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ORIGINAL ARTICLE

Synergy of GHK-Cu and hyaluronic acid on collagen IV upregulation via fibroblast and ex-vivo skin tests

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Abstract

Introduction: GHK-Cu and HA are two commonly used skin care ingredients, both of which were reported to enhance collagen synthesis. This work aims to investigate their co-effect on collagen regulation.

Materials and Methods: In cell experiments, human dermal fibroblasts were treated by a series of GHK-Cu and HA combinations, and the expressions of collagen I, IV, and VII were measured by qRT-PCR. The best formula screened out from cell experiments were further studied by ex-vivo skin model, and the content of collagen IV was quantified by immunofluorescence method.

Results: The combination GHK-Cu and HA was found to promote the generation of collagen I, IV, and VII. Especially, they form a synergy on collagen IV. At the ratio of 1:9, GHK-Cu and LMW HA deliver the strongest effect to elevate collagen IV synthesis by 25.4 times in cell test and 2.03 times in ex-vivo skin test.

Conclusion: The co-effect of GHK-Cu and HA was revealed. Their synergy brings an insight to anti-aging technology: choosing proper molecular weight of HA and managing its ratio with GHK-Cu could enhance DEJ health via stimulating collagen IV synthesis.

KEYWORDS
collagen, hyaluronic acid, skin care, synergy

1 | BACKGROUND

GHK (glycyl-L-histidyl-L-lysine) is a tripeptide, occurring in human serum, plasma, saliva, and urine,[1] which has a strong affinity for copper ions (Cu^{2+}) to form GHK-Cu complexes. In subsequent studies, its benefits for skin health were revealed. Miller D. M. and Park J. R.'s research[2,3] indicated that GHK-Cu could reduce the production of TNF-α and IL-6 by blocking the activation of NF-κB p65 and p38 MAPK to inhibit excessive inflammatory responses. In Hong's study,[4] GHK-Cu was found to affect gene expression, including YWHAB, MAP3K5, LMNA, APP, GNAQ, F3, NFATC2, and TGM2, to regulate the immune system and tissue regeneration. Furthermore, Siméon A. and Wegrowski Y.[5,6] discovered that GHK-Cu could stimulate the synthesis of glycosaminoglycans (GAGs), increase the accumulation of chondroitin sulfate, and boost collagen I production. Moreover, GHK-Cu was found to increase skin clarity and reduce wrinkles.[7,8] Therefore, GHK-Cu has been widely used in skin care products as a key active ingredient.

Another commonly used skin care compound is hyaluronic acid (HA). As an endogenous substance, it distributes throughout human body, including eye, cavum articulare, and skin.[9] In skin, HA is synthesized by hyaluronan synthases (HAS1, HAS2, HAS3),[10] and its level declines dramatically during aging. For example, the epidermal HA content decreases from 0.03% in 19–47-year-old women down

to 0.015% in 60-year-old women.[11] Therefore, it is necessary to supplement HA to maintain skin hydration and elasticity.[12] As a natural moisturizing factor (NMF), not only does HA form hydrogen bonds with water, but also it controls water mobility by interacting with AQP3.[13] Moreover, the interaction between HA and CD44 evokes the signal for self-renewal and maintenance,[14] which regulates multiple bioreactions. LMW HA (MW < 10 K Da) was found to stimulate the migration and proliferation of endothelial cells, promote the synthesis of collagen,[15] and upregulate inflammatory mediators. While, HMW HA (MW > 1000 K Da) was reported to inhibit the production of pro-inflammatory mediators,[16-19] decrease oxidative stress, and inhibit angiogenesis.[20-22] In addition, chemically modified HA, such as acetylated HA (AcHA), shows better bioavailability than normal HA,[23] being able to decrease MMP expression and slow down ECM degradation induced by chronobiological and extrinsic aging.[24]

However, the co-effect of GHK-Cu and HA was rarely reported. On one hand, HA has affinity for copper ions, thanks to its carboxyl groups, and thus, it may interact with GHK-Cu molecules. On the other hand, both HA and GHK-Cu could regulate collagen synthesis. Next, they might form a co-effect on collagen regulation, and this work aims to reveal this phenomenon via cell and ex-vivo skin model tests.

