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ORIGINAL ARTICLE

Synergistic effect of copper and amino acid mixtures on the production of extracellular matrix proteins in skin fibroblasts

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Abstract

The stimulation of extracellular matrix (ECM) protein production is an interesting target to maintain normal skin structure and delay skin aging. Copper has been shown to stimulate ECM protein synthesis by activating lysyl oxidase. Although copper increases elastin and collagen synthesis, the effect of copper and amino acid mixtures on gene expression and protein synthesis changes relating to the ECM have not been fully investigated. In this study, we showed that copper ions (Cu^{2+}) and amino acid mixtures significantly increased the expression of genes and proteins related to the ECM in human dermal fibroblasts. The expression of genes involved in ECM production was evaluated through quantitative polymerase chain reaction in the presence of amino acid mixtures containing different Cu^{2+} concentrations. Cu^{2+} dose-dependently increased the gene expression of elastin and collagen I. In addition, a mixture of amino acids and Cu^{2+} increased the protein expression of elastin and collagen I. We further evaluated the effect of Cu^{2+} with or without amino acids. Although Cu^{2+} treatment increased the expression of genes encoding ECM proteins, the Cu^{2+} treatment without amino acids did not increase protein expression in the ECM. Our results demonstrated the synergistic effects of amino acids and a Cu^{2+} mixture on ECM protein synthesis in dermal fibroblasts.

Keywords Copper · Elastin · Collagen I · Extracellular matrix · Fibroblast

Introduction

Skin aging is a complex biological process influenced by a combination of intrinsic and environmental factors [32]. In addition to natural aging caused by time lapse, environmental stresses, including ultraviolet radiation, pollution, chemicals, and toxins, are known to cause accelerated skin aging [14]. One of the changes during skin aging is the increased presence of wrinkles characterized by an abnormal balance between extracellular matrix (ECM) protein synthesis and degradation [17]. Chronic exposure to environmental stresses causes increased ECM protein degradation, while ECM protein synthesis is decreased. Gradual changes in ECM proteins lead to skin aging phenotypes, including deep wrinkles, laxity, dullness, and sagging [32]. Thus, stopping the degradation and stimulating the synthesis of ECM proteins is an interesting approach to delay skin aging.

The dermis is mainly composed of ECM-producing cells called dermal fibroblasts [19]. These cells produce primary structural constituents of the skin, including various types of collagens, elastin, and fibronectin [30]. The ECM proteins themselves and the interactions between ECM proteins and cell surface receptor proteins play major roles in maintaining the basal structure of the skin dermis [27]. Collagen proteins are the main constituents of the skin dermis. As the main component of connective tissue, collagen is the most abundant protein in mammals, accounting for 25%–35% of the whole-body protein content [18]. Collagen fibers account for 90% of all dermal fibers, thus playing a pivotal role in the maintenance of skin homeostasis [26]. Elastin is another ECM protein that plays an important role in maintaining the structure of the skin [31]. Compared to collagen proteins, it is highly elastic and helps the tissue regain its shape after stretching or contracting [31]. Collagen and elastin have an unusual amino acid

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composition compared to other proteins, which makes them perform their unique role as ECM proteins [24, 29]. The triple-helical structure of collagen arises from three amino acids: glycine, proline, and hydroxyproline. These amino acids make up the repeating motif Gly-Pro-X, where X is any amino acid. Elastin also has a similar amino acid composition to that of collagen in terms of glycine and proline residues, but with a specific enrichment of the hydrophobic residues valine and alanine.

Due to the unusual composition of amino acids, attempts to increase ECM protein levels have been focused on treating cells with different amino acid compositions [9, 11]. Treatment with amino acids that contribute to collagen and elastin synthesis is the main strategy for increasing ECM proteins in fibroblasts [22]. For example, proline concentration is critical for collagen synthesis in skin fibroblasts [6]. Glutamine also stimulates collagen synthesis through the conversion to proline [5]. De Servi et al. showed that mixture of various amino acids (glycine, proline, lysine, leucine, valine, and alanine) with hyaluronic acid increased ECM genes in cultured human fibroblasts [4]. They found that optimal ratios between the amino acids were important for the production of ECM proteins. Other strategies also aimed to increase ECM protein levels in skin fibroblasts. Treatment with hyaluronic acid significantly increase collagen expression in cultured human skin fibroblasts by affecting various stages of the synthesis [1, 3]. The post-translational modification of collagen is also important for synthesis. For instance, ascorbic acid is necessary for the hydroxylation of prolyl and lysyl residues, and in this process, copper ions (Cu^{2+}) are also important for lysyl oxidase activity [2, 10, 20].

