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Chitooligomers inhibit UV-A-induced photoaging of skin by regulating TGF-β/Smad signaling cascade

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ABSTRACT

In the present study, effect of low molecular weight chitooligomers on prevention of photoaging in UV-A irradiated in vitro human dermal fibroblasts was investigated.

The results indicated that chitooligomers suppressed family of collagenase (MMP-1, MMP-8 and MMP-13) and gelatinase (MMP-2 and MMP-9) MMPs expression. These levels were inhibited by increase in expression of TIMP-1 and TIMP-2 genes. In addition, treatment with chitooligomers enhanced the expression of collagen synthetic markers such as procollagen and type I collagen in UV-A irradiated dermal fibroblasts. Furthermore, we confirmed that the UV-A-induced transcriptions of AP-1 and TGF-β/Smad signaling cascade were regulated by chitooligomers treatment in UV-A irradiated dermal fibroblasts.

Therefore, our study suggests that chitooligomers can be developed as topical application of natural anti-aging cosmeceuticals since reduced the skin collagen decomposition by enhancement of collagen synthesis.

1. Introduction

Numerous structural changes occur in skin connective tissue as a consequence of the aging process. Related with age, changes include decrease in a number of interstitial fibroblasts, a decomposition of collagen and thinning of connective tissue fiber. These changes have been concerned as an evidence of skin aging in many of the individuals and skin damage could be accelerated by environmental factors such as UV exposure (El-Domyati et al., 2002; González, Fernández-Lorente, & Gilaberte-Calzada, 2008). It has been thought that these alterations in the dermal connective tissue are mainly responsible for wrinkle formation of skin. Fibroblasts are major cell type of skin dermis which can differentiate into adipocytes, chondrocytes and osteocytes since differentiation potential (Fisher et al., 2002). Because of these reason, many studies have been conducted using dermal fibroblasts as an in vitro model to study photoaging (Bae et al., 2005).

UV irradiation induces decomposition of skin connective tissue by activating matrix metalloproteinases (MMP) that are responsible for the degradation of skin collagen and inhibition of collagen synthesis of extracellular matrix in connective tissues (Wlaschek et al., 1994). Therefore, inhibition of collagenase MMPs expression by activating collagen synthesis might be an effective strategy to prevent wrinkle formation after UV irradiation.

Retinoic acid is known as a metabolite of retinol that mediates the functions of vitamin A required for growth and development. It has been reported that retinoic acid could repair the appearance of photo damaged skin observed from clinical study (Rosenthal et al., 1990). Previous studies also have shown that treatment with retinoic acid to human skin suppress the activation of MMP-1 by UV irradiation, which is involved in the decomposition of the extracellular matrix (Fisher et al., 2001). However, the therapeutic use of retinoic is limited because retinoic acid is extremely unstable in the presence of UV light, air, and oxidizing agents that give to the appearance of the toxic side effects as well as skin irritants mutagenicity (Okano et al., 2006). Thus, we try to find novel bioactive compound from marine resources with the goal to develop safe and suitable for the treatment of photoaging.

Chitooligmers are made from chitin or chitosan via chemical or enzymatic depolymerization and possess water solubility, biocompatibility and biodegradability (Muzzarelli, Stanic, & Ramos, 1999; Muzzarelli, Terbojevich, Muzzarelli, & Francescangeli, 2002). In addition, chitooligmers have been shown to have anti-microbial, anti-tumoral, anti-fungal, anti-viral, and anti-oxidative profiles according to their molecular size (Kim & Rajapakse, 2005; Ngo, Kim, & Kim, 2008). Nevertheless, effect of chitooligmers on anti-photoaging has not been well established.

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Therefore, the purpose of this study is to determine the anti-photoaging effect of chitooligmers with low molecular weight on skin collagen improvement and to elucidate its mechanism action using cultured human dermal fibroblasts caused by UV-A irradiation.