2 | METHOD AND MATERIALS

2.1 | Materials

Three different HA materials were employed for experiments: LMW HA (1 K–5 K Da), HMW HA (1000 K–1200 K Da), and AcHA (75% acetylated, MW 30 K–50 K Da). HA materials were > 99.5% purity, supplied by Bloomage Biotechnology Co., Ltd. The material of GHK-Cu was a chemically synthesized reagent with >99% purity, supplied by Zhejiang Peptites Biotech Co., Ltd. Before tests, HA and GHK-Cu were prepared into solutions with DI water, according to the formulas in Tables 1 and 2.

Human dermal fibroblasts (HDFs, lot# Fb19052002) and skin explants (lot# EX221011) were used for cell and ex-vivo skin model tests, respectively. Accordingly, two different mediums, KcGrowth medium (Cat. # PY3011), and EpiGrowth medium (Cat. # PY3021), were employed. The materials were supplied by Guangdong Biocell Biotechnology Co., LTD.

In cell experiments, the following reagent kits were employed: Total RNA extraction reagent kit (RNAiso Plus, Takara®), reverse transcription reaction kit of cDNA synthesis (PrimeScript™ RT reagent Kit, Takara®), green fluorescence reagent kit (SYBR Premix EX Taq™ II, Takara®), and recombinant human TGF-beta1-Mammalian (TGF-β1, Peprotech®). The following chemicals were used: MTT (Sigma-Aldrich®), Dimethyl sulfoxide (DMSO, Sigma-Aldrich®), and fetal bovine serum (FBS, Cat. # RY-F22-05, Royacel®).

In ex-vivo skin model test, VC, VE (Sigma-Aldrich®), paraformaldehyde solution (Biosharp®), anti-collagen IV antibody (Cat. # ab6311, Abcam), and the conjugated antibody (Cat. # ab150117, Abcam) were used.

TABLE 1 Experimental design of cell tests.

Case	Test formulas
Cell-BC	–
Cell-NC	–
Cell-PC	100 ng/mL TGF-β1
Cell-1	300 μg/mL GHK-Cu
Cell-2	300 μg/mL LMW HA
Cell-3	300 μg/mL AcHA
Cell-4	300 μg/mL HMW HA
Cell-5	10 μg/mL GHK-Cu, 290 μg/mL LMW HA
Cell-6	30 μg/mL GHK-Cu, 270 μg/mL LMW HA
Cell-7	90 μg/mL GHK-Cu, 210 μg/mL LMW HA
Cell-8	10 μg/mL GHK-Cu, 290 μg/mL AcHA
Cell-9	30 μg/mL GHK-Cu, 270 μg/mL AcHA
Cell-10	90 μg/mL GHK-Cu, 210 μg/mL AcHA
Cell-11	10 μg/mL GHK-Cu, 290 μg/mL HMW HA
Cell-12	30 μg/mL GHK-Cu, 270 μg/mL HMW HA
Cell-13	90 μg/mL GHK-Cu, 210 μg/mL HMW HA

TABLE 2 Experimental design of ex-vivo skin model tests.

Case	Test formulas
Tissue-BC	–
Tissue-NC	–
Tissue-PC	100 μg/mL VC, 7 μg/mL VE
Tissue-1	300 μg/mL GHK-Cu
Tissue-2	300 μg/mL LMW HA
Tissue-3	30 μg/mL GHK-Cu, 270 μg/mL LMW HA

2.2 | Cell experiment

First, cell viabilities were assessed by GHK-Cu and HA solutions, where GHK-Cu concentration was from 0.0391 to 5 mg/mL, and HA concentration was from 0.0781 to 10 mg/mL. A series of HDF cell samples were cultured in KcGrowth medium with 10% FBS for 24 h. The culture condition was 37°C and 5% CO_2, the same below. Then, GHK-Cu and HA solutions were spiked into the mediums, respectively. The samples were cultured for another 24 h. After that, a mixture of KcGrowth medium and MTT (0.5 mg/mL in the medium) was dosed into the samples for another 4-h culture. Finally, the microplate reader (BioTek®) was used to measure optical density (OD) for cell viability calculations.

In the formal test, different combinations of GHK-Cu and HA with the identical total concentration were studied, as shown in Table 1. In Cell-5–13, the ratio of GHK-Cu and HA varies from 1:29 to 3:7 (wt:wt). In Cell-1–4, single-compound legs were tested. Blank, negative and positive controls were placed as the reference, that is, Cell-BC, -NC, and -PC.