Previous studies have shown the effect and role of Cu^{2+} supplementation on the synthesis of ECM proteins [16]. A recent report also suggested the effect of Cu^{2+} on pro-collagen 1 and elastin expression in an ex vivo human skin model [12]. Furthermore, the effects of amino acid supplementation on ECM synthesis has been shown in the previous studies. Although the effects of amino acids supplementation and Cu^{2+} supplementation on ECM synthesis has been reported separately, however, the synergistic effect of Cu^{2+} and amino acid supplementation on ECM synthesis and the exact mechanisms has not been investigated. Here, we tested various doses and compositions of formulations consisting of amino acids and Cu^{2+} ions. Using human dermal fibroblasts, the synergistic effects of amino acids and Cu^{2+} ions on the expression of genes and proteins involved in ECM production were analyzed.

Materials and methods

Preparation and composition of tested formulations

Different formulations of amino acids and Cu^{2+} were tested in this study. The concentrations of the different formulations were determined from previous study [4]. The compositions of the mixtures are shown in Table 1.

Cell culture

HS27 human skin fibroblasts (CRL-1634, American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle medium (DMEM; Welgene, Korea) supplemented with 20% fetal bovine serum (FBS; Welgene), 233.6 mg/mL glutamine, 100 µg/mL penicillin–streptomycin, and adjusted to pH 7.4–7.6 with $NaHCO_3$ in a 5% CO_2 atmosphere. All experiments were conducted using sub-confluent, low-passage human skin fibroblasts (passages 18–25). The cells were treated with different formulations for each experiment.

Evaluation of cytotoxicity

A cell viability assay was conducted using a commercially available kit (EZ-1000, Dogen Bio, Korea) to evaluate the effect of the formulation on cell viability. Briefly, the cells were cultured in 48-well plates and treated with the different formulations shown in Table 1. After the indicated time, the absorbance was read at 450 nm using a microplate reader. All cell experiments were performed at least three times to ensure reproducibility.

Table 1 Composition of the formulations tested

	D	EL1	EL4	EL11	EL12
Glycine (µg/mL)	302	266.7	266.7	266.7	266.7
L-proline (µg/mL)	227	266.7	266.7	266.7	266.7
L-alanine (µg/mL)	228	200	200	200	200
L-valine (µg/mL)	168	166.7	166.7	166.7	166.7
L-leucine (µg/mL)	42	56.7	56.7	56.7	56.7
L-lysine HCl (µg/mL)	33	43.3	43.3	43.3	43.3
Cupric sulfate pentahydrate (µg/mL)	0	0	3.3	1.67	0.33

RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction (qPCR)

Total RNA from cells was obtained using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as previously described. Briefly, cells (6×10^5) were seeded in a 60 π dish with medium and incubated for 24 h. Then, the cells were washed with PBS and treated with different formulations. After 72 h, the cells were harvested and RNA was extracted using 1 mL of TRIzol reagent. Aliquots of 0.2 mL of chloroform per 1 mL homogenate were added and samples were shaken vigorously for 30 s. The aqueous phases were transferred to fresh tubes and an equal volume of isopropanol was added. Samples were incubated at 4 °C for 15 min and centrifuged at 12,000×*g* for 15 min at 4 °C. Supernatants were removed and RNA pellets were washed once with 75% ethanol by vortexing and then centrifuged at 7500×*g* for 8 min at 4 °C. Pellets were dried for 10–15 min and dissolved in diethyl pyrocarbonate-treated water. mRNA (2.0 µg) was reverse-transcribed using the cDNA synthesis kit from GenDEPOT (Katy, TX, USA). The synthesized cDNA was used for qPCR. qPCR was performed using SYBR Green Master Mix (Bioline, Taunton, MA, USA) and the CFX Connect System (Bio-Rad Laboratories Inc., Philadelphia, PA, USA). The primers used were as follows: COL1A1-F: CCA AAT CTG TCT CCC CAG AA, COL1A1-R: TCA AAA ACG AAG GGG AGA TG, ELN-F: CCA TGT CCA CAC AAG GAC AG, ELN-R: GCC AGA GTG GCT TTC TCA AC, COL4A1-F: GCT TGA AAA GGG TTG AGC AG, COL4A1-R: TTG AGT CCC GGT AGA CCA AC, ACTB-F: GAG GCC TGG ACT CTC AAC TG, ACTB-R: AAT GAA TGG GGG TTG AAT GA.