2. Materials and methods

2.1. Materials and Cell culture

The < 1 kDa chitooligomers in admixture were donated by Kitto Life Co. (Seoul, Korea). Chitooligomers were prepared using an UF membrane reactor system that had molecular weight cut off (MWCO) 10, 5, 3, and 1 kDa, respectively (Kim & Rajapakse, 2005). Molecular weight of chitooliogomers was determined by previous method (Rinaudo, Milas, & Le Dung, 1993). Human dermal fibroblasts were purchased from Promo cell (Heidelberg, Germany) which was isolated from the dermis of adult skin. Dermal fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD USA) containing 10% fetal bovine serum (FBS), 2 mM glutamine and 100 μg/ml penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD, USA) at 37 °C humidified atmosphere of 5% CO_2. Dermal fibroblasts (passage 2) were maintained for 6 additional passages and sub-cultured for experiment at about 90–95% confluence by detaching with trypsin-EDTA solution.

2.2. UV-A irradiation

Dermal fibroblasts were seeded in 24-well plate at a density of 1×10^5 cells/well with DMEM containing 10% FBS, 2 mM gluramine and 100 μg/ml penicillin-streptomycin at 37 °C humidified atmosphere of 5% CO_2. After incubation for 24 h, the cells were exposed to UV-A energy at 6 J/cm^2 (365 nm UV-A light source, Bio-Sun lamp, Vilber Lourmat, Marine, France) in 200 μl of phosphate buffered saline (PBS) to each well. After UV-A irradiation, the cells were incubated for 24 h in serum-free DMEM.

2.3. Cell cytotoxicity and protection of photo-damage determination using MTT assay

Cytotoxic influence of chitooligomers on cultured dermal fibroblasts was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Kim, Kong, Seo, & Kim, 2010). The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were treated with the control medium or the medium supplemented with various concentrations of chitooligomers and retinoic acid. After incubation for 24 h, the cells were incubated with 100 μl of MTT (1 mg/ml) for 4 h. Finally, 100 μl DMSO was added to dissolve the formazan salt and cell viability was measured at the absorbance at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). Furthermore, effect of chitooligomers on protection of photo-damage was also determined. After UV-A irradiation, various concentration of samples were treated in cultured cell and incubated with 24 h following above procedure. Relative cell viability was quantified as a percentage compared to the control group.

2.4. Determination of the fibroblast protection against photo-damage

Dermal fibroblasts are exposed to UV-A light in the presence of chitooligomers and retinoic acid. Various concentrations of chitooligomers and retinoic acid were treated in cultured cells with UV-A irradiation then further incubated for 24 h. Photocytotoxicity was expressed as a decrease in cell viability as determined by MTT assay following Organisation for Economic Co-operation and Development (OECD) guideline for testing of chemicals (OECD, 2002).

2.5. MMP-1 activation determined by enzyme-linked immunosorbent assay (ELISA)

In order to determine MMP-1 secretion, dermal fibroblasts were incubated with the different concentration of chitooligomers in the absence of FBS followed by UV-A irradiation. After 24 h, the cell-free supernatants of dermal fibroblasts were collected and used for MMP-1 assay. This assay was designed to assess and quantify the levels of MMP-1 using Biotrak^{TM} ELISA kits (Amersham Pharmacia Biosciences) as per manufacturer's instructions. The standard curve of MMP-1 was established by plotting the pg/ml concentrations versus absorbance values of standard wells. It was used to quantify the amount of MMP-1 secreted from the cells.