In Cell-PC and Cell-1–13, the cells were cultured for 24 h after formula dose, and exposed to a 365-nm-wavelengthed UV light with the energy of 30 J/cm^2 for 26 mins. The negative control, Cell-NC, underwent the same UV exposure but without formula treatment. The blank control, Cell-BC, was not treated by formulas or UV light.

After another 24-h culture, measurements were carried out. The levels of collagen- I, IV, and VII were detected by real-time reverse transcription PCR (qRT-PCR) assay with specific primers. Total RNA was reverse-transcribed to cDNA with PrimeScipt™ RT reagent kit. PCR reaction was performed by using specific primers and SYBR Premix EX Taq™ II kit. Then, the fluorescence was quantified by the Bio-Rad detector (BioRad®).

2.3 | Ex-vivo skin model test

In the ex-vivo skin model test, two single-compound cases and one combination case were studied. Detailed information was shown in Table 2. The ratio of GHK-Cu and HA in the combination case was the one screened out by cell experiments. Blank, negative, and positive controls were included, that is, Tissue-BC, NC, and PC.

Except for Tissue-BC, all the other explants were first exposed to UV light (365 nm UV with 30 J/cm^2 for 1600 s, and then, 302 nm UV with 50 mJ/cm^2 for 21 s). After that, the explants were soaked in fresh mediums, and treated by test formulas. Then, they were cultured for 24 h to complete one treatment cycle. The cycles were repeated four times, and the mediums were refreshed in each cycle. After 4 cycles, the explants were cultured for another 72 h in the incubator before harvest. To be noted, the blank control, that is, Tissue-BC, was cultured for 7 days without UV or formula treatment.

Finally, the explants were stained supravitally. Collagen expression was detected by a primary antibody that binds to the target and a secondary conjugated antibody with fluorescent chromogenic labeling that binds to the primary. The nuclei were counterstained by 4′,6-diamidino-2-phenylindole. The immunostaining was captured by a fluorescence microscope (Leica® DM2500) and analyzed by ImageJ software. The value of integrated optical density (IOD) was calculated to present the content of collagen IV.

2.4 | Statistical analysis

In each case, the average result and standard deviation were calculated from six replicates. A double-tailed *t*-test ($\alpha = 0.05$) was used for data comparison, and the results were presented as letters, shown in Figures 2–4. If two cases contain identical letter(s), they are considered to be parity; while, if the letter(s) of two cases are totally different, they are considered to be significantly different.

3 | RESULTS AND DISCUSSION

3.1 | Cell test results

The results of cell viability were plotted in Figure 1. It can be seen that the curves of HA treatment tend to be higher than that of GHK-Cu treatment. For example, when HA concentration (three types of HA) is below 1.25 mg/mL, cell viability remains higher than 90%. While, the cell viability begins to decline dramatically when the concentration of GHK-Cu exceeds 0.31 mg/mL. Therefore, in the following cell experiments, the total concentration of test formulas was set to be 0.3 mg/mL (i.e., 300 μg/mL) to ensure all cell viabilities were greater than 90%.

The gene amplification results of collagen-I, IV, and VII were summarized in Figures 2–4, respectively. By comparing the data between test formulas and negative controls, one can conclude the formula impact on collagen regulation.

First, the results of single-compound cases in Figures 2–4, that is, Cell-1–4, indicate that GHK-Cu could significantly boost collagen I, IV, and VII generation. While LMW HA, HMW HA and AcHA can only enhance the generation of collagen I and VII. Among the HA materials, LMW HA and AcHA show better boosting effect on collagen I synthesis but worse on collagen VII, versus MW HA.

Second, by comparing the results between combination and single-compound cases in Figures 2–4, that is, Cell-5–13 versus Cell-1–4, the co-effect of GHK-Cu and HA can be discovered. For collagen I and VII regulation, there is no synergy effect, but a certain antagonism. For example, in Figure 2, the results of Cell-5–10 are lower than those of Cell-1–3, which means the mixture of GHK-Cu and LMW HA/AcHA delivers a weaker boosting effect on collagen I versus single compounds. Similar situations can be found in collagen