Protein extraction

To evaluate the effect of the formulation on the expression of proteins involved in ECM production, proteins were extracted from the cells. All solutions, tubes, and centrifuges were maintained at 0–4 °C. CETi protein extraction solution (Translab, Daejeon, Korea) containing a protease inhibitor cocktail was used to extract the total protein from the cells according to the manufacturer's instructions. Briefly, cells (6×10^5) were seeded in a 60 π dish with medium and incubated for 24 h. Then, the cells were washed with PBS and treated with different formulations. After 72 h, the cells were harvested, and proteins were extracted using lysis buffer. The protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, MA, USA).

Western blotting

Western blot analysis was performed as described previously, with minor modifications. Briefly, proteins (20–100 µg) were boiled with gel-loading buffer (0.3125 M Tris–HCl pH 6.8, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 0.05% bromophenol blue, and 25% glycerol) for 5 min at a volume ratio of 4:1. Proteins in each sample were then separated on 8–15% acrylamide gels through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred using a Bio-Rad Western system (Philadelphia, PA, USA) to polyvinylidene difluoride (PVDF; Millipore, Burlington, MA, USA) membranes, which were immediately placed in blocking buffer (5% nonfat milk) containing 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20. Membranes were then washed with Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.6)–Tween buffer (10 mM Tris pH 7.5, 100 mM NaCl, and 0.1% Tween 20) for 30 min, incubated with specific primary antibodies (1:500–1:2000 dilution) against collagen I: sc-59772, elastin: sc-58756, β-actin: sc-47778 (Santa Cruz Biotechnology, Dallas, TX, USA) at 4 °C overnight, washed three times with TBS-Tween buffer for 10 min, and incubated with horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat antibodies (1:10,000, Genetex, Irvine, CA, USA) at 25 °C for 1 h. The immunoblots were visualized using Western Bright Peroxide solution (Advansta, San Jose, CA, USA) and a Davinch-chemi CAS-400 imager (Davinch-K, Korea) according to the manufacturer's instructions.

Statistical analyses

The Student's *t* test was used to analyze differences between the two groups. The statistical significance was set at $p < 0.05$. The analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Results

Cytotoxicity of different formulations

The cytotoxic effect of the formulations was tested on Hs27 human dermal fibroblasts (HDFs). As shown in Fig. 1, the mixture treatment did not have significant cytotoxicity after 48 h and 72 h. However, a Cu^{2+} concentration of more than 3.3 µg/mL had significant cytotoxicity (data not shown). Therefore, to avoid cytotoxicity, a Cu^{2+} concentration of less than 3.3 µg/mL was used in further experiments.

Effect of the mixture on gene expression involved in ECM production

Next, to compare the difference between amino acids supplementation with or without Cu^{2+} supplementation, the effect of the mixture on gene expression involved in ECM