2.6. RT-PCR analysis

RT-PCR analysis was performed by our previous method (Kim et al., 2010). Target cDNA was amplified using the forward and reverse primer sequences: forward 5′-GAT-GTG-GAG-TGC-CTG-ATG-TG-3′ and reverse 5′-TGC-TTG-ACC-CTC-AGA-GAC-CT-3′ for MMP-1; forward 5′-TCT-GCA-AGG-TTA-TCC-CAA-GG-3′ and reverse 5′-TAT-TCC-TGG-AAA-GGC-ACC-TG-3′ for MMP-8; forward 5′-GGA-GCC-TCT-CAG-TCA-TGG-AG-′ and reverse 5′-TTG-AGC-TGG-ACT-CAT-TGT-CG-3′ for MMP-13; forward 5′-TGA-AGG-TCG-GTG-TGA-ACG-GA-3′ and reverse 5′-CAT-GTA-GCC-ATG-AGG-TCC-ACC-AC-3′ for MMP-2; forward 5′-CAC-TGT-CCA-CCC-CTC-AGA-GC-3′ and reverse 5′-CAC-TTG-TCG-GCG-ATA-AGG for MMP-9; forward 5′-AAT-TCC-GAC-CTC-GTC-ATC-AG-3′ and reverse 5′-TGC-AGT-TTT-CCA-GCA-ATG-AG-3′ for TIMP-1; forward 5′-TGA-TCC-ACA-CAC-GTT-GGT-CT-3′ and reverse 5′-TTT-GAG-TTG-CTT-GCA-GGA-TG-3′ for TIMP-2; forward 5′-GAG-TCA-ACG-GAT-TTG-GTC-GT-3′ and reverse 5′-GAC-AAG-CTT-CCC-GTT-CTC-AG-3′ for β-actin.

2.7. Western blot analysis

Protein expression levels of MMPs and signal transductions were examined by Western blot analysis as described previously (Kim et al., 2010).

2.8. Statistical analysis

Data were expressed as mean ± SD ($n = 5$) and analyzed using the analysis of variance (ANOVA) procedure of Statistical Analysis System (SAS v9.1, SAS Institute Inc., Cary, NC, USA). Significant differences between treatment means were determined using Duncan's multiple range tests at the $p < 0.05$ level.

3. Results and discussion

3.1. Chitooligomers protect dermal fibroblasts against photo-damage

Dermal fibroblasts were co-incubated with or without chitooligomers and retinoic acid at the concentrations of 0.2, 0.5, 1 mg/ml and 1, 2, 5 μM, respectively. None of the chitooligomers exhibited any significant cytotoxicity as shown in Fig. 1(A). Results obtained from MTT assay revealed that chitooligomers is safe compound for in vitro experiments. Furthermore, we checked the protective effect of chitooligomers on UV-A irradiated damage. The result has shown to Fig. 1(B), treatment with chitooligomers

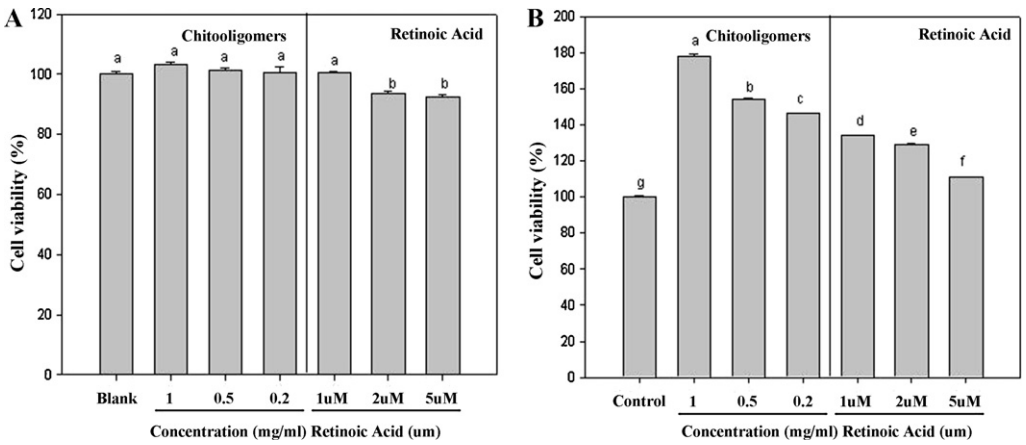

Fig. 1. Effect of chitooligomers on cell viability (A) and photo-damage protection (B) in human dermal fibroblasts was determined by MTT assays. (a–g) Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test. Blank: no UV-A exposure, Control: only UV-A irradiated group.

was significantly protected cell damage in a dose-dependent manner.

3.2. Chitooligomers have not shown phototoxicity in dermal fibroblasts

Cells were co cultured with chitooligomers and retinoic acid with UV-A irradiation in order to determine phototoxic ability of chitooligomers. As shown in Fig. 2, none of the chitooligomers exhibited any phototoxicity whereas retinoic acid showed a little phototoxic effect at the concentration of 5 μM. Therefore, we selected concentration of 1 uM retinoic acid as a positive control for further experiment to compare effect of chitooligomers on UV-A mediated photoaging.

