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Effects of GHK-Cu on MMP and TIMP Expression, Collagen and Elastin Production, and Facial Wrinkle Parameters

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Abstract

Background: Glycyl-L-histidyl-L-lysine-copper (GHK-Cu) is an endogenous tripeptide-copper complex involved in collagen synthesis and is used topically as a skin anti-aging and wound healing agent. However, its biological effects are yet to be fully elucidated.

Objectives: To investigate the effects of GHK-Cu on gene expression of metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), and on production of collagen and elastin by human adult dermal fibroblasts (HDFa); and to investigate the effectiveness of topical application of GHK-Cu on wrinkle parameters in volunteers.

Methods: Cultured HDFa were incubated with GHK-Cu at 0.01, 1 and 100 nM in cell culture medium. Gene expression (mRNA) for MMP1, MMP2, TIMP1 and TIMP2 in treated and control HDFa was measured by RT-PCR. Cellular production of collagen and elastin was measured colourmetrically using commercial assay kits. Correlations between gene expression and collagen and elastin production were determined. A randomised, double-blind clinical trial involving twice daily application of GHK-Cu, encapsulated in lipid-based nano-carrier, to facial skin of female subjects (n= 40, aged 40 to 65) was run over 8 weeks. The formulation vehicle (a serum) and a commercial cosmetic product containing Matrixyl® 3000, a lipophilic GHK derivative, were used as controls.

Results: GHK-Cu significantly increased gene expression of MMP1 and MMP2 at the lowest concentration whilst simultaneously increasing the expression of TIMP1 at all the tested concentrations. All examined concentrations of GHK-Cu increased both collagen and elastin production. An increase of the mRNA expression ratio of TIMPs to MMPs was associated with an increase in collagen/elastin production. Application of GHK-Cu in nano-carriers to facial skin of volunteers significantly reduced wrinkle volume (31.6%; $p=0.004$) compared to Matrixyl® 3000, and significantly reduced wrinkle volume (55.8%; $p<0.001$) and wrinkle depth (32.8%; $p=0.012$) compared to control serum.

Conclusions: GHK-Cu significantly increased collagen and elastin production by HDFa cells depending on the relative mRNA expression of their TIMP(s) over MMP. Topical application of GHK-Cu with the aid of nano-carriers reduced wrinkle volume to a significantly greater extent than the vehicle alone or a commercial product containing Matrixyl 3000®, a GHK lipophilic derivative.

Keywords: GHK-tripeptide; Metalloproteinases (MMP); Tissue inhibitors of metalloproteinases (TIMP); Cell culture; Delivery; Skin barrier

Introduction

The peptide glycyl-L-histidyl-L-lysine-copper (GHK-Cu) is gaining interest as an anti-aging and wound healing bioactive agent [1-3]. GHK is capable of up and downregulating over 4000 genes [4]. Previously, the effects of GHK-Cu on collagen production and metalloproteinase (MMP) expression have been investigated in cultured rat fibroblasts and in rat wound healing models [5-7]. GHK-Cu functions as an activator of tissue remodelling and increases secretion of MMP2 and a number of tissue inhibitors of metalloproteinases (TIMP1 and TIMP2) in cultured fibroblasts [7,8]. MMP1, 8 and 13 degrade mainly fibrillar collagens whilst gelatinases MMP2 and 9, act on type IV collagen in the basement membrane and elastin. Increased expression of MMPs typically occurs in heightened inflammatory responses that are usually marked by opposing inhibitory processes [9]. TIMPs tightly control MMP activities through competitive irreversible inhibition, thereby controlling the breakdown and re-synthesis of the extracellular matrix [10,11].

Therefore, increased TIMP expression in the skin may have anti-wrinkle benefits. While this has yet to be determined, topical application of GHK-Cu has resulted in beneficial effects on wrinkles [12]. A 12 week trial of topically administered GHK-Cu in 71 volunteers demonstrated improvements in fine lines, viscoelastic properties, thickness and density of the skin, without irritation [12]. Other trials report significant improvements in skin appearance [13],

increased dermal keratinocyte proliferation and increased pro-collagen synthesis [14]. Maquart et al. found dose related effects of GHK-Cu, including increases in dry weight, total protein, collagen and glycosaminoglycan content in rat skin [15].