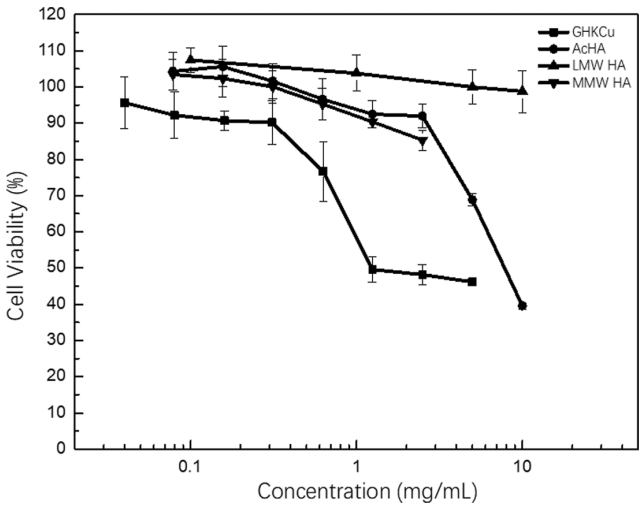

FIGURE 1 Results of cell viabilities.

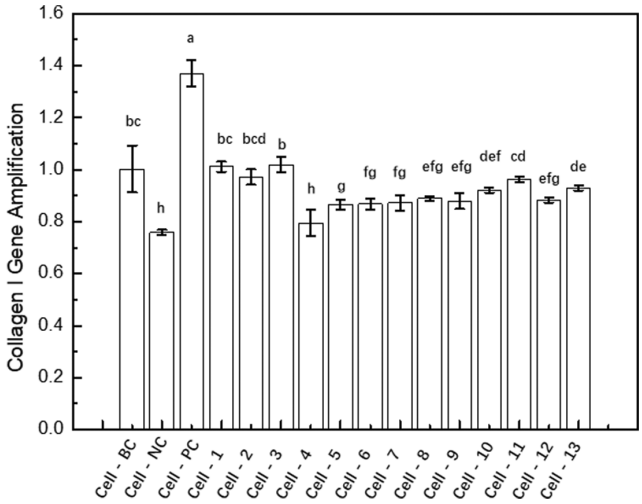

FIGURE 2 Results of collagen I gene amplification in cell tests. (AVE ± SD, $n = 6$).

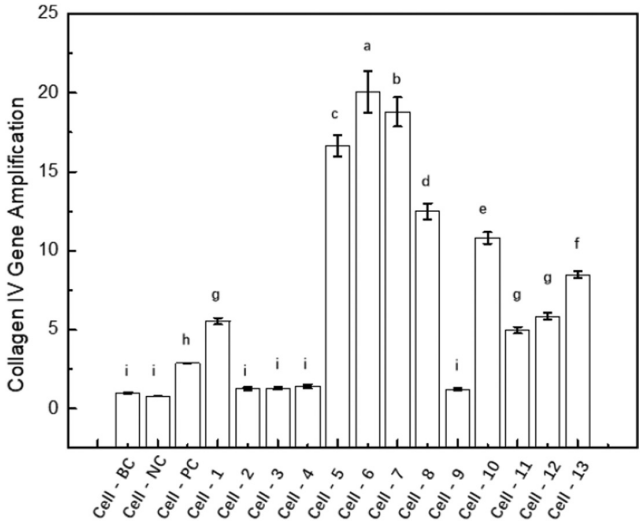

FIGURE 3 Results of collagen IV gene amplification in cell tests. (AVE ± SD, $n = 6$).

VII results, that is, Cell-11–13 versus Cell-1, 4 in Figure 4. In the other test conditions, GHK-Cu and HA present additive effect on collagen I and VII enhancement.

While, a significant synergy between GHK-Cu and HA was discovered in collagen IV regulation, that is, Figure 3. The most outstanding data are Cell-5–7, that is, the mixture of GHK-Cu and LMW HA. As its reference, the collagen boosting rate of GHK-Cu is only 7.01 times versus negative control, and LMW HA does not show any boosting effect. However, when these two compounds are combined at the ratio of 1:29–3:7, they elevate collagen IV generation by 21.08 ~ 25.42 times versus negative control. Similar synergies can be achieved by mixing GHK-Cu and HMW HA, that is, Cell-11 ~ 13, but the absolute values are not as high as Cell-5–7. The co-effect between GHK-Cu and AcHA is slightly different, for they only present synergy at the boundary conditions of the mixing range, but no synergy at the ratio of 1:9.