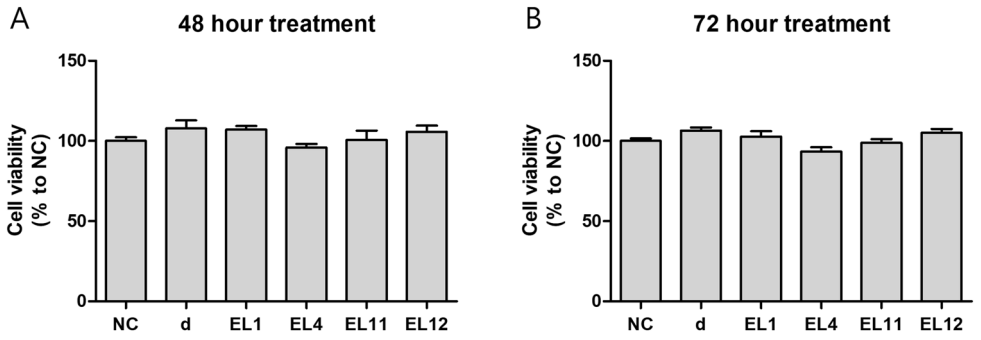

Fig. 1 Determination of the formulation cytotoxicity on Hs27 dermal fibroblasts. **A** The cell viability was determined 48 h after treatment with different formulations. The results are expressed as the percentage of viable cell compared to non-treated cells. **B** The cell viability was determined 72 h after treatment with different formulations. The results are expressed as the percentage of viable cells compared to non-treated cells

production was evaluated. The treatment with an amino acid mixture without Cu^{2+} did not have any effect on *COL1A2* and *ELN* gene expression levels (Fig. 2A, B). The copper-containing EL4 and EL11 mixture increased gene expression of *COL1A2* and *ELN* in a dose-dependent manner (Fig. 2A, B, *COL1A2* $^{**}p < 0.01$ vs. non-treated control, *ELN* $^{***}p < 0.001$ vs. non-treated control.). EL12, which has a low dose of Cu^{2+}, had no effect on gene expression of *COL1A2* and *ELN*. In contrast to the gene expression of *COL1A2* and *ELN*, there were no changes in *COL4A1* expression. These results indicate that a mixture of amino acids and Cu^{2+} increases gene expression involved in ECM production, and a certain amount of Cu^{2+} is needed to exert this effect.

Effect of the mixture on protein expression in the ECM

To investigate whether increased gene expression involved in ECM production is associated with protein expression, the protein expression of the ECM was analyzed. Similar to the gene expression results, the treatment with EL1 and EL4 significantly increased collagen I and elastin protein expression (Fig. 3, $^{*}p < 0.05$ vs. non-treated control.). Interestingly, EL12, which did not affect elastin gene expression, significantly promoted the expression of elastin (Fig. 3B). These results show that a mixture of amino acids and Cu^{2+} increased collagen I and elastin protein expression.

Effects of copper ions on ECM changes and comparison with mixtures

To determine whether the effect of the mixture is dependent on Cu^{2+}, we compared the effect of Cu^{2+} with or without amino acids. Treatment with EL4 or the same amounts of copper (3.3 µg/mL) increased *COL1A2* and *ELN* gene expression in fibroblasts (Fig. 4A, B, $^{*}p < 0.05$ vs. non-treated control.). These results implied that increased gene expression involved in ECM production was dependent on Cu^{2+}, regardless of the amino acid. We further measured the ECM protein levels under the same experimental conditions. Treatment with EL4 or the same Cu^{2+} amounts (3.3 µg/mL) increased collagen I and elastin protein expression; however, the extent of increase was higher under EL4 treatment

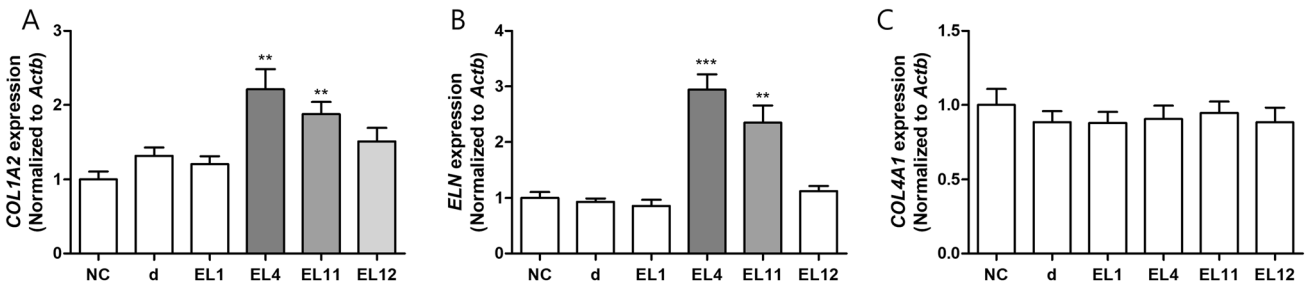

Fig. 2 Effect of formulations on gene expression involved in ECM production in Hs27 dermal fibroblasts. **A** Gene expression of collagen I (*COL1A2*) was analyzed through qPCR. $^{**}p < 0.01$ vs. non-treated control. **B** Gene expression of elastin (*ELN*) was analyzed through qPCR. $^{***}p < 0.001$ vs. non-treated control. $^{**}p < 0.01$ vs. non-treated control. **C** Gene expression of elastin (ELN) was analyzed through qPCR