Topical application of GHK-Cu, amongst numerous other peptides, is widely promoted in the cosmetic industry [16]. Our previous pre-formulation studies, however, showed the logD of GHK-Cu at pH 4.5 and 7.4 to be -2.49 ± 0.33 and -2.49 ± 0.35, respectively, suggesting the tripeptide is highly hydrophilic [17]. Therefore, while this peptide may have considerable biological potential, the efficient trans-epidermal delivery of GHK-Cu is challenging based on its physicochemical properties. Ideally, compounds should have a moderate oil-water partition coefficient (log P) of between 1-3 and few polar centres in order to permeate into the skin [18]. To overcome the epidermal barrier, in this present study GHK-Cu was formulated into a lipophilic nano-carrier that improves delivery into the skin. Increased lipophilicity may also be achieved by combining GHK with a lipophilic moiety. For example, Matrixyl 3000®, containing a chemical combination of GHK and palmitic acid, is currently used as an active component in commercial cosmetic products.

There are potential differences associated with GHK-Cu application to rat and human fibroblasts [19]. This study therefore aimed to determine the effect of GHK-Cu, for the first time, on human adult dermal fibroblasts (HDFa), using collagen, elastin, and mRNA expression of MMP1, MMP2, TIMP1 and TIMP2 as biological markers. Further, the effect of GHK-Cu on wrinkle parameters was evaluated in volunteers, comparing a product containing Matrixyl 3000®, and a serum vehicle control, in a randomised double-blind, split-face, trial. Wrinkle depth and volume changes were used as endpoints. Given our previous study [17] demonstrated that GHK-Cu is highly hydrophilic, a lipid-based nano-carrier system was employed for delivery into the skin. This trial is the first to investigate the biological effect of GHK-Cu formulated into a nano-carrier.

Materials and Methods

Materials

GHK-Cu was purchased from Salkat Ltd (Auckland, New Zealand). Snowberry New Zealand Limited provided the New Radiance Face Serum (NRFS - nano-carrier containing GHK-Cu in a serum vehicle) and CONTROL (the serum vehicle without GHK-Cu or nano-carrier). Strivectin SD Advanced Intensive Concentrate (SSID) was purchased online. All Taqman gene expression assay kits, reagents, 18S housekeeper genes, SuperScript complimentary DNA Synthesis kit and PureLink RNA mini kit were purchased from Applied Biosystems (Life technologies, Auckland, New Zealand). Reagents for agarose preparation (SeaKem LE agarose) were purchased from Lonza (Auckland, New Zealand). Human adult primary dermal fibroblasts (HDFa) were purchased from Invitrogen (Invitrogen, USA). All phosphate buffered saline (PBS, 10 mM) was freshly prepared (8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4 in 1 litre water and adjusted to pH 7.4 with HCl. All water used was prepared by reverse osmosis.

Cell culture of primary human dermal fibroblasts

Fibroblasts, passage numbers between 4 and 8, were seeded in 6-well growth plates at a density of 30,000 cells per well. Cells were incubated in 3 mL DMEM with 1.5% FBS and 50 U ml^{-1} each of penicillin and streptomycin for 24 h at 37°C in a Heracell 150i incubator (Thermo Fisher Scientific, Victoria, Australia) maintaining a 5% CO2 atmosphere with >80% relative humidity. The cells were incubated with GHK-Cu solutions in DMEM for 24 hours. The final concentrations of GHK-Cu were 0.01, 1 and 100 nM, a range previously reported to produce a response in MMP and TIMP expression in *in vitro* rat fibroblasts [7]. Control cultures received an additional volume of water (without the GHK-Cu) at the same time as the treated cultures. Each condition was examined in triplicate, with each culture tested three times. Following removal of medium, cells were processed for RNA extraction.