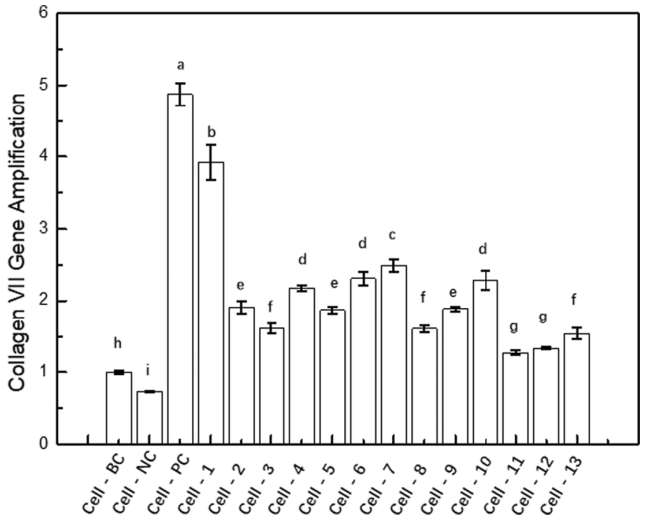

FIGURE 4 Results of collagen VII gene amplification in cell tests. (AVE ± SD, $n = 6$).

The synergy of GHK-Cu and HA on collagen IV upregulation may result from the collaborative effect of generation stimulation and degradation protection, as illustrated by Figure 6. GHK-Cu increases the production of glycosaminoglycans (GAGs) in fibroblasts, stimulating the synthesis of chondroitin sulfate (CS) to accelerate the production of collagen.[25,26] It is hypothesized that different cellular pathways are engaged to stimulate gene expression such as TGF-β and TIMPs pathways. Meanwhile, the over-expressed matrix metalloproteinases (MMPs) stimulated by ROS could accelerate the degradation of collagens. HA presents the ability to inhibit MMP synthesis by scavenging ROS[27] and activating thioredoxin reductase (TRXR), so as to protect collagen degradation.

However, there exists an optimum HA amount to achieve this synergy. Obviously, when HA level is low, the degradation protection is insufficient, but if its level is too high, HA may weaken the interaction of GHK-Cu and receptors through forming a hydration layer around cells. Meanwhile, HA MW also plays a key role in the mechanism. The chelating effect of HA elevates when MW increases, and as a result, HMW HA would form excess force with GHK-Cu to reduce its connection with receptors. Additionally, LMW HA shows better antioxidant capability than HMW HA to quench ROS. Therefore, the best synergy in fibroblast test was achieved by mixing GHK-Cu and LMW HA at the ratio of 1:9.

3.2 | Ex-vivo skin model results

As found in cell experiments, the mixture of GHK-Cu and LMW HA at the ratio of 1:9 could form the most significant synergy to boost collagen IV generation. This benefit was further verified by the ex-vivo skin model, results shown in Figure 5. In the fluorescence photos, blue color represents cell nuclei and green color indicates collagen IV. The intensity of green fluorescence quantitatively reflects the concentration of collagen IV. To be noted, the green line located in

