# The effect of L-carnitine in the expression of matrix metalloproteinases by human dermal fibroblasts

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Abstracts

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* MMP inhibition and activity and expression of UVA-induced MMP 1 in human skin fibroblasts. Fluorometric assays of the proteolytic activities of MMP-1 were performed using fluorescent collagen substrates. ELISA (enzyme linked immuno sorbent assay), gelatin-substrate zymography, and RT-PCR ELISA techniques were used for the effects of L-carnitine on MMP expression and activity, MMP mRNA expression in UVA irradiated fibroblast. L-carnitine inhibited the activities of MMP-1 in a dose-dependent manner and the $IC_{50}$ values calculated from semi-log plots were 2.45mM, and L-carnitine showed strong inhibition on MMP-2 (gelatinase) activity in UVA irradiated fibroblast by zymography. Also, UVA induced MMP expression was reduced 40% by treated with L-carnitine, and MMP-1 mRNA expression was reduced dose-dependent manner. Therefore L-carnitine was able to significantly inhibition the MMP activity, regulation of MMP expression in protein and mRNA level. All these results suggest that L-carnitine may be useful as new anti-aging cofactor for protection against UVA induced MMP expression and activity.

## INTRODUCTION

Skin aging is influenced by several factors, including genetics, environmental

exposure (ultraviolet (UV) irradiation, xenobiotics, mechanical stress), hormonal changes, and metabolic processes (generation of reactive chemical compounds such as activated oxygen species, sugars, and aldehydes) (1). Especially besides of many beneficial effects including vitamin D metabolism and melanin synthesis, solar ultraviolet (UV) irradiation also gives notorious effects leading to skin erythema, skin aging including premature wrinkle formation and ultimately skin carcinogenesis (2). Thus, study concerned about UV-irradiated and chronologically aged human skin were increased and UV-induced skin aging seems to be provides help in development of new clinical strategies to impede chronological aging. It is well known that a decrease in collagen is shown with photoaging of human skin. Collagen, the predominant component of dermal connective tissue, is biosynthesized by dermal fibroblasts. 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 (3-6). In the first, MMP-1 is interstitial collagenase that degrades type 1 and 3 fibrillar collagens into specific N-terminal (three-quarters) and C-termianl (one-quarter) length fragments which are then susceptible to further hydroysis by other MMPs such as MMP-2 and MMP-9. And MMP-2 is gelatinase that degrades denatured collagens and elastin, especially basal layer between epidermis and dermis. Therefore, MMP-1 and MMP-2, which can be produced by both epidermal keratinocytes and dermal fibroblasts, appears to play a key role in dermal remodeling (7). 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 matrix-metalloproteinase (MMP) family members and type 1 procollagen (8-11). The activity of these enzymes is controlled by tissue inhibitor metalloproteinase (TIMP). However, the biological equilibrium existing between MMP and TIMP can be destroyed by UV radiation, hormonal imbalances, local inflammation and normal aging processes (12, 13). Matrix metalloproteinase inhibitors or regulators represent new and interesting tools that the cosmetic formulation chemist can use to protect the skin against environmental insults. L-carnitine ($\beta$-hydroxy-$\gamma$-trimethyl-ammoniumbutyric acid)is a small water-soluble cofactor molecules and it's main cellular functions are

participation in the transport of long-chain fatty acids into the mitochonderial matrix for β -oxidation to provide cellular energy and especially intra-mitochondrial acyl-CoA: CoA ratio. The modulation of the cellular and especially intra-mitochondrial 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 (14). Recently, it has been reported that L-carnitine alters nitric oxide synthase activity in fibroblasts depending on the peroxisomal status and L-propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector (15). But, 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. Our results suggest that it is possible to apply L-carnitine for a new anti-aging skin care.