RNA extraction and PCR

RNA extraction was performed using a RNA extraction kit based on the manufacturer's protocol (Biocolor, Carrickfergus, Ireland). Briefly, control and treated cells were washed with PBS three times, cells transferred to RNase-free tubes and centrifuged at 2000 x g for 5 minutes to obtain a cell pellet. The supernatant was removed and 0.6 mL lysis buffer added along with 1% 2-mercaptoethanol to re-suspend the pellet. The dispersion was mixed with a 70% ethanol solution at 1:1 (v/v) and centrifuged in a spin cartridge tube and attached collection tube. The collection tube was washed twice with buffer to purify the RNA, which was eluted after a final wash with RNase-free water.

RNA quality and quantity assessment

Concentration and integrity of extracted RNA was quantified using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA). Purity criteria was >1.8 for 260/280 ratio (the *ratio* of absorbance at 260 nm and 280 nm) and >2 for the 260/230 ratio (Nanodrop technical support documentation). Confirmation of quality was by Agarose gel electrophoresis. To prepare the gel substrate, 2.6 g of SeaKem LE agarose was dissolved in 65 ml tris-borate-EDTA (TBE) buffer at 80°C, placed onto the frame, and allowed to set for a minimum of 30 minutes. TBE buffer was added, covering the gel completely, before PCR product (10 µL) was added to the wells along with 5 µL Blue Juice Loading Buffer. A 100V potential difference was placed across the gel and left for 60 minutes. The gel was stained with 100 ml TBE buffer containing 5 µL of a 10 mg mL^{-1} ethidium bromide (EtBr) solution. After 15 minutes the EtBr was washed off with water and imaged using a GelDoc EZ Imager (Biorad Laboratories, Auckland, New Zealand).

Reverse transcription (cDNA synthesis) and RT-qPCR

The extracted RNA was reverse transcribed using the SuperScript complimentary DNA (cDNA) Synthesis kit using the manufacturer's protocol (Applied Biosystems). Briefly, the reaction mixture comprised 14 µL of RNA free water, 4 µL of 5× VILOTM master mix and 2 µL of 10× SuperScript® Enzyme Mix. Negative reverse transcription (RT) samples were produced as a control. The samples were incubated in an Applied Biosystems Gene Amp PCR 9700 for 10 min at 25°C, 120 min at 42°C and 5 min at 85°C, sequentially.

To quantify cDNA, real time quantitative polymerase chain reaction (RT-qPCR) was used [20,21]. RT-qPCR was performed with a predesigned mix (RTMIX) consisting of 5 µL Master Mix, 0.5 µL Gene Expression Assay, 0.5 µL 18S (a housekeeper gene), and 2 µL of RNase-free water.

The RTMIX (8 µl) was combined with 2 µL of cDNA sample and placed into a 384-well MicroAmp® plate. Each cDNA sample was prepared in triplicate and each condition was measured 3 times in 3

separate culture samples. After loading all the samples, the MicroAmp® plate was sealed and centrifuged for 1 minute at 2000 rpm. The plate was placed into a Sequence Detection System (Life technologies, Auckland, New Zealand) for DNA amplification. 6-Carboxyfluorescein (FAM) was used as the fluorophore for each Taqman gene expression probe whilst 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC) was used for the 18S housekeeper gene [22]. Amplification consisted of four-cycle stages: 50°C for 2 minutes, 60°C for 30 seconds, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. To calculate the relative gene expression the results from RT-qPCR were normalized using the $2^{-\Delta\Delta Ct}$ method [23].

Collagen and elastin quantification

Cells were seeded in 12-well plates (surface area 3.77 cm^2 per well) at a density of 1×10^5 cells per well. Each well contained 3 mL cell culture medium without bovine serum. After the initial 24 h to allow cell settling and attachment, each well was supplemented with GHK-Cu solution (in water, 100 µL). Cells were treated for 48 and 96 h. Control samples were supplemented with water. Following treatment, cell culture medium was collected for collagen measurement, while the cells were detached by incubation with 250 µL of trypsin for 10 minutes at 37°C for elastin measurement.