3.3. Chitooligomers inhibits collagenase MMP activation by UV-A irradiation

Collagens are known to as main component of skin and degraded by collagenase MMPs. MMP-1 is family of collagenase which is capable of degrading triple-helical collagens. The main reason for wrinkle formation is known to decomposition of collagen by MMP (Fisher et al., 2002). As shown in Fig. 3, MMP-1 activation was suppressed by chitooligomers treatment in a dose-dependent manner and its effect was similar to the inhibitory effect of retinoic acid at the concentration of 1 μM. This result indicated that chitooligomers exerted adequate protective effect on collagen degradation by inhibition of MMP-1 expression in UV-A irradiated dermal fibroblasts.

3.4. Chitooligomers regulate expression of collagenase genes and proteins

In photoaging process, decomposition of dermal collagen is closely related to UV-A irradiation by activating the matrix degrading enzymes as well as MMPs. Among MMPs, most of researches have been focus on the gelatinases and collagenases which are considered to be involved in photoaging in relation to winkle formation (Nishimori et al., 2001). It has been reported that over-expression of gelatinases and collagenases by UV irradiation resulted in collagen destruction in human conjunctivochalasis fibroblasts (Inomata et al., 2003; Zhang, Liu, Cui, Zhou, & Cao, 2008). As shown in Fig. 4, the levels of gelatinase (MMP-2 and MMP-9) and collagenase MMPs (MMP-1, MMP-8 and MMP-13) were down-regulated by chitooligomers treatment in both gene and protein level. These MMPs levels are regulated by tissue inhibited tissue inhibitor of metalloproteinases (TIMPs). Thus the expression of TIMP-1 and TIMP-2

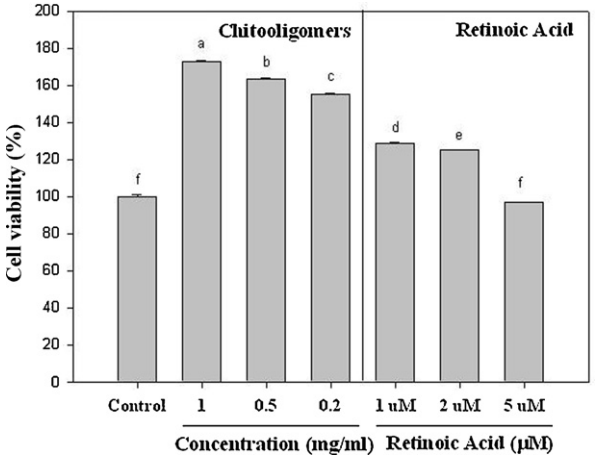

Fig. 2. Phototoxicity of exposed to 6 J/cm^2 of UV-A irradiation and cytotoxicity level was determined by MTT assays. (a–f) Means with the different letters are significantly different ($p < 0.05$) by Duncan's multiple range test. Control: only UV-A irradiated group.

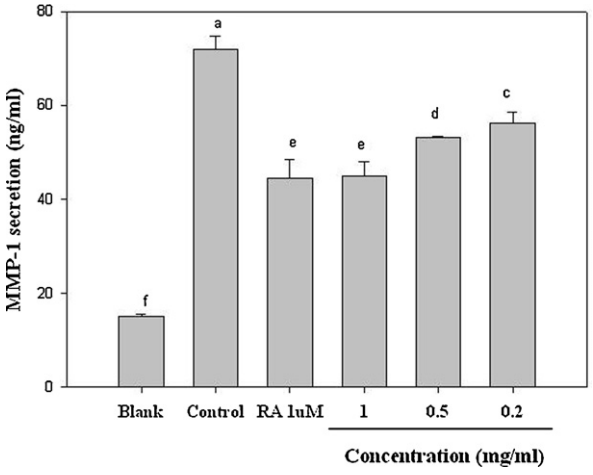

Fig. 3. Effect of chitooligomers on collagen degrading MMP-1 secretion in human dermal fibroblasts exposed to 6 J/cm^2 of UV-A irradiation. The level of MMP-1 was determined by ELISA assay. (a–f) Means with the different letters are significantly different ($p < 0.05$) between samples and blank or control at each concentration. Blank: without UV-A irradiation, Control: only UV-A irradiated group.

