP 발현에 대한 영향을 확인하였다. MMP inhibition assay는 콜라겐을 이용한 형광분석법을 실시하였고 자외선 조사에 의해 유도된 MMP 발현양은 ELISA로 정량하였으며 그 활성은 젤라틴 기질 zymography로 확인하였고 MMP mRNA 발현양은 RT-PCR ELISA로 확인하였다. 또한, 사람을 이용한 임상 실험을 통하여 주름 개선 효과를 평가하였다. L-carnitine은 농도 의존적으로 MMP 저해 활성을 나타났으며 IC_{50}값은 2.45 mM이었으며 자외선 조사에 의해 발현된 MMP 활성을 강하게 저해하였다. 자외선 조사에 의해 발현되는 MMP에 대해 단백질의 양적인 변화는 40% 정도 감소되었으며 L-carnitine 처리에 의해 농도 의존적으로 MMP mRNA의 발현양은 감소되었다. 이러한 실험결과를 통하여 L-carnitine은 MMP 효소의 저해능 뿐만 아니라 자외선 조사에 의해 유도되는 MMP 단백질 발현과 mRNA 유전자 수준에서의 조절이 가능함을 확인하였다. 사람을 이용한 임상 실험에서는 1% 카르니틴을 함유하는 화장품을 약 3개월간 사용 후에는 유의적으로 주름 개선 효과를 확인하였다. 결론적으로 L-carnitine은 광노화에 관여하는 MMP 활성과 발현 조절 메커니즘을 통하여 광손상에 대응하는 항노화 소재로서의 화장품에 매우 효과적이었음을 확인하였다.

Abstract: L-Carnitine (β-hydroxy-γ-trimethyl-ammoniumbutyric acid) is a small water-soluble molecule important in mammalian fat metabolism. It is essential for the normal oxidation of fatty acids by the mitochondria, and is involved in the trans-esterification and excretion of acyl-CoA esters. In this paper, to investigate the relationship between aging and L-carnitine, we investigated the effects of *in vitro* matrix-metalloproteinase (MMP) inhibition and activity and expression of UVA-induced MMPs in human skin fibroblasts. Also, we studied to develop as anti-aging cosmetics with L-carnitine. Fluorometric assays of the proteolytic activities of MMP-1 (collagenase) were performed using fluorescent collagen substrates. ELISA (enzyme linked immuno sorbent assay), gelatin-substrate zymography, RT-PCR ELISA techniques were used for the effects of L-carnitine on MMP expression, activity, and MMP mRNA expression in UVA irradiated fibroblast (5 J/cm^2), respectively. In addition, we performed clinical study with L-carnitine cream. L-carnitine inhibited the activities of MMP-1 in a dose-dependent manner and the IC_{50} values calculated from semi-log plots were 2.45 mM, and L-carnitine showed strong inhibition on MMP-2 (gelatinase) activity in UVA irradiated fibroblast by zymography. Also, UVA induced MMP-1, 2 expression was reduced 43%, 53% by treated with L-carnitine at 1.25 mM, and MMP-1 mRNA expression was reduced dose-dependent manner. Therefore L-carnitine was able to significantly inhibit the MMP activity, and regulate MMP expression in protein and mRNA level. The results of clinical study showed that 1.0% L-carnitine treated group reduced wrinkle significantly compared with placebo treated group ($P < 0.05$). All these results suggest that L-carnitine may be useful as new anti-aging cosmetics for protection against UVA induced MMP expression and activity.

Keywords: *L-carnitine, MMP, anti-aging, cosmeceuticals, human dermal fibroblast*

† 주 저자 (e-mail: bclee@hanbul.co.kr)