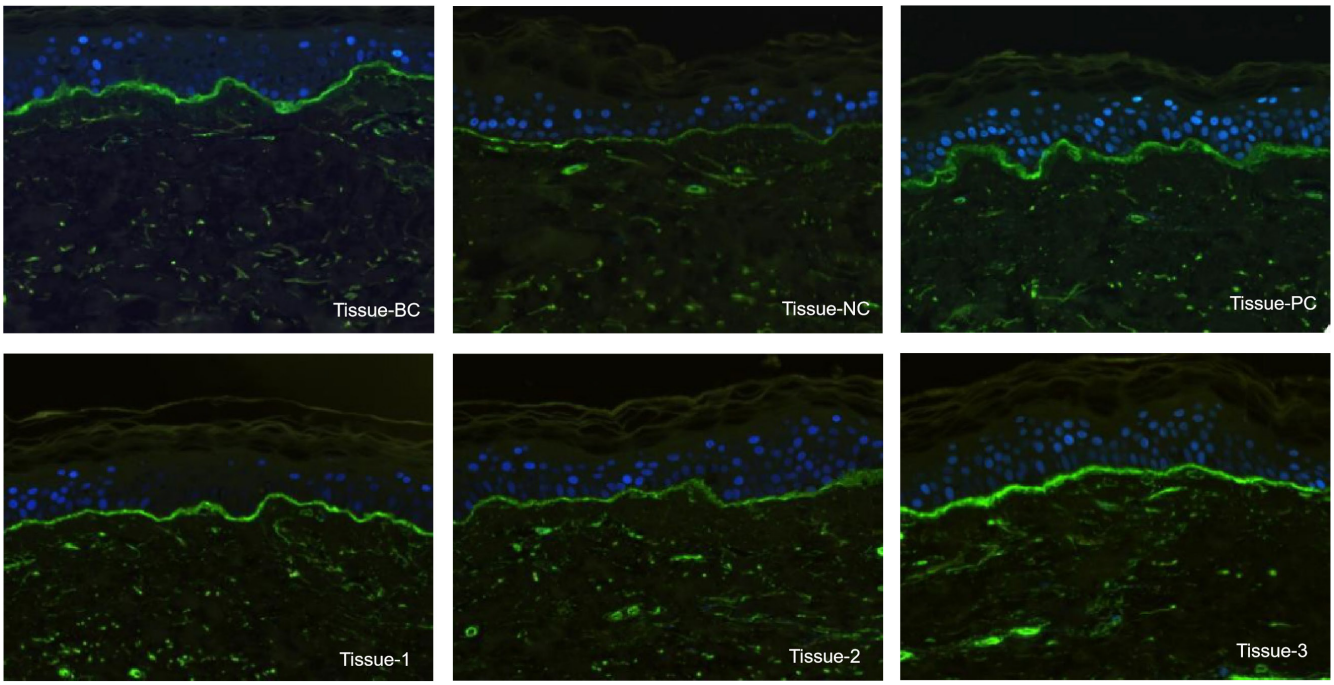

FIGURE 5 Immunofluorescence photos of collagen IV content in ex-vivo skin model tests. (green color: collagen IV; blue color: nuclei).