## MATERIALS AND METHODS

### *Reagents*

L-carnitine, biotin, myo-inositol, 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 Cal- biochem. Collagenase inhibitory assay kit was from molecular probes (Eugene, OR, USA). Green tea extract was obtained from Bioland Co.(Chungnam, KOREA)

### *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 (0.2ug/ml) in a final volume of 200ul reaction buffer in 96-well microplates. The enzymatic assays were optimized with 0.1 units of MMP-1. Digested products from DQ collagen substrates have absorption maxima at ~495nm and fluorescence emission maxima at ~ 515nm. For all the MMPs tested the activities under these conditions were linear for at least

15min. For each time point, correct for background fluorescence by subtracting the values derived from the no-enzyme control.

*Culture of Human dermal fibroblast*

HDFs from new born foreskin 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.

*Cell treatment and UVA irradiation*

HDFs ($1.5\times10^5$/well) were seeded into 35ø plates and cultured overnight. Prior to UV irradiation, the cells were washed twice with phosphate buffered saline (PBS). 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. UVA irradiation doses were 5J/$cm^2$ and the radiation intensity was measured using UV radiometer (EKO, JAPAN). The culture media, DMEM without serum, containing the L-carnitine was immediately added to the cells (concentrations and times of incubation are described in the results).

*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 (16). The samples were mixed with SDS sample buffer in the absence of reducing agent, incubated at 37℃ for 20min, and electrophoresed on 10% polyacrylamide gels at 4℃. After electrophoresis, the gels were washed in 2.5% Triton X-100 for 1hr to remove SDS and incubated for 12hr at 37℃ in 50mM Tris-HCl, pH 7.6, 0.15 NaCl, 10mM $CaCl_2$, 0.02% $NaN_3$ and then stained with 0.1% Coomassie Brilliant Blue R250.

*Determination of MMP-1 and-2 by ELISA*

The expression of MMP-1 and -2 was assayed by enzyme-linked immunosorbent assay (ELISA). HDFs ($8\times10^3$/well) were seeded into 96-well plates and cultured overnight. The culture media were replaced with DMEM containing L-carnitine. After 24 hours' 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 hours. The supernatants were removed and the coated well was washed with PBS-T for 3 times and followed by blocking with 5% skim milk in PBS for 1 hour at 37℃. After washing 3 times with PBS containing 0.05% Tween 20 (PBST), 50㎕ of 1/1000 diluted primary antibody, Ab-5 or Ab-3 in PBST were added into each well and incubated for 40 min. Washing the wells with PBST 3 times, 50㎕ of 1/1000 diluted secondary Ab, anti-mouse IgG conjugated with alkaline phosphatase in PBST was added and incubated for 40 min. After 5 times washing with PBST, 100㎕ of 1mg/ml pNPP (p-nitrophenyl phosphate) in diethanolamine buffer was added. The optical density was measured at 405nm after 30 min. Cytotoxicity of supplemented chemicals was measured by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

*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 immunoenzymatic assay (ELISA) method (17). Firstly, cDNA was mixed with 10× buffer, 10mM dNTPs, 1U Taq DNA polymerase (Bioneer, Korea) and MMP-1, primers in a final volume of 50㎕. The primer sequences and relative predicted PCR product sizes are given below.

| | |
|---|---|
| MMP1 | 5'-AAAGGGAATAAGTACTGGGC-3' (sense) |
| | 5'-AATTCCAGGAAAGTCATGTG-3' (antisense) |
| β-actin | 5'-CGAGCTGCCTGACGGCCAGG-3' (sense) |
| | 5'-ATTTGCGGTGGACGATGGAG-3' (antisense) |

A sample containing all reaction reagents except cDNA was used as PCR negative control in any amplification. The mixtures were incubated for the indicated cycles