1. Introduction

Skin aging is influenced by several factors, including genetics, environmental exposure (ultraviolet (UV) irradiation, xenobiotics, and mechanical stress), hormonal changes, and metabolic processes (generation of reactive chemical compounds such as activated oxygen species, sugars, and aldehydes)[1]. UVA radiation plays a major role in altering the dermis and activating a family of degradative enzymes called matrix metalloproteinase (MMPs). These enzymes target the components of the extracellular matrix (ECM) such as collagen, laminin, fibronectin, and proteoglycan[2]. The expression of MMPs in UV irradiated fibroblasts is known to be initiated by reactive oxygen species and by activation of cell surface growth factor and cytokine receptors. These stimulates mitogen-activated protein (MAP) kinase signal transduction pathways and transcription factor AP-1. Among genes whose expression is regulated by AP-1 are several MMP family members and type 1 procollagen[3]. MMPs inhibitors or regulators represent new and interesting tools that the cosmetic formulation chemist can use to protect the skin against environmental insults. L-carnitine (β-hydroxy-γ-trimethyl-ammoniumbutyric acid) is a small water-soluble cofactor and it's main cellular functions are participation in the transport of long-chain fatty acids into the mitochondrial matrix for β-oxidation to provide cellular energy and especially intra-mitochondrial acyl-CoA: CoA ratio. It did not become known well that the effect of the L-carnitine on the production of MMPs and skin aging. In this study, we evaluated the effects of L-carntine on MMPs from human dermal fibroblasts (HDFs) irradiated with UVA and applied the anti-wrinkle cosmetic formulation.

2. Materials and Methods

2.1. Reagents

L-Carnitine, anti-MMP-1 antibody (Ab-5), anti-mouse IgG conjugated with alkaline phosphatase was purchased from Sigma chemical Co. (St. Louis, MO, USA). Anti-MMP-2 antibody (Ab-3) was obtained from Calbiochem. Collagenase inhibitory assay kit was from molecular probes (Eugene, OR, USA).

2.2. Collagenase Inhibition Assays

The MMP-1 activity assay, which is based upon fluorescence measurement of collagen fragments upon cleavage by MMP-1, was performed according to the manufacturer's protocol. The enzymes were mixed with quenched fluorescent substrates (0.2 μg/mL) in a final volume of 200 μL reaction buffer in 96-well microplates. Digested products from DQ collagen substrates have absorption maxima at ~495 nm and fluorescence emission maxima at ~515 nm.

2.3. Culture of Human Dermal Fibroblast and UVA Irradiation

Human dermal fibroblasts (HDFs) were acquired from Korea Cancer Center Hospital. HDFs were maintained in Dulbecco's Modified Eagle's Media (DMEM) with 10% FBS and kept in a humidified 5% CO_2 atmosphere at 37℃. HDFs from passage 6 to 10 were used in the experiments. HDFs (1.5×10^5/well) were seeded into 35 ϕ plates and cultured overnight. The cells were irradiated from a distance of 15 cm by a UV source (UVA simulator, Jhonsam, KOREA) emitting wavelengths in the range of 340~450 nm.

2.4. Zymography

Zymography in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.15% gelatin was performed according to the method of Demeule *et al.*[4]. The samples were mixed with SDS sample buffer in the absence of reducing agent, incubated at 37℃ for 20 min, and electrophoresed on 10% polyacrylamide gels at 4℃. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1 h to remove SDS and incubated for 12 h at 37℃ in 50 mM Tris-HCl, pH 7.6, 0.15 NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 and then stained with 0.1% Coomassie Brilliant Blue R250.

2.5. Determination of MMP-1 and -2 by ELISA

HDFs (8×10^3/well) were seeded into 96-well plates and cultured overnight. The culture media were replaced with DMEM containing L-carnitine. After 24 h incubation, the supernatants were transferred into a 96 well plate and the coating buffer (Na_2CO_3 1.59%, $NaHCO_3$ 2.93%, NaN_3 0.20%, $MgCl_2$ 1.02%, pH 9.6) was added 1 : 1 (v/v) and incubated for 12 h. The expres-

sion of MMP-1 and -2 was assayed by enzyme-linked immunosorbent assay (ELISA).

2.6. RT-PCR-ELISA

Total RNA was extracted from cultured cells using the Promega RNAgent Total RNA isolation System. PCR amplification is carried out using 5′ biotinylated primers (sense) to generate biotinylated PCR products detectable by digoxygenin-labelled probes in an ELISA method[5,6]. The primers used were as follows; MMP-1 sense primer, 5′-AAAGGGAATAAGTACTGGGC-3′ and antisense primer, 5′-AATTCCAGGAAAGTCAT-GTG-3′; β-actin sense primer, 5′-CGAGCTGCCTGA-CGGCCAGG-3′ and antisense primer, 5′-ATTTGCG-GTGGACGATGGAG-3′.