Collagen measurement: Total soluble collagen content in culture medium was measured using the Sircol soluble collagen assay kit as described by manufacturer's protocol (Biocolor, Carrickfergus, Ireland) [24]. To isolate the collagen, 1 mL of cell culture medium was placed into a 1.5 mL Eppendorf tube and mixed with a 200 µL aliquot of the provided isolation and concentration reagent (polyethylene glycol TRIS-HCl buffer, pH 7.6). Following vortex mixing for 30 seconds, samples were incubated at 4°C overnight to allow collagen to precipitate. Samples were then treated as per manufacturer's instructions (Biocolor, Carrickfergus, Ireland) and transferred to a 96 well plate for absorbance measurement at 555 nm. Reagent blanks were also measured for absorbance. The concentration of collagen was then calculated using a standard curve.

Elastin measurement: The cell suspension was transferred to a microcentrifuge tube and pelleted at 12,000 × g. 1.0 M oxalic acid solution (100 µL) was added to the pellet making a final concentration of 0.25 M oxalic acid solution and heated to 100°C for 1 h to convert insoluble elastin to soluble α-elastin. The samples were then treated as per manufacturer's instructions (Biocolor, Carrickfergus, Ireland) and transferred to a 96 well plate for reading at 513 nm [24]. Reagent blanks (250 µL) including oxalic acid, PBS and water served as controls. Absorbance at 513 nm from these control reagents was subtracted from the final readings of the sample, providing a reading for elastin. Absorbance values were converted into concentrations using a standard curve.

Effects on human facial wrinkle parameters

The trial was conducted at Dermatest GMBH, a cosmetic research institute, with 40 female volunteers (aged from 40 to 65 years) over the course of 8 weeks. All test products were supplied as identically packaged coded containers with investigators and subjects blinded to treatments. Prior to application all the subjects underwent a dermatological examination and a 10-day period of no cosmetic use. All participants gave informed consent.

Participants were randomly placed into two treatment groups: Group 1) NRFS serum, the Nano-carrier enhanced GHK-Cu serum and a product containing Matrixyl® 3000 (SSID) as a positive control, and Group 2) NRFS serum and CONTROL, the latter contained no GHK-Cu or nano-carrier, acting as a negative control. All subjects applied the NRFS serum twice each day (in the morning and again in the evening) to the right side of the face, and either the SSID (Group 1) or CONTROL (Group 2) to the left side of the face according to the same instructions. The participants were instructed to not apply other skin care products to the test areas during the course of the trial.

Measurement of wrinkle depth and volume

The Phaseshift Rapid *in vivo* Measurement of the Skin system (PRIMOS, GF Messtechnik GmbH, Teltow, Germany) was used as a three-dimensional analytical instrument for this study. The principle of this instrument has been previously described by Jaspers et al. [25]. Briefly, a digital stripe projection technique is used as an optical measurement process. A parallel stripe pattern is projected onto the skin over the wrinkle area and depicted on the CCD chip of a digital camera connected to an evaluation computer. The measurement head is then moved close to the immobilized head of the participant (Canfield Scientific Inc., NJ, U.S.A). The parallel projections are then distorted by the elevation differences on the skin and a three-dimensional effect recorded. The distortions provide a qualitative and quantitative measurement of the skin profile. They are digitised and quantitatively evaluated using software attached to the PRIMOS system. The measurement area was a 30 × 40 mm region, located as close as possible to the corner of the eye. A single large wrinkle was identified on each subject and measured for wrinkle depth and volume at 4 (NRFS and SSID) and 8 weeks (NRFS, SSID and CONTROL). Measurements of the same wrinkle at 4 and 8 weeks after treatment were compared with initial measurements.