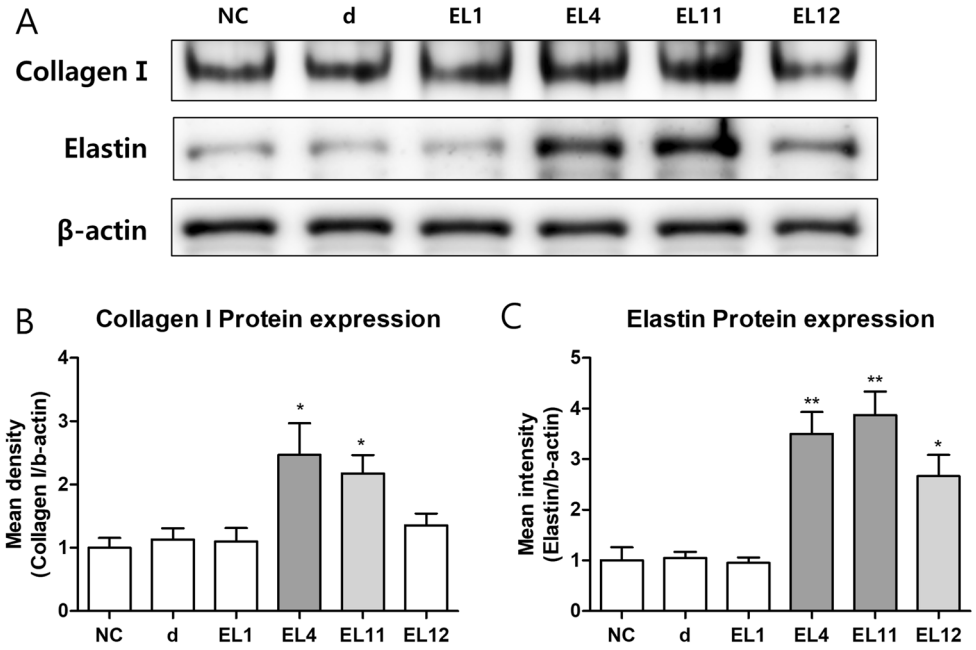

Fig. 3 Effect of formulations on protein expression of the ECM in Hs27 dermal fibroblasts. **A** Protein expression of collagen I and elastin were analyzed through western blot. β-actin was used as internal control. **B** Fold change difference in collagen I protein expression was determined through densitometric analysis. *p < 0.05 vs. non-treated control. **C** The fold change difference in elastin protein expression was determined through densitometric analysis. **p < 0.01 vs. non-treated control. *p < 0.05 vs. non-treated control