2.7. Clinical Study of L-Carnitine on Facial Wrinkle

In total 50 healthy woman volunteers (25 in the 1.0% L-carnitine treatment group and 25 in the placebo group) were studied. Volunteers were asked to apply the cream to the entire face twice a day for 3 months. A silicone replica of the crow's feet area was taken at baseline and at weeks 12. Skin replicas were then analyzed by skin visiometer SV 600. Standard wrinkle and roughness were calculated on these profiles, as follows- R1 (Skin roughness), R2 (average roughness), R3 (maximum roughness), R4 (smoothness depth), R5 (arithmetic average roughness) - the features were then calculated and statistically analyzed.

2.8. Statistical Analysis

Results were presented as means±standard error (SE). Experimental results were statistically analyzed by using Student's t-test (SigmaPlot 2000). P values <0.05 were regarded as indicating significant differences.

3. Results and Discussion

3.1. Collagenase Inhibition Assays

Collagenase activity were measured using fluorescein-conjugated collagen as substrate and collagenase purified from *Clostridium histolyticum* is provided with the assay kit to serve as a control enzyme. L-carnitine caused the strongest inhibition of collagenase activity, producing an inhibition of 80% at 5 mM (Figure 1).

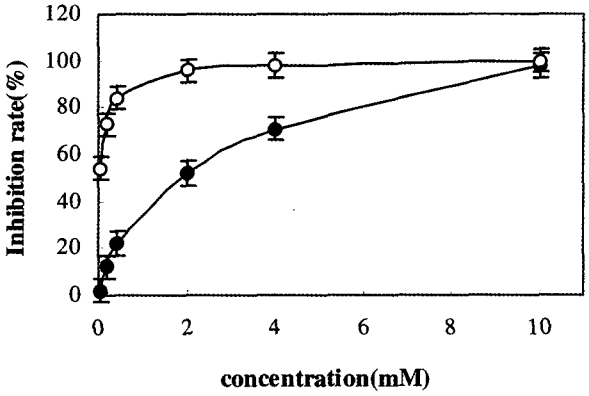

Figure 1. Determination of IC_{50} values for the inhibition of MMP-1. Fluorometric assays of the activities of MMP-1 was performed in the presence of increasing concentrations of L-carnitine (●) and 1,10-phenanthroline (○).

The activities of MMP-1 were inhibited in a dose-dependent manner. The IC_{50} values calculated from semi-log were 2.5 mM, and 1,10-phenanthroline is 0.05 mM for MMP-1.

3.2. Zymography of L-Carnitine on MMPs from UVA Irradiated HDFs

The effects of L-carnitine on MMP-2 (gelatinase) activities were confirmed by zymography using supernatant from UVA irradiated HDFs (Figure 2). The gels were regenerated, washed and incubated (18 h) with or without of L-carnitine (0.625, 2.5, 5 mM) or 1,10-phenanthroline (0.4 mM) in the incubation buffer. When treatment of 2.5 mM above concentration were added to the incubation buffer, the gelatinolytic activities of MMP were strongly inhibited like as a 1,10-phenanthroline. These data indicated that L-carnitine showed not only the inhibitory activity of MMP-1 (collagenase) but also MMP-2 (gelatinase) from UVA irradiated HDFs.

3.3. Effect of L-Carnitine on the Production and Activity of MMPs

To estimate the effect of L-carnitine on MMP expression from UVA irradiated HDFs, ELISA were used to quantify protein respectively for MMP-1 and 2 in the culture medium of HDFs and their gelatinase activity were confirmed by gelatin-substrate zymography. We found that MMP-1 and MMP-2 protein production in the supernatants of HDFs was increased 2.5-fold

Figure 2. The effect of L-carnitine on MMP-2 (gelatinase) activity from UVA irradiated human dermal fibroblasts. Gelatin zymography was performed on MMP-2 in the presence of L-carnitine and 1,10-phenanthroline. 1. 5 J/cm^2 UVA irradiated MMP, 2. L-carnitine 0.625 mM, 3. 2.5 mM, 4. 5 mM, 5. 0.4 mM 1,10-phenanthroline.