Data analysis of *in vivo* trial data

R package lme (R version 3.0.2) was used to calculate statistical significance using regression analysis. The significance level was set at 0.05. For the primary analysis, comparing the NRFS serum and SSID, a multiple linear mixed model was used with random intercepts for each patient, to take account of the grouping by participants in the data. In the multiple linear mixed model, the groups being compared (NRFS vs SSID and NRFS vs CONTROL), with the right side of subject's face belonging to one group and the left side of the same subject's face belonging to the other treatment were made exactly comparable with regard to starting wrinkle depth, and eliminated the individual biological variation of the subjects. The equivalent analysis was performed for wrinkle volume.

Percentages reported in this study were calculated by averaging the percentage changes for each individual in the trial. The individual changes were calculated from the relative change percentages; e.g., starting skinfold depth (833.4 µm) was compared against the 8-week depth measurement (722.1 µm). The difference (111.3 µm) was expressed as a 13.35% change.

Results

RNA quality and quantity

All samples for RNA analysis met the purity criteria confirmed by Agarose gel electrophoresis. The 260/280 ratio was 1.95 ± 0.15 and the 260/230 ratio was 2.12 ± 0.08, over all the samples examined.

Effects of GHK-Cu on mRNA expression of MMPs and TIMPs

After 24 hours, expression of MMP1, MMP2, TIMP1 and TIMP2 in the GHK-Cu treated cell lines, relative to untreated controls, all showed a concentration dependency effect (Figure 1) with increased expression at lower GHK-Cu concentrations except for the TIMP2. For MMP1 and MMP2, interestingly, only the lowest concentration (0.01 nM) treatment resulted in significantly increased expression (p=0.03). TIMP1 expression was significantly increased at all concentrations, with a concentration effect, while TIMP2 expression was significantly decreased at higher concentrations (1 and 100 nM).

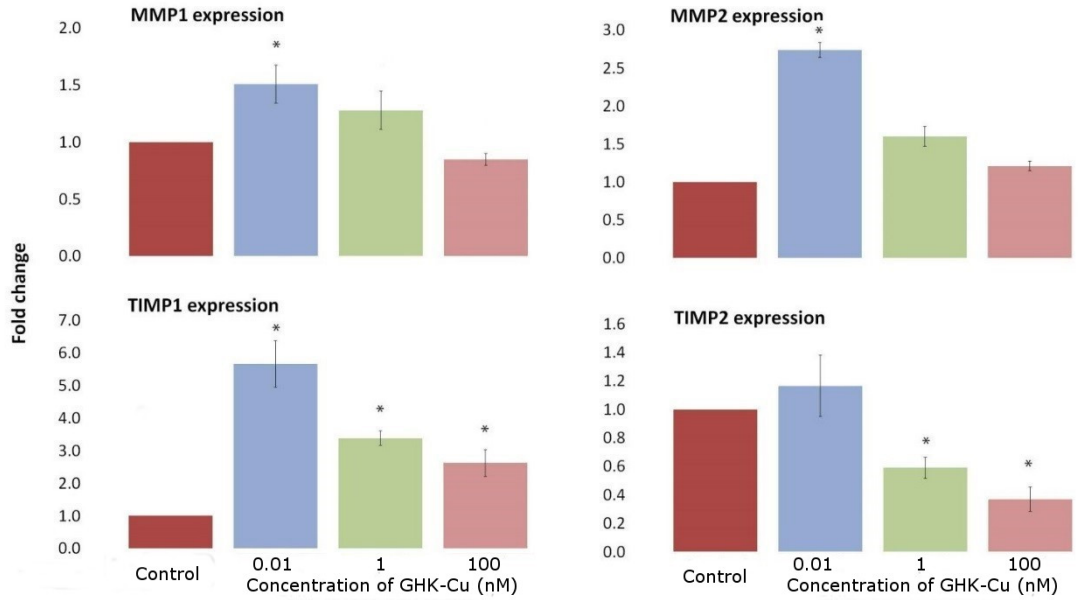

Figure 1: Effect of GHK-Cu on gene expression of MMP1, MMP2, TIMP1 and TIMP2 in HDFa cultures after incubation for 24 hours. Data are means ± SD, n=3 from three individual experiments. * Denotes p<0.05 statistical difference from control (untreated cells).