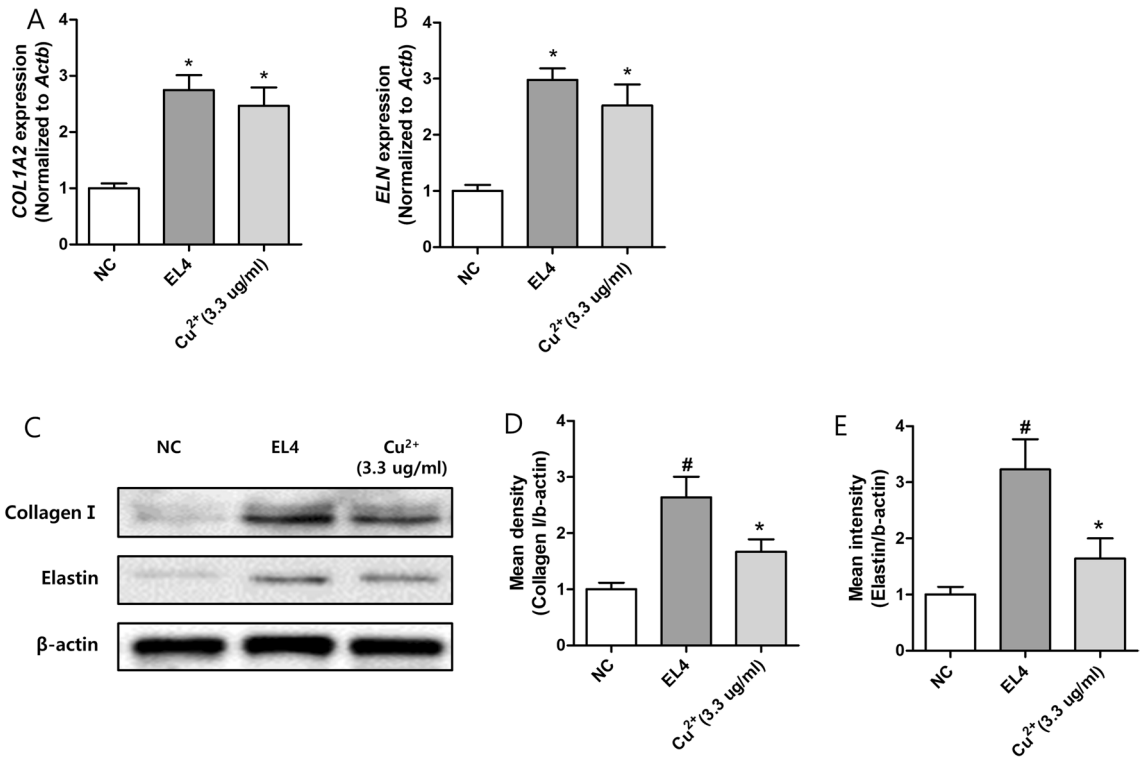

Fig. 4 Comparison of the effect of Cu^{2+} with or without amino acids on ECM proteins of Hs27 dermal fibroblasts. **A** Gene expression of collagen I (COL1A2) was analyzed through qPCR. *p < 0.05 vs. non-treated control. **B** Gene expression of elastin (ELN) was analyzed through qPCR. *p < 0.05 vs. non-treated control. **C** Protein expression of collagen I and elastin were analyzed through western blot. β-actin was used as internal control. **D** The fold change difference in collagen I protein expression was determined through densitometric analysis. #p < 0.05 vs. non-treated control. *p < 0.05 vs. EL4 group. **E** The fold change difference in elastin protein expression was determined through densitometric analysis. #p < 0.05 vs. non-treated control. *p < 0.05 vs. EL4 group

conditions (Fig. 4C, D, E, *$p < 0.05$ vs. non-treated control. #$p < 0.05$ vs. EL4 group). These results indicate that Cu^{2+} and amino acids have synergistic effects on ECM protein synthesis.

Discussion

Skin aging is a complex biological process influenced by a combination of intrinsic and extrinsic factors. The dermis, which is primarily composed of the ECM and fibroblasts, undergoes the most significant changes during aging. The most notable changes include an imbalance between ECM synthesis and degradation, which promotes age-associated wrinkle formation [13, 28]. The total amount of collagen decreases with age and it is also fragmented by several enzymes. The reduction in the amount and function of collagen leads to the deterioration of fibroblast function and a further decrease in the amount of dermal collagen [23]. Elastic fibers are another fibrous component of dermal ECM. Elastin levels also decrease with age, leading to a reduction in the number of functional components [31]. Maintaining adequate amounts of ECM proteins is an interesting approach to delay skin aging.

Changes in ECM proteins during aging are accompanied by various alterations in biological processes, including changes in intracellular signaling, increases in enzymes associated with ECM protein cleavage, and decreases in de novo synthesis of ECM proteins [8]. Based on these mechanisms, several anti-aging strategies have been investigated to promote young and healthy skin. A representative approach for the maintenance of ECM proteins includes supplying adequate precursors of ECM proteins and creating proper conditions for cells to produce more ECM proteins. In this regard, supplying sufficient amino acids that constitute ECM proteins is an interesting approach. Indeed, supplying additional amino acids, including proline and glutamine, has been shown to increase ECM proteins under several experimental conditions [5, 6, 9]. Supplementation of other components associated with ECM protein synthesis also showed positive effects on ECM protein synthesis. However, the synergistic effect of mixtures of Cu^{2+} and amino acids have not been investigated. Here, we report the synergistic effect and mechanism of action of Cu^{2+} and amino acid mixtures on HDFs.