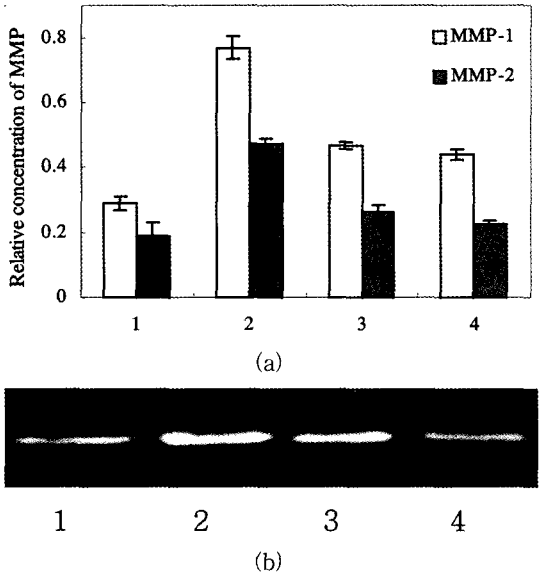

Figure 3. The effect of L-carnitine on the production (a) and activity (b) of MMP-1 and MMP-2 by human dermal fibroblasts. 1. non irradiated, 2. 5 J/cm^2 UVA irradiated, 3. UVA + L-carnitine 2.5 mM, 4. UVA + L-carnitine 1.25 mM.

over control levels (Figure 3(a), Lane 1, 2) and also the gelatinase activities were proportionally increased with the UV irradiation of HDFs using gelatin zymography. To determine whether L-carnitine could modulate the production of MMP-1 by irradiated HDFs, L-carnitine was applied for 24 h after UVA irradiation to the cells. In the presence of added L-carnitine at 1.25 mM, MMP-1 and MMP-2 production were decreased 43, 53% compared with UVA irradiated cells (Lane 4). Also we found that gelatinase activity is reduced by added L-carnitine (Figure 3(b)). We confirmed that L-carnitine regulate protein synthesis and activity of MMP from UVA irradiated HDFs.

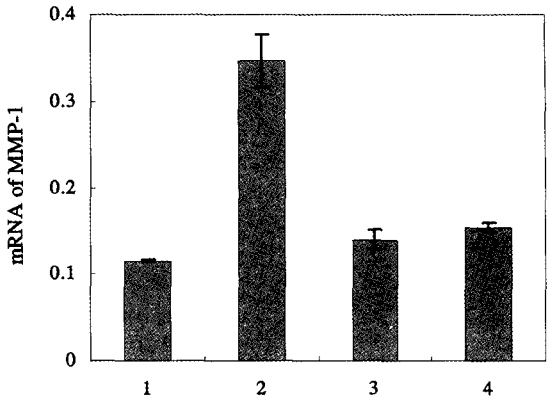

Figure 4. The effect of L-carnitine on the production of MMP mRNA from UVA irradiated human dermal fibroblasts. 1. non-irradiated, 2. 5 J/cm^2 UVA irradiated, 3. UVA + L-carnitine 10 mM, 4. UVA + L-carnitine 5 mM.

3.4. The Effect of L-Carnitine on the Production of MMP mRNA

After irradiated UVA, HDFs were incubated with the L-carnitine. UVA irradiation of fibroblasts cells led to 3-fold rise in MMP-1 mRNA expression (Figure 4 Lane 2), which was reduced by 1.3-fold in the presence of L-carnitine (5 mM) (Figure 4. Lane 4). As the MMP-1 mRNA levels are known to be correlated with MMP-1 protein expression, reduction of MMP-1 mRNA expression would be expected to be an inhibition of MMP-1 protein production. We found that MMP mRNA and protein expression from UVA irradiated human dermal fibroblast is regulated by L-carnitine. And further studies are required to investigate the fine signaling pathways affected by L-carnitine.

3.5. Wrinkle Reduction of L-Carnitine

Topically applied L-carnitine prevented UV-induced MMP-mediated collagen destruction, therefore we investigated 3 months pilot study to determine the efficacy and safety of L-carnitine on facial wrinkle in comparison with vehicle lacking L-carnitine. Volunteers were asked to apply the cream to the entire face twice a day for 3 months. L-carnitine showed significant decrements of two skin roughness parameters (R2, R3) compared with placebo emulsion (Figure 5). No subjects dropped out of the study because of adverse events. These data suggest that L-carnitine significantly decreased facial wrinkle compared with vehicle alone after 3 months of use.