Effects of GHK-Cu on collagen and elastin production

Both collagen (0.0726x+0.05, R^2=0.983) and α-elastin (0.0178x + 0.05, R^2=0.984) standard curves showed a linear relationship between the absorbance and concentration within the ranges tested (5-15 μg/ml and 12.5-50 μg/ml respectively).

Production	Concentration of GHK-Cu (nM)	Treatment (hours)		
		0	48	96
Collagen	0 (control)	6.97 ± 1.0	7.55 ± 0.3	15.29 ± 0.4
	0.01		8.64 ± 0.3	18.04 ± 1.8*
	1		8.14 ± 0.7	17.07 ± 1.4*
	100		8.53 ± 0.5	16.63 ± 1.6*
α-Elastin	0 (control)	36.57 ± 3.8	79.03 ± 1.5	200.4 ± 2.5
	0.01		82.84 ± 4.0	257.97 ± 2.5**
	1		80.48 ± 5.6	271.09 ± 4.3**
	100		84.08 ± 2.4	268.2 ± 2.6**

Table 1: Collagen and elastin levels (μg/ml) of HDFa cells treated with GHK-Cu. Data are means ± SD, n=3. * p<0.05 and ** p<0.017 compared to the control.

After treated with GHK-Cu solutions, the production of collagen or α-elastin by fibroblasts only slightly increased at 48 hours compared with the non-treated cells (Table 1). For both collagen and α-elastin, GHK-Cu significantly increased secretion over the controls at 96 hours. There was an inverse dose dependent response for collagen production at 96 hours. Alpha-elastin was increased by approximately 30% at all concentrations but without a clear concentration dependency.

As the expression of MMP1 and TIMP1 reduced, with increasing concentration of GHK-Cu, there was a corresponding decrease in collagen. A similar trend did not occur for α-elastin.

Effect of GHK-Cu on wrinkle parameters

The Snowberry New Radiance Face Serum (NRFS), SSID (Strivectin SD Advanced Concentrate), and CONTROL (Control vehicle) were well tolerated by 39 of the 40 subjects throughout the eight-week application period. One participant experienced minor unwanted skin reactions on both the right and left side of their face after application of the NRFS serum and SSID. This subject ceased application and symptoms resolved without medical treatment. Wrinkle depth and volume changes at four and eight-weeks are summarised in Table 2. Comparisons and statistical significance between treatment groups are given in Table 3.

	Treatment	Wrinkle parameter (weeks)	Percent change from baseline
	NRFS serum	Depth (4)	-18.3 ± 10.3
	(n=19)	Depth (8)	-26.8 ± 12.8
		Volume (4)	-17.2 ± 8.1
Group 1		Volume (8)	-25.8 ± 9.4
	SSID	Depth (4)	-15.9 ± 8.6
	(n=19)	Depth (8)	-22.4 ± 8.5
		Volume (4)	-16.8 ± 8.6
		Volume (8)	-20.0 ± 7.8
	NRFS serum	Depth (8)	-20.3 ± 8.7
Group 2	(n=20)	Volume (8)	-24.1 ± 8.6
	CONTROL	Depth (8)	-15.3 ± 7.2
	(n=20)	Volume (8)	-15.0 ± 5.2

Table 2: Percentage changes in wrinkle depth and volume over the trial period from 39 volunteers (mean ± SD).

At 8 weeks the NRFS decreased wrinkle volume by 31.6% more than the SSID product ($p<0.01$). Wrinkle depth of the NRFS serum decreased by 23.4% more so than the SSID product ($p=0.0577$). The difference between the two treatments was most marked from weeks 4 to 8. SSID decreased wrinkle volume by 18.85% over the last 4-week period while NRFS serum decreased volume by 49.59% (percentage change in the mean individual percentage changes from the end of week 4 to the end of week 8) indicating that change was slowing in the SSID group at a faster rate than that of the NRFS serum. Compared to Control there was a 55.8% relative reduction wrinkle volume with the NRFS serum ($p<0.01$) and a 32.8% decrease in wrinkle depth ($p=0.0123$).