(predenaturation 5min at 95℃; denaturation 50sec at 95℃; annealing 20sec at 56℃; extension 20sec at 72℃) in a GeneAmp PCR System 2400 (Perkin-Elmer, USA). The correct size of all PCR products was confirmed by comparing with a DNA standard on agarose gel. After a given cycle of PCR, the amount of amplified cDNA was determined by the ELISA method. Firstly, microplates were coated with 50㎍/㎖ of avidin in coating buffer (CB; 15mM $Na_2CO_3$, pH 9.6), and incubated 2hr at 37℃. After incubation, free sites were saturated with 2% blocking solution in CB. Biotinylated PCR products diluted in PBS containing 3% bovine serum albumin (PBSB) were distributed onto microplates (100㎕ per well) and incubated 1h at room temperature. After incubation the microplates were washed three times with PBST. Amplified cDNA was denatured using 0.25M NaOH at room temperature for 10min. Following the washing, 100㎕ per well of 10pmol/㎖ digoxygenin-labelled probes in hybridization buffer [6.25× SSC, 0.625% Blocking reagent, 0.125% Tween 20, 0.5M $NaH_2PO_4$(pH 6.5)] were added and incubated at 42℃ for 2hr. Anti-digoxygenin AP-conjugated antibody was added (1:3000 in PBSB) and incubated 1hr at 37℃. The reaction was developed by nitrophenyl phosphate (pNPP; 1M diethanolamine buffer, pH 9.6). The amount of amplified products was measured as optical density at 405nm using a microplate reader.

*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.

## RESULTS AND DISCUSSION

### *In-vitro* 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. 1,10-phenanthroline used as a control to optimize conditions for screening potential gelatinase or collagenase inhibitors. We measured collagenase

inhibitory of some soluble cofactors as a new MMP inhibitor. Among soluble cofactor tested, L-carnitine caused the strongest inhibition of collagenase activity, producing an inhibition of 80% at 5mM (Fig. 1). But biotin and myo-inositol were not shown inhibition of collagenase. 1, 10-phenanthroline is known as the metal chelator and general inhibitor of metalloproteinase and green tea catechin showed the inhibition of matrix metalloproteinase (18,16). We found that 1,10-phenanthroline at 0.4mM effectively inhibits the activity of *Clostridium* collagenase and MMP inhibitory activity of green tea extract at 94 % at 0.02%(w/v). But relatively little is known about the inhibitory activity of L-carnitine on MMPs. Only L-propionyl-carnitine as superoxide scavenger, antioxidant, and DNA cleavage protector was showed by Vnella et al. Collagenase activity were measured in the presence of increasing concentrations of L-carnitine to estimate $IC_{50}$ value for MMP-1(Fig. 2). The activities of MMP-1 were inhibited in a dose-dependent manner. The $IC_{50}$ values calculated from semi-log were 2.5mM, and 1,10-phenanthroline is 0.05mM for MMP-1. L-carnitine showed less effectively inhibition of MMP-1 than 1,10-phenanthroline, but the above data indicate that L-carnitine is a new MMP inhibitor as a soluble cofactor.

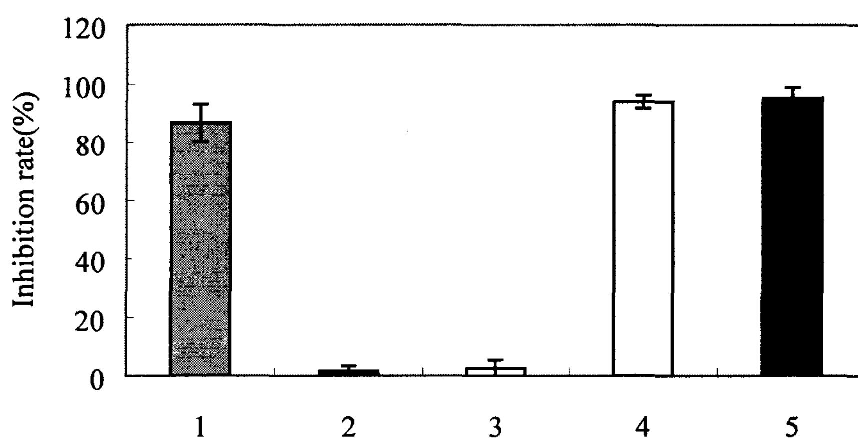


Fig. 1. The effect of L-carnitine on MMP-1 activities. Collagenase activity were measured using a fluorometric assay in the presence of inhibitor and compared with no inhibitor control. 1 - 5mM L-carnitine, 2 - 5mM Biotin, 3 - 5mM myo-inositol, 4 - 0.02% (v/v) green tea extract, 5 - 0.4mM 1,10-phenanthroline