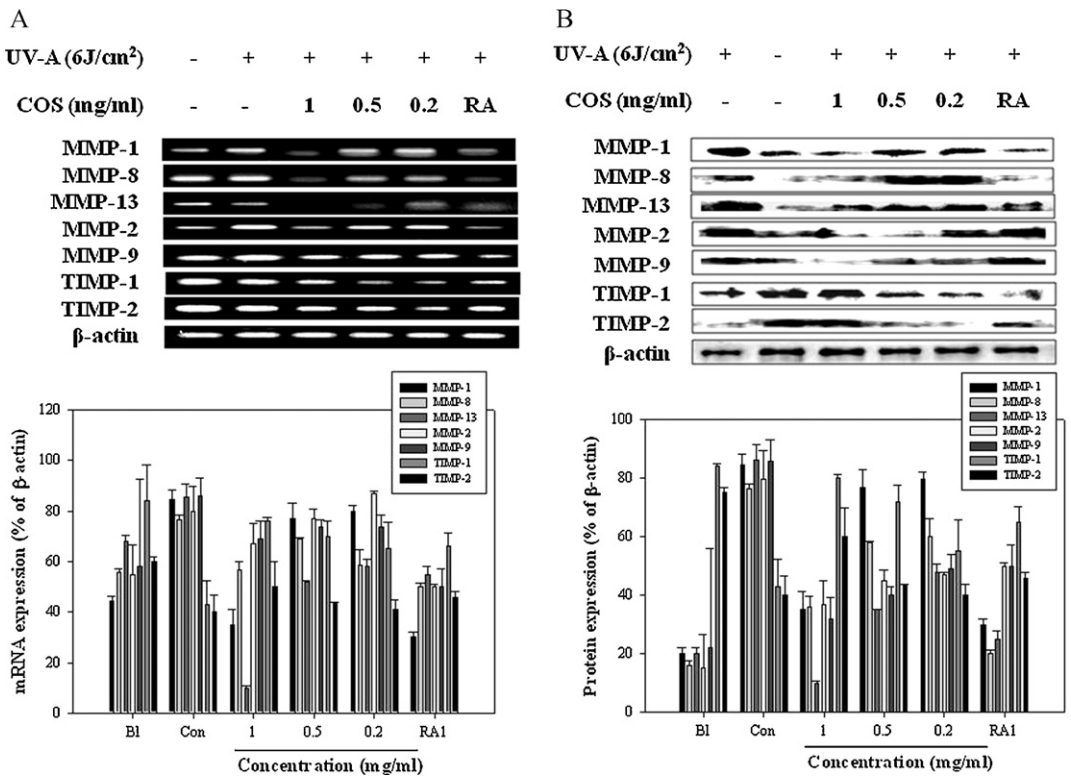

Fig. 4. Effect of chitooligomers on the levels of collagen degrading MMPs in UV-A irradiated human dermal fibroblasts. Gene (A) and protein (B) expression levels were determined by RT-PCR and Western blot analysis, respectively. Cells were exposed to 6 J/cm^2 of UV-A irradiation and incubated with chitooligomers for 24 h. β-actin was used as an internal standard. RA: retinoic acid.

were also evaluated. The result indicated that chitooligomers increased expression of TIMP-1 and TIMP-2 gene similar with retinoic acid. Following the above results, chitooligomers has protective effect on UV-A induced collagen decomposition via negative regulation of MMPs expression.

3.5. Chitooligomers can promote collagens synthesis

In fibroblasts, the collagen molecules are synthesized and secreted into the extracellular space as procollagens. Until now many of collagen has been identified, but over than 90% of the collagen in the body is type I collagen (Rittié & Fisher, 2002). Type I collagen is belong to a group of proteins supplying tensile strength and stability due to disulphide bonds give skin the elastic properties (Wiestner et al., 1982). Treatment with chitooligomers enhanced collagen type I level in a dose dependant manner (Fig. 5). In addition, interstitial collagens are synthesized and secreted out of the cells as procollagens. Expression of procollagens was decreased in UV-A alone irradiated cells whereas these decreased cellular levels of procollagen result in UV-A exposure were enhanced in the presence of chitooligomers. These result indicated that chitooligomers might be involved in collagen synthesis by regulation of collagen degrading MMP expressions.