Group	Parameter	Intergroup difference [a]	Improvement (%) [b]	p-value
NRFS serum versus SSID	Wrinkle Depth	-32.8 µm	23.4%	0.0577
	Wrinkle Volume	-0.3 m^3	31.60%	0.0044
NRFS serum versus CONTROL serum	Wrinkle Depth	-28.3 µm	32.8%	0.0123
	Wrinkle Volume	-0.4 m^3	55.80%	<.0001

Table 3: Comparisons between treatments after 8 weeks. a The Intergroup difference is the average depth or volume change compared between the treatments within the same group. A negative result indicates that change was greater for the NRFS as compared to the other treatment. b The average improvement of NRFS compared with the other treatment within the group.

Discussion

In the present paper we report on the effects of GHK-Cu on synthesis of collagen and elastin, and expression of MMP1, MMP2, as well as tissue inhibitors of metalloproteinases (TIMP), TIMP1 and TIMP2, by human adult dermal fibroblasts (HDFa). These data are presented alongside the results of a human clinical trial investigating effects of GHK-Cu on wrinkle depth and volume.

MMPs/TIMPs expression

Application of GHK-Cu at all the tested concentrations to cultured human dermal fibroblasts increased mRNA expression of both MMP1 and MMP2. TIMP1 mRNA expression increased and to a greater extent than MMP1, suggesting net inhibition of proteolytic activity for collagens. GHK-Cu, however, did not change TIMP2 significantly at 0.01 nM, and at higher concentrations decreased expression ($p<0.05$) (Figure 1). The findings of increased MMPs and TIMP1 mRNA following exposure to GHK-Cu are consistent with the findings of Simeon et al. [7] who also examined effects of GHK-Cu on MMPs of rat fibroblast cells. The inverse dosage dependent response results from this study, however, do differ from a study showing that increasing the free copper concentration increases both MMP1 and TIMP1 expression [25]. Our study with GHK-Cu shows that at higher doses there is generally little effect or inhibition of both MMPs and their TIMPs. It is thus likely that GHK-Cu effects that we observed are not due to free copper.

Irrespective to the dose response, on the other hand, there was clear correlation between MMPs with their TIMP levels in the GHK-Cu treated cells; a high concentration of MMP was accompanied with a high level of its TIMP (Figure 1). A simultaneous increase and decrease of various MMPs and TIMPs has been previously reported [6]. It is important to note that TIMPs regulate the proteolytic activity of MMPs by direct interaction with these enzymes, and not by regulation at a transcriptional level [26]. It is also important to note that TIMP1 acts against all members of both collagenase and gelatinase classes [27], thus the no change, or decrease in TIMP2 at higher concentrations of GHK-Cu, does not necessarily signal a shift in favour of inflammatory changes and increased degradation. The relative increase in TIMP1 following exposure to GHK-Cu is consistent with a shift to matrix production and growth, and both TIMP1 and TIMP2 are considered to have growth factor-like functions [27].

Collagen/elastin production

Marquart et al. [5] reported a dosage dependent increase in the amount of collagen produced by human fibroblasts incubated with GHK-Cu. The response peaked at 1 nM with higher concentrations resulting in less collagen synthesized. In this present study the collagen levels were above non-treated cells at 96 hours at concentrations of 0.01-100 nM ($p<0.05$). Increasing GHK-Cu concentrations did not significantly increase the response. In contrast, the production of elastin, measured as α-elastin, was 30% higher than that found in the untreated cells regardless of the GHK-Cu concentrations (0.01-100 nM) (Table 1).

The increase in collagen production, albeit modest, supports the conclusion that GHK-Cu stimulates tissue growth and repair. The increase in elastin similarly supports the conclusion that GHK-Cu stimulates tissue growth and repair.