The effect of Cu^{2+} on ECM protein synthesis has been demonstrated by several studies. Rucker et al. first showed the effect of a copper-deficient diet on bone collagen [21]. These researchers found that collagen was more easily solubilized under copper-deficient conditions, and that copper was required to maintain proper amine oxidase activity. Siegel et al. identified lysyl oxidase as a copper-dependent enzyme that promotes the cross-linking of collagen and elastin [25]. Other studies have demonstrated the role of copper in the biology of skin fibroblasts. Copper stimulates dermal fibroblast proliferation in the skin and upregulates the protein expression of collagen (types I, II, and V) and elastin produced by fibroblasts [7, 15, 16]. More recently, in an ex vivo human skin model, the effect of copper on pro-collagen1 and elastin expression was examined [12]. These studies suggested that some of these effects are indirectly affected by other proteins, including HSp-37, and TGF-β expression.

Based on previous results, we aimed to determine the synergistic effects of Cu^{2+} and amino acids. We prepared several formulations containing amino acids with different Cu^{2+} concentrations. Our results are similar to those of previous reports. Cu^{2+} treatment of dermal fibroblasts significantly increased the protein expression of collagen I and elastin. Interestingly, treatment with amino acids without Cu^{2+} did not affect collagen I and elastin expression. We further compared the effect of Cu^{2+} with and without amino acids. We found that the gene levels of collagen I and elastin were increased under copper-treated conditions, even without amino acids. From this result, we conclude that Cu^{2+} induces the gene expression of collagen I and elastin. However, when we checked the protein levels, formulations containing both amino acids and Cu^{2+} showed higher expression of ECM proteins compared to the Cu^{2+}-only treated group. These results suggest that amino acids and copper (as Cu^{2+}) have synergistic effects on the translation of mRNA involved in ECM production, thereby increasing protein expression. Collectively, our results suggest that Cu^{2+} increases the gene expression of collagen I and elastin, and the combination with amino acids further increased protein translation, suggesting the therapeutic potential of Cu^{2+} and amino acid mixtures on skin aging (Fig. 5).

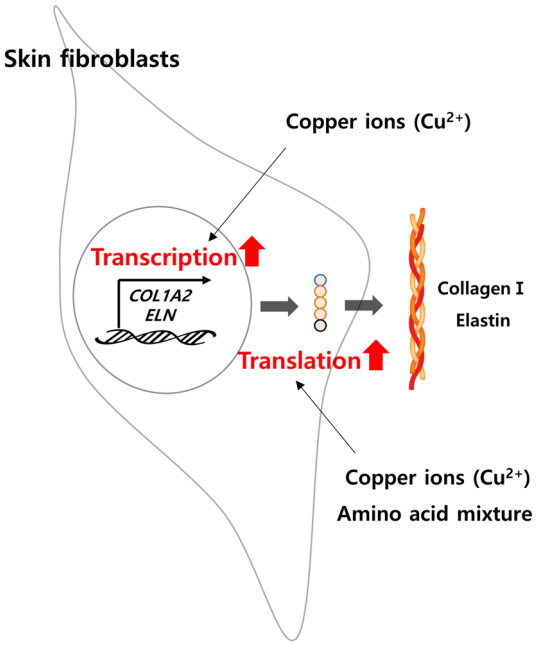

Fig. 5 Mechanisms of copper and amino acid mixtures on the production of extracellular matrix proteins in skin fibroblasts

Author Contributions KWC: Performed experiments, writing, funding acquisition. SHS: Methodology, performed experiments, materials provided. MK: Conceptualization, funding acquisition.

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Declarations

Conflict of interest None.

Consent to participate All the authors consent to publish this manuscript.

Consent for publication The manuscript is approved by all authors for publication.

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