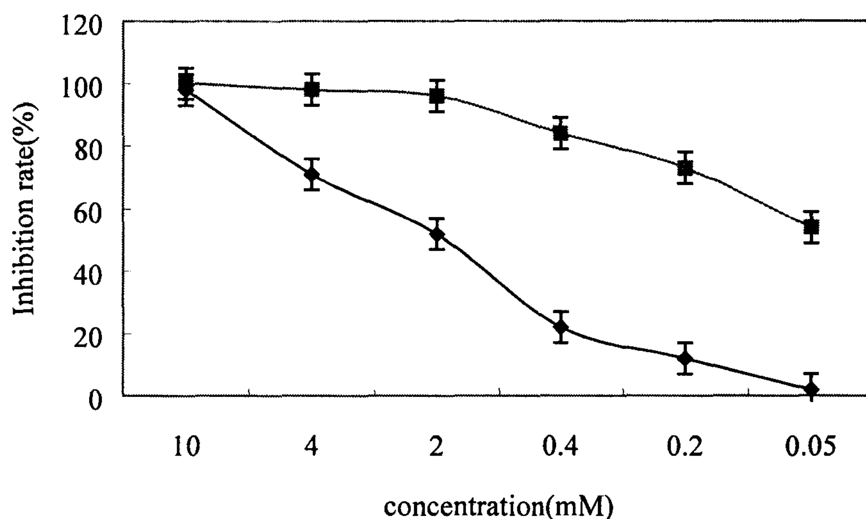


Fig. 2. Determination of $IC_{50}$ values for the inhibition of MMP-1. Fluorometric assays of the activities of MMP-1 was performed in the presence of increasing concentrations of L-carnitine (◆) and 1,10-phenanthroline (□).

**Zymography of L-carnitine on MMPs from UVA irradiated HDFs**

Also, The effects of L-carnitine on MMP-2 gelatinase activities were confirmed by zymography using supernatant from UVA irradiated human dermal fibroblasts (Fig. 3). MMPs, the supernatant from UVA irradiated HDFs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gel containing 0.15% gelatin. The gels were regenerated, washed and incubated (18hr) with or without of L-carnitine (0.625, 2.5, 5mM) or 1,10-phenanthroline (0.4mM) in the incubation buffer. When treatment of 2.5mM 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 collagenase from *C. histolyticum* but also MMP-2 (gelatinase) from UVA irradiated human dermal fibroblasts.

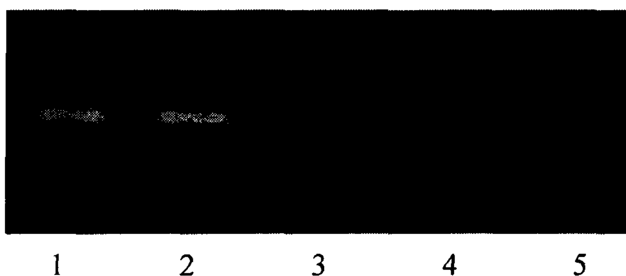


Fig. 3. The effect of L-carnitine on MMP-2 gelatinase activity from UVA irradiated human dermal fibroblasts. Gelatin zymography was performed on MMP-2 in the presence of L-carnitine and 1,10-phenanthroline. 1. 4.2J/cm$^2$ UVA irradiated MMP, 2. L-carnitine 0.625mM, 3. 2.5mM, 4. 5mM, 5. 0.4mM 1,10-phenanthroline.

**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, Enzyme-linked immunosorbent assay (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. It is well documented that UVA irradiation stimulates the production of interstitial collagenase and gelatinase mRNA and protein in cultured HDFs (4,19,20). Consistent with these findings, we found that MMP-1 and MMP-2 protein production in the supernatants of HDFs was increased 2.5-fold over control levels (Fig. 4. (A), Lane 1.2) and also the gelatinase activities were proportionally increased with the UV irradiation of HDFs using gelatin zymography. But At higher UVA-doses, a decrease in secreted MMP-1 was observed as a result of cytotoxic effects of the UVA radiation (data not shown). To determine whether L-carnitine could modulate the production of MMP-1 by irradiated HDFs, L-carnitine was applied for 24hr after UVA irradiation to the cells. In the presence of added L-carnitine at 1.25mM, MMP-1 and MMP-2 production were decreased 43, 53% compared with UVA irradiated cells (Lane 4). But the reduction of MMP by L-carnitine was not shown in a dose dependent. Above super-physiological concentration (2.5mM), the reduction of MMP was slightly decreased (Lane 3). Thus, The physiological concentration of L-carnitine in cellular status on MMP regulation is seem to be 1.25mM. Also we found that gelatinase activity is reduced by