3.6. Chitooligomers suppress AP-1 signaling cascade

In addition to cause of collagen decomposition, UV exposure impairs new type I collagen synthesis (Fisher et al., 1996, 1997). Negative regulation of type I collagen synthesis is mediated in part by UV-induced AP-1 which is composed of Jun and Fos family proteins. It has been reported that AP-1 inhibits transcription of type I procollagen by induction of MMP expression (Bornstein, 1996; Chung, Agarwal, Uitto, & Mauviel, 1996). The result addressed in Fig. 6(A), AP-1 transcription was down-regulated by treatment with chitooligomers in a concentration dependent manner. This result indicated that chitooligomers has protective effect against UV-A exposure like retinoic acid.

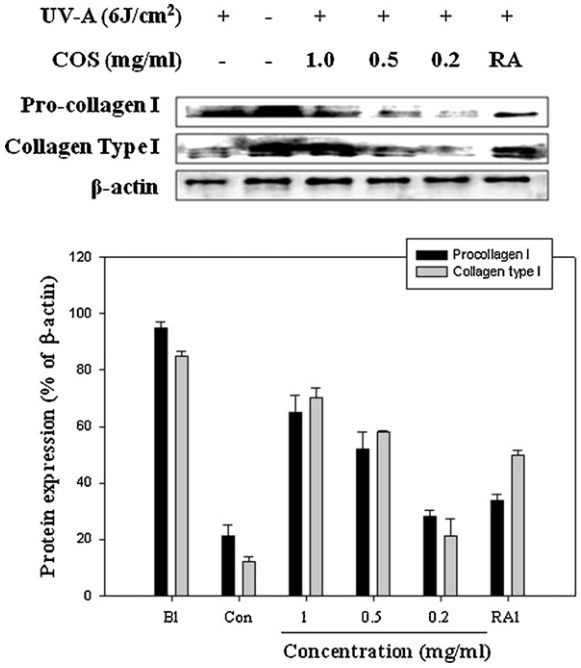

Fig. 5. Effect of chitooligomers on collagen synthesis in UV-A irradiated human dermal fibroblasts. Cells were exposed to 6 J/cm^2 of UV-A irradiation and incubated with chitooligomers for 24 h. β-actin was used as an internal standard. RA: retinoic acid.