Relationship of MMPs/TIMPs expression and collagen/elastin production

As expected MMPs and TIMPs mRNA expression, and collagen and elastin production were markedly affected by exposure of the cells to GHK-Cu. An increase of TIMP1 and TIMP2 suggested an inhibition of

proteolytic activity of MMP1 and MMP2 and thus decreased fibrillar collagen (collagen) and elastin degradation which is consistent with the observed increase in collagen/elastin in this study (Table 1). Surprisingly, the increase in either collagen or elastin appeared to have little concentration-dependence on GHK-Cu although an inverse dosage dependent response with TIMP1 and TIMP2 was observed. More interestingly, although MMP2 increased and TIMP2 decreased with the treatment with GHK-Cu (0.01-10 nM), which in theory mean a decreased elastin level in the treated cells, an increase in elastin level was observed, and again with a little dose-dependence. This means single factor, either MMP or TIMP cannot determine the level of the matrix protein.

To fully understand the mRNA results, the ratios of TIMP/MMP expression were calculated (Table 4). High (>1) and relatively consistent ratios of TIMP1/MMP1 at all GHK-Cu contractions were found. This could explain the observation with cellular secretion of collagen. The low ratio of TIMP2 to MMP2 would predict a shift towards degradation. Considering however that TIMP1 acts against all members of the gelatinase classes (MMP2) and that TIMP2 also acts on MMP2, the ratio of (TIMP1+TIMP2)/MMP2 was used as a measure of prediction (Table 4).

Ratio	GHK-Cu concentration (nM)		
	0.01	**1**	**100**
TIMP1/MMP1	3.76	2.65	3.1
TIMP2/MMP2	0.43	0.37	0.31
(TIMP1+TIMP2)/MMP2	1.72	1.39	1.07

Table 4: TIMP/MMP ratios on HDFa following 24 hour treatment with GHK-Cu at difference concentrations (calculated from the mean values from data in Figure 1).

Clinical study

The effects on MMP and TIMP expression with the associated increase in collagen and elastin production supported the investigation of topical GHK-Cu treatment in human volunteers. Due to the physicochemical properties of GHK-Cu hindering topical absorption, and given previous studies have evaluated topical GHK-Cu delivery *in vivo* [12,13], this present study used nano-carriers to facilitate delivery of GHK-Cu to the dermis. The clinical trial investigated the effect of GHK-Cu on facial wrinkle volume and depth. The application of GHK-Cu in nano-carriers (NRFS serum) resulted in a significant and improved reduction in facial wrinkle volume and depth compared to the CONTROL (serum only) and a reduction in wrinkle volume compared to the SSID, a commercially available product containing a lipophilic derivative of GHK (Matrixyl 3000®). This confirms the NRFS, containing GHK-Cu in a carrier system, is more effective than the SSID product for total wrinkle volume reduction. It also shows that improvement can be seen with continued use, observed as wrinkle depth and volume reductions between 0-4 and 4-8 weeks. The NRFS serum also significantly outperformed the CONTROL (vehicle only) in regards to wrinkle volume and depth. This evidence proves that it was the nano-carrier that impacted significantly on the total wrinkle reduction and not the formulation. Of particular importance was that all participants using NRFS showed a reduction in wrinkle volume and depth.

Importantly the nano-carrier encapsulated GHK-Cu was well tolerated and similar to SSID with only one participant reacting to both. Side-effects of anti-wrinkle agents such as tretinoin are relatively common and include peeling, dryness and erythema [28]. None of these were experienced by participants in the GHK-Cu trial, notwithstanding the aforementioned reaction.

Conclusion

GHK-Cu significantly increased cellular production of collagen and elastin by HDFa cells with little concentration dependency (0.01-10 nM). Incubation with the tripeptide also correlated with a relative higher mRNA expression of the respective TIMP(s) than the respective MMP in the cells.

Topical application of GHK-Cu with the aid of nano-carrier delivery systems reduced wrinkle volume to a significantly greater extent than the vehicle alone or a commercial product containing Matrixyl 3000®, a GHK lipophilic derivative.

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