added L-carnitine (Fig. 4. (B)). These findings indicate that L-carnitine was able to regulate MMP production and activity. Recently Fisher *et al.* showed that all-trans retinoic acid(tRA) acts to inhibit induction of c-Jun protein by ultraviolet irradiation (6), thereby preventing increased matrix metalloproteinases. Also, Roberta *et al.* showed that age-dependent increase of collagenase expression can be reduced by α-tocopherol via protein kinase C (21). MMP activity is regulated at three levels : synthesis (primarily transcription), activation of the zymogen, and inhibition of proteolytic activity, by specific endogenous protein inhibitors called tissue inhibitor of metalloproteinases (TIMPs). We confirmed that L-carnitine regulate protein synthesis and activity of MMP from UVA irradiated HDFs.

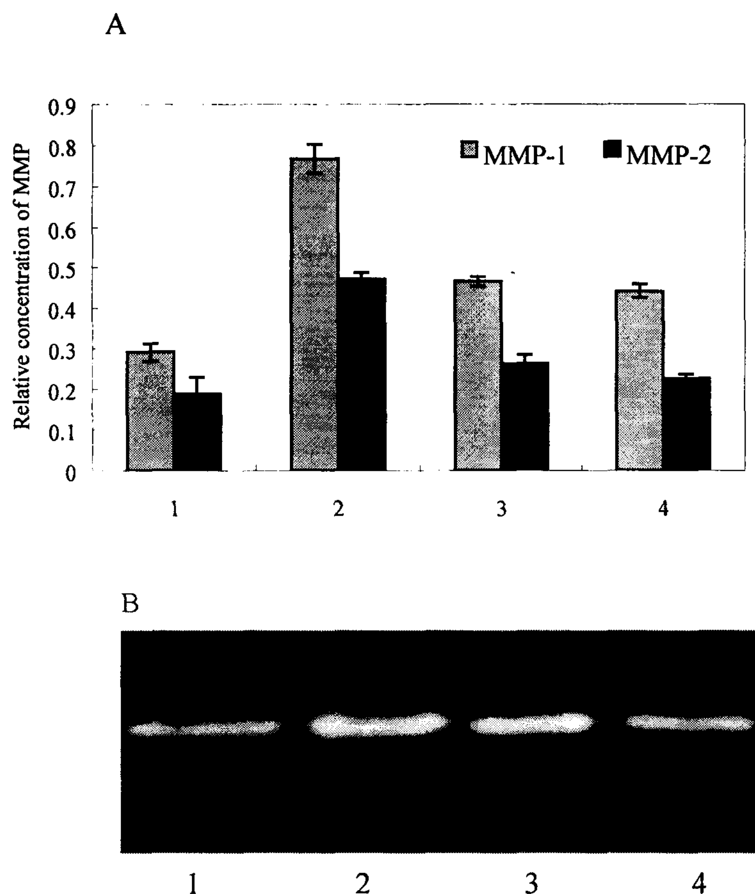


Fig. 4. The effect of L-carnitine on the production (A) and activity (B) of MMP-1 and MMP-2 by human dermal fibroblasts. After UVA irradiated, fibroblast cultured for 24hr in

the presence of increasing concentrations of L-carnitine. MMP protein and activity were quantified by ELISA and Zymography. 1. non irradiated, 2. 5J/cm$^2$ UVA irradiated, 3. UVA + L-carnitine 2.5mM, 4. UVA + L-carnitine 1.25mM