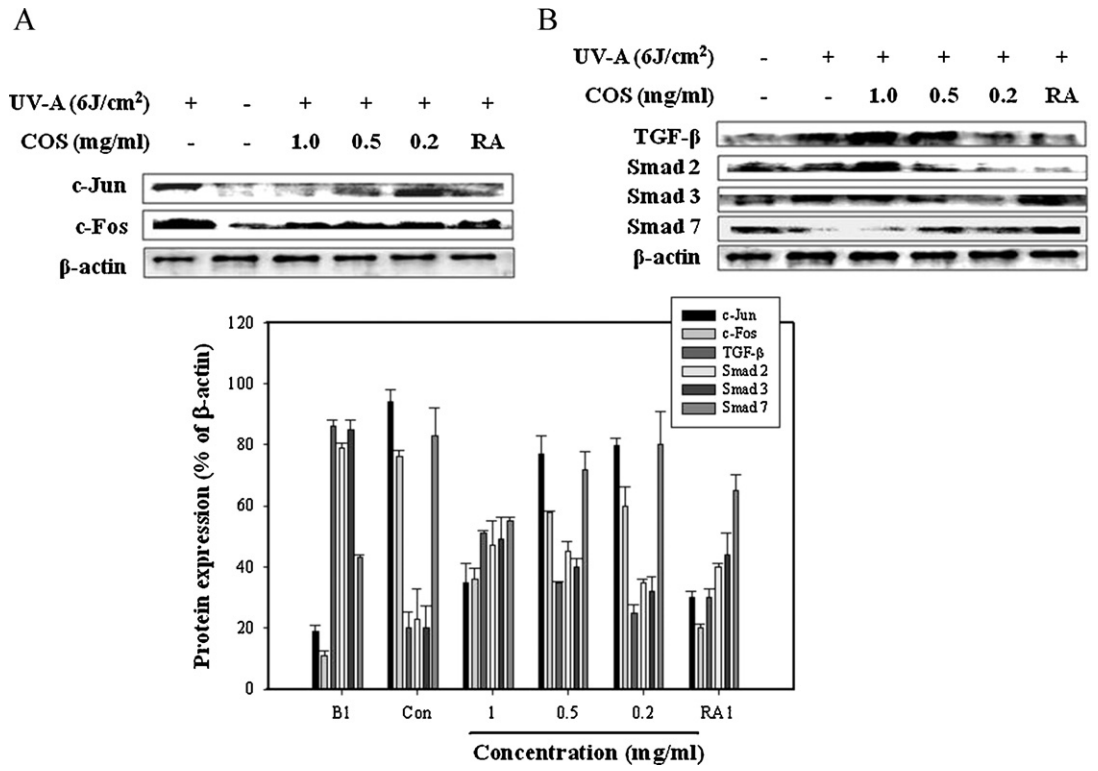

Fig. 6. Effect of chitooligomers on AP-1 (A) and TGF-β/Smad (B) signaling cascade in UV-A irradiated human dermal fibroblasts. Cells were exposed to 6 J/cm^2 of UV-A irradiation and incubated with chitooligomers for 24 h. Transcription levels of signaling cascades were determined by Western blot analysis. β-actin was used as an internal standard. RA: retinoic acid.

3.7. Chitooligomers induce TGF-β/Smad signaling cascade

In relation to skin aging process, TGF-β/Smad pathway plays a major role in cell growth and collagen regulation, little study is known regarding altered TGF-β/Smad signaling in UV-mediated skin aging. Therefore, we evaluated the transcriptional level of TGF-β/Smad signaling in UV-A irradiated human fibroblasts in vitro.

In addition, previous study observed TGF-β activation is decreased during cellular aging in cultured human dermal fibroblasts (Zeng, McCue, Mastrangelo, & Millis, 1996). According to previous research, our results suggest that expression of R-Smads such as Smad 2 and Smad 3 in UV-A irradiated skin cells can respect to suppress the TGF-β expression. On the other hand, Smad7 mediated pathway is partially abolished the negative regulation of MMP-1 activity by TGF-β signaling (Yuan & Varga, 2001). Suppressed R-Smads expressions are probably response to UV irradiated damage, increased AP-1 activity, increased MMP expression which are constitute collagen deficiency during dermal photoaging. As shown in Fig. 6(B), TGF-β expression was significantly increased in the presence of chitooligomers in a concentration dependent manner and its effect was similar with retinoic acid treatment.

Unlike TGF-β, AP-1 inhibits positive regulation of procollagen gene transcription preventing the Smad 2, 3, 4 transcriptions (Kang et al., 2003; Verrecchia and Mauviel, 2002). Therefore, effects of chitooligomers on Smads protein expression were observed in UV-A exposed dermal fibroblasts compared with retinoic acid treatment group.

The result obtained from Western blot analysis, the levels of Smad 2 and Smad 3 proteins were dramatically increased in UV-A irradiated dermal cells by treatment with chitooligomers whereas Smad 7 was down-regulated in UV-A irradiated dermal cells. According to these results, UV-A irradiation causes an impairment of the TGF-β/Smad signaling cascade. However, these regulations in respect to collagen deficiency during the UV-induced photoaging process were successfully modulated by treatment with chitooligomers.

4. Conclusions

The present study indicated that chitooligomers has protective effect on UV-enhanced decomposition of matrix component may result in an increase of collagen synthesis and a decrease of matrix degrading enzymes expression in human dermal fibroblasts. Treatment with chitooligomers results in opposing changes by regulation of AP-1 and TGF-β/Smad pathway.

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