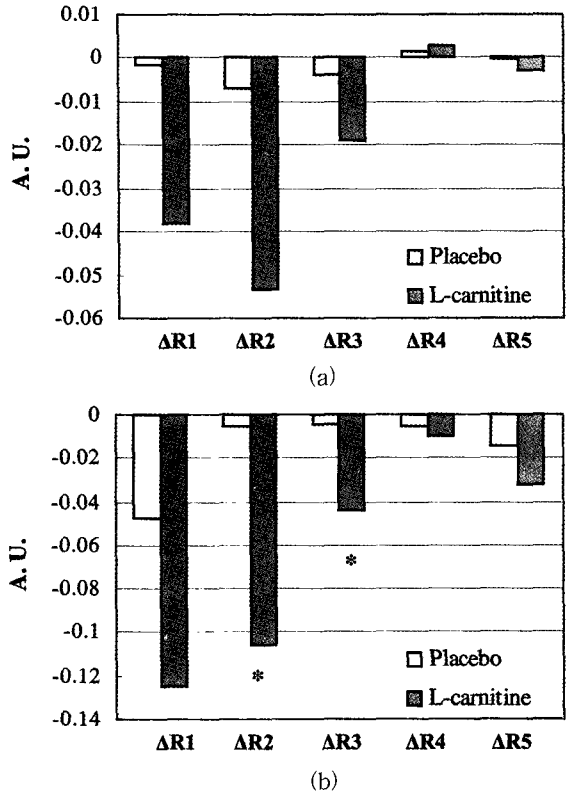

Figure 5. Comparison of the volunteer's eye area before and after the treatment of the o/w emulsion containing L-carnitine (*, P<0.05). (a) The wrinkle index (ΔR1 ~ ΔR5) of the volunteer's eye area 8 weeks after the treatment. (b) The wrinkle index (ΔR1 ~ ΔR5) of the volunteer's eye area 12 weeks after the treatment.

4. Conclusions

In this study, L-carnitine showed effectively inhibition on MMP-1 (collagenase) and MMP-2 (gelatinase) human dermal fibroblasts. MMP-1 and MMP-2 production were decreased 43, 53% compared with UVA irradiated cells by L-carnitine. Also gelatinase activity is reduced by added L-carnitine. UVA irradiation of fibroblasts cells led to an 3-fold rise in MMP-1 mRNA expression, which was reduced by 1.3-fold in the presence of L-carnitine (5 mM). We concluded that the expression of MMP-1 and -2 by the UV irradiated HDF is regulated by L-carnitine. Clinical study showed that 1.0% L-carnitine treated group reduces wrinkle significantly compared with placebo treated group ($P < 0.05$). All these results suggest that L-carnitine may be useful as new anti-wrinkle cosmetics for protection against UVA induced MMP expression and activity.

References

1. L. Rittie and G. J. Fisher, UV-light-induced signal cascades and skin aging, *Aging. Research. Reviews*, **1**, 705 (2002).
2. A. Vanella, A. Russo, R. Acquaviva, and A. Campisi, UVA irradiation induces collagenase in human dermal fibroblasts *in vitro* and *in vivo*, *Arch. Dermatol. Res.*, **283**, 509 (1999).
3. G. J. Fisher, H. S. Talwar, J. Lin, P. Lin, F. McPhillips, Z. Wang, X. Li, Y. Wan, S. W. Kang, and J. J. Voorhees, Retinoic acid inhibits induction of c-Jun protein by ultraviolet radiation that occurs subsequent to activation of mitogen-activated protein kinase pathways in human skin *in vivo*, *J. Clin. Invest.*, **101**, 1432 (1998).
4. M. Demeule, M. Brossard, M. Page, D. Gingras, and R. Beliveau, Matrix metalloproteinase inhibition by green tea catechins, *Biochimica et Biophysica Acta*, **1478**, 51 (2000).
5. S. Lauwers, Y. Vander Heyden, and B. Rombaut, Screening of an enterovirus specific RT-PCR ELISA method for the quantification of enterovirus genomes in human body fluids by means of a three-level experimental design, *J. Pharmaceutical and biomedical analysis*, **25**, 131 (2001).
6. T. B. Choe, B. C. Lee, I. C. Park, and S. K. Hong, Inhibition of matrix metalloproteinase-1 and 2 expression using nitric oxide synthase inhibitors in the UV irradiated human dermal fibroblasts, *J. Cosmetic Science*, **54**, 229 (2003).