**The effect of L-carnitine on the production of MMP mRNA**

After irradiated UVA, Human dermal fibroblasts were incubated with the L-carnitine. UVA irradiation of fibroblasts cells led to an 3-fold rise in MMP-1 mRNA expression (Fig. 5 Lane 2), which was reduced by 1.3-fold in the presence of L-carnitine (5mM) (Fig. 5 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. E.A.Offored et al. reported that UVA irradiation of fibroblast cells led to an 8-fold rise in MMP-1 mRNA expression, which was reduced by 4-fold in the presence of vitamin E (2uM) and 2.5-fold in the presence of vitamin C (14uM) by northern blot (21). And Fisher et al. showed that tRA is an important regulator of cellular homoestasis in many, tissues, including human skin. The actions of tRA are mediated by nuclear retinoid receptors that are ligand-activated transcription factors. tRA inhibits many of the effects of phorbol esters. This inhibition occurs principally through antagonism of AP-1 and tRA blocks increases in AP-1 DNA binding and induction of MMPs by UV radiation in human skin *in vivo* (8). Actually, Above mentioned retinoic acid, Vitamin E and Vitamin C act as a powerful antioxidant and their mechanisms concerning about aging are well known relatively. But, We tested antioxidant activity of L-carnitine was not exhibited. Although the L-carnitine studied here collagenase inhibitory properties *in vitro*, their overall biological functions on MMP regulation *in vivo* have yet to be elucidated. Only we found that MMP mRNA and protein expression from UVA irradiated human dermal fibroblast is regulated by L-carnitine. Whether L-carnitine act as enzyme inhibitor or inducers, how regulates MMP gene expression, has yet to be clarified but perhaps it could be linked to their regulation of nitric oxide synthase activity (14) and it's ability as superoxide scavenger, antioxidant, and DNA cleavage protector (15). And further studies are required to investigate the fine signaling pathways affected by L-carnitine. In conclusion, Our results suggest that L-carnitine may be useful as new anti-aging photoprotectors against UVA induced MMP expression and activity.

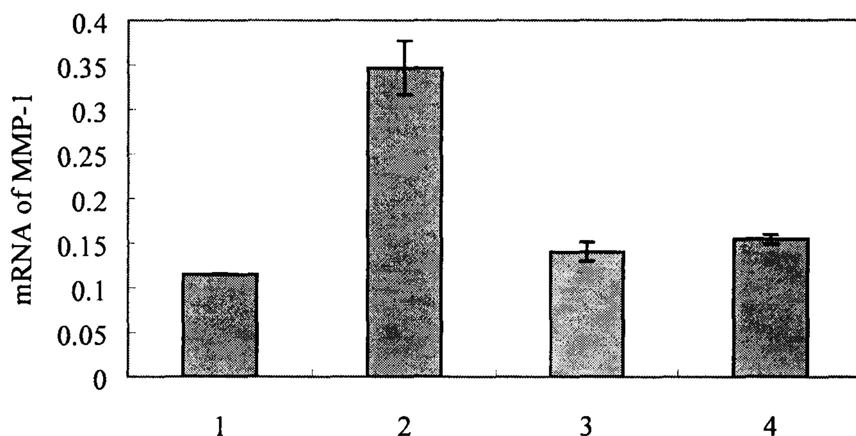


Fig. 5. The effect of L-carnitine on the production of MMP mRNA from UVA irradiated human dermal fibroblast. The intracellular mRNA of MMP was determined using RT-PCR-ELISA method. 1. non-irradiated, 2. 5J/cm2 UVA irradiated, 3. UVA + L-carnitine 10mM, 4. UVA + L-carnitine 5mM

**CONCLUSIONS**

L-carnitine is a small water-soluble cofactor molecules and it's main cellular functions are participation in the transport of long-chain fatty acids into the mitochonderial matrix for β-oxidation to provide cellular energy. In this study, L-carnitine showed effectively inhibition on MMP-1 (collagenase) from *C. histolyticum* and MMP-2 (gelatinase) human dermal fibroblasts. And 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 (5mM). >From these results, we concluded that the expression of MMP-1 and -2 by the UV irradiated HDF is regulated by L-carnitine.

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