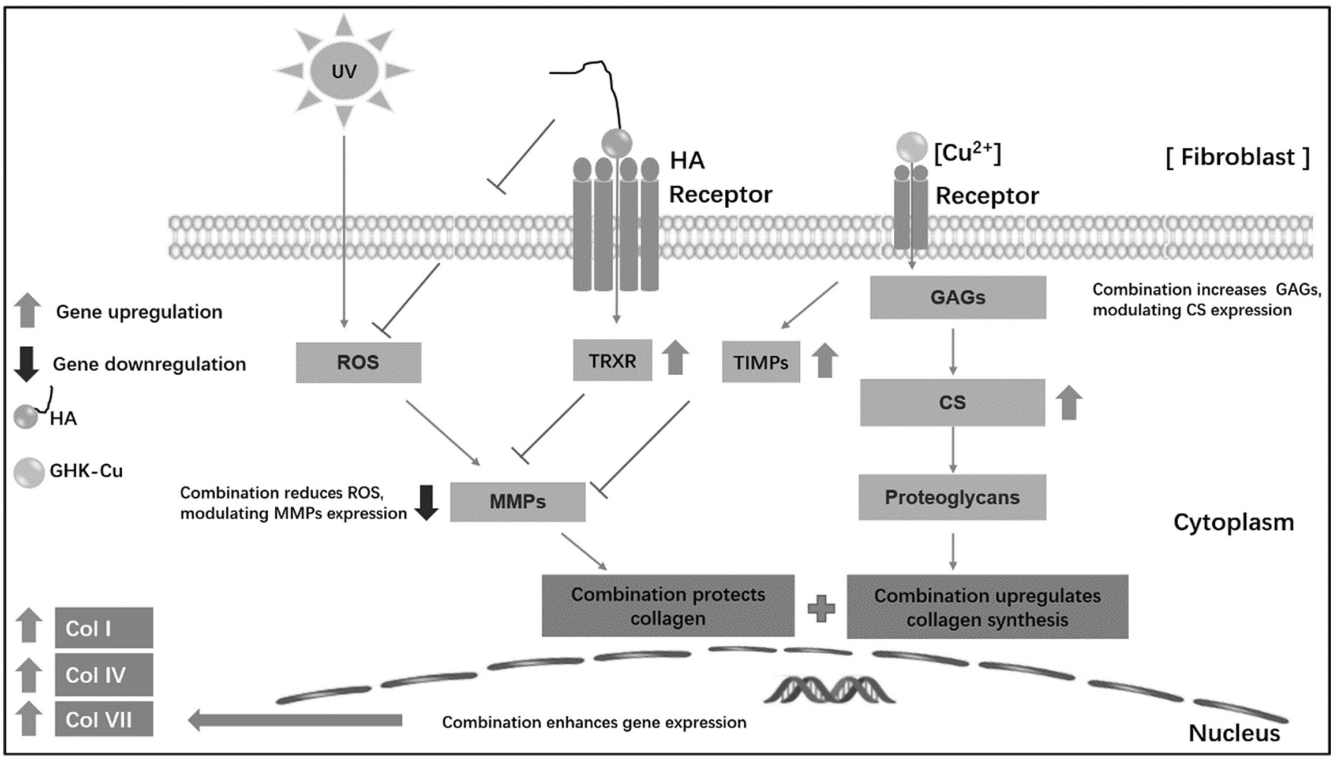

FIGURE 6 Explanation of the synergy mechanism between GHK-Cu and HA on collagen upregulation.

the middle of each photo represents the dermal-epidermal junction (DEJ) which contains the highest content of collagen IV.

Obviously, the concentration of collagen IV in Tissue-1, 2, 3 is much higher than that of negative control Tissue-NC, which is consistent with the learning of cell experiments. Among 3 test formulas, the combination case Tissue-3 shows the highest fluorescence intensity, which clearly verified the synergy of GHK-Cu and HA to upregulate collagen IV at skin level.

Table 3 converts the fluorescence intensity into IOD values and calculates the upregulation rate of each formula. The combination

TABLE 3 Collagen IV IOD values and upregulation rates in ex-vivo skin model tests.

Case	IOD values, (AVE ± SD)	Collagen IV upregulation rates vs. negative control, (times)
Tissue-BC	1.000 ± 0.09	1.40
Tissue-NC	0.715 ± 0.03	–
Tissue-PC	0.962 ± 0.08	1.35
Tissue-1	1.117 ± 0.08	1.56
Tissue-2	1.066 ± 0.11	1.49
Tissue-3	1.452 ± 0.04	2.03

of GHK-Cu and HA delivers a higher rate than single compounds, that is, 2.03 times versus 1.56/1.49 times. Interestingly, the absolute value of collagen upregulation is lower than what discovered in cell test. The reason may lay on two facts: (1) the diffusion speeds of GHK-Cu and HA in skin is different, leading to a deviation from the best ratio when they stimulate the fibroblasts; (2) GHK-Cu and HA are consumed by other physiological processes, diluting their effect on fibroblasts. To be noted, although the synergy of GHK-Cu and HA was revealed by cell and ex-vivo skin tests, their actual benefit in skin care products requires additional technology assistance.

On one hand, their penetration speeds in human skin need to be matched to ensure the fibroblasts are treated by desired species ratios. Then, encapsulation or penetrating enhancement technologies would be employed, such as liposome or supermolecules.

On the other hand, formula conditions need to be precisely adjusted to keep the active stable. For example, the impact of formula pH on GHK-Cu stability was discovered in this work. Only when the formula pH is within 5.5–6.5, GHK-Cu can survive the accelerated aging test, that is, 50°C—1 month condition, with less than 5% degradation.

Since collagen IV is one of the key components in DEJ, upregulating its level could effectively maintain and improve DEJ functionality. Particularly in aged skin, DEJ becomes thin and flat, leading to a poor transfer of nutrients and biological information.[28] Thus, it can be inferred that the synergetic combination of GHK-Cu and HA would be beneficial for skin anti-aging.

4 | CONCLUSION

The co-effect of GHK-Cu and HA on collagen regulation was revealed. For collagen I and VII, the combination presents boosting effect, but it lowers the upregulation ability of single compounds. For collagen IV, GHK-Cu and HA form a synergy to elevate collagen synthesis. Especially, at the ratio of 1:9, GHK-Cu and LMW HA deliver the strongest synergy to increase collagen IV generation by 25.42 times in cell test and 2.03 times in ex-vivo skin test. This learning brings an insight to skin anti-aging technology: choosing proper molecular weight of HA and managing its ratio with GHK-Cu could enhance DEJ health by stimulating collagen IV synthesis. In practical applications, formulation technologies, such as encapsulation and penetration enhancement, are recommended to assist this benefit delivery.

AUTHOR CONTRIBUTION

F. J., M. H., and Y. H. performed the experiments; Z. L., Y. W. provided the idea and designed the experiments; Z. L., F. J., and Y. W. wrote the paper.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL APPROVAL

Authors declare human ethics approval was not needed for this study.

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