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Beneficial Regulation of Fibrillar Collagens, Heat Shock Protein-47, Elastin Fiber Components, Transforming Growth Factor-β 1, Vascular Endothelial Growth Factor and Oxidative Stress Effects by Copper in Dermal Fibroblasts

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Abstract

Skin aging is associated with the loss of the structural collagens and the elastin fiber components that form the extracellular matrix (ECM). It is associated with reduced transforming growth factor-β (TGF-β), angiogenesis and increased oxidative stress. Copper has been incorporated into cosmetics for anti-skin aging. This research investigated the mechanism for the anti-skin aging effect copper ions, from cuprous oxide powders. Dermal fibroblasts were exposed to copper and examined for expression (protein and/or promoter levels) of types I, III, V collagen, heat shock protein-47 (HSP-47), elastin, fibrillin-1, and fibrillin-2, TGF-β 1, vascular endothelial growth factor (VEGF), and in addition for membrane damage and lipid peroxidation. The direct antioxidant activity of copper was also determined. The research indicates that copper's anti-skin aging and skin regeneration potential is through its stimulation of ECM proteins, TGF-β 1, VEGF, and inhibition of oxidative stress effects at physiological concentrations; and supports its use in cosmetics.

Keywords: fibrillin, copper oxide, extracellular matrix, antioxidant, skin aging, wound repair

INTRODUCTION

The structural integrity of the extracellular matrix (ECM) is compromised in skin aging. The structural ECM proteins are primarily fibrillar collagens, in order of predominance types I, III, and V that provide structure; and elastin fibers, formed of elastin and fibrillins that give firmness and elasticity to skin [1-4]. The dermal fibroblasts are primarily responsible for the synthesis of the ECM proteins [1-5]. The formation of collagen is closely associated with the expression of heat shock protein-47 (HSP-47) [6,7]. HSP-47 is a collagen-specific chaperone that is essential for the formation of its triple helical structure, as well as its maturation and secretion [6,7]. Further, collagen formation is closely associated with the expression of HSP-47 [7]. A primary regulator of the ECM proteins is TGF-β , which stimulates ECM formation, and that of angiogenesis is vascular endothelial growth factor (VEGF) [2-5,8]. With skin aging there is loss/atrophy of structural ECM, reduced expression of TFG-β , and clinical manifestations of wrinkles, diminished structural integrity, and impaired wound healing [1-5,9,10].

The essence of copper to ECM structure has been in association with its role as a cofactor to lysyl oxidase, which oxidizes lysine and hydroxyllysine residues in collagen and elastin for the formation of cross-links [11-14]. An alteration in the expression of lysyl oxidase is associated with abnormalities in the ECM, TGF-β activity, aging, and senescence [12-14]. Copper in a complex with a matrix-derived tripeptide (glycyl-histidyl-lysine or GHK) (Cu-GHK) stimulates expression of collagen and elastin in vivo and in vitro, inhibits clinical manifestation of skin aging, and exhibits anti-inflammatory actions [15-18]. Copper chloride stimulates total collagenous proteins in oral fibroblasts, and copper oxide nanoparticles promote formation of

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elastin matrices in vascular smooth muscle cells [19,20]. Wound dressings with copper oxide particles are safe and induce the expression of proangiogenic factors, including TGF-β and VEGF, in diabetic mice wounds [21,22]. Copper is absorbed from topical applications of copper-containing ointments, and the use of copper oxide-containing pillow cases improves skin appearance through the release of copper ions in the presence of moisture [23,24].

Copper is a trace metal, with physiological relevance in nM concentrations, and has clinical in vivo anti-skin aging effects [22,25]. The primary hypothesis of this research was the anti-skin aging potential of copper through the beneficial regulation of the expression of the predominant structural ECM genes in dermal fibroblasts, which are the primary cells responsible for the maintenance of the ECM. Specifically, we evaluated the efficacy of copper ions, from copper oxide particles, in nM concentrations to (1) increase expression of types I, III and V collagens, and HSP-47; (2) stimulate expression of elastin, fibrillin-1, and fibrillin-2; (3) enhance expression of TGF-β 1 and VEGF; and (4) inhibit membrane damage and lipid peroxidation.

EXPERIMENTAL DESIGN AND METHODS

Cell Culture and Dosing

Human neonatal dermal fibroblasts from three donors (Cascade Biologics) were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin (P/S), and 1% *L*-glutamine. Four independent experiments, in replicates of 3-4, were performed by exposing dermal fibroblasts at passages 19-21 (in vitro aged) in experimental media (DMEM containing 1% serum replacement and 1% P/S) (10) with or without copper ions from copper oxide particles.

Our initial studies were with copper ions released from suspensions of cuprous oxide particles of different sizes (22, 2.5, 1 μm: SCM Metal Products Inc., Research Triangle park, NC, USA; Yogi Dye Chem Pvt. Ltd., Mumbai, India) or from 1 μm copper oxide containing wound dressing (polypropylene nonwoven fabric: Cupron Scientific, Israel) in experimental media. The concentration of the released copper ions was measured by several procedures, which include those that had been used in our previously reported work with copper in which the quantitation of copper was not described: electrogravimetric analysis; QuantiChrom copper assay kit (BioAssay Systems); in-house spectrophotometric measurement following reactions with 2,2′-biquinoline (Sigma) or bicinchoninic acid (Thermo Fischer Scientific Inc.), and copper test strips (EMD Chemicals) (4). The results were similar from the different procedures to measure the concentration of copper ions; and hence the copper concentrations of the experimental dilutions were determined using copper test strips in further experiments (EMD Chemicals). In addition, initial research indicated similar effects of the copper ions released from the copper particles of the different sizes or from the copper dressing, into the experimental media, on the regulation of the ECM proteins in fibroblasts. Further research studies were performed with the 1 μm copper oxide powder that has been carefully analyzed, of more than 99% purity, and has been incorporated into wound dressing and pillowcases, shown to have anti-skin aging effects in vivo (22, 24).

The experiments consisted of seeding fibroblasts in at 2×10^5 cell density in 33 mm dishes overnight, transfecting each dish with 5 μg each of type I collagen or elastin promoter-reporters and 0.5 μg control (TK) promoter-reporter complexed with ESCORT (Sigma, 7 μg per dish) for 24 hr before treatment with 0-0.5 nM of copper for 24 hr; and examining for cell viability {CellTiter 96® Aqueous One or MTS assay [tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) + electron coupling reagent (phenazine ethosulfate; PES)] (Promega)}, and the expression of the reporters for promoter activity. The experiments also consisted of seeding fibroblasts at 2×10^5 cell density in 33 mm dishes for 2 days, treatment with 0-0.5 nM of copper for 24 hr; and examining for cell viability, and the expression of fibrillar collagen (types I, III, V) proteins, elastin fiber proteins (elastin, fibrillins), TGF-β 1, VEGF and HSP-47 proteins, membrane damage, and lipid peroxidation. The cell viability was not altered at these concentrations of copper, which has been reported (4).

The direct antioxidant activity of the copper ions was measured through the inhibition of ABTS oxidation.

Collagen (Types I, III, V), HSP-47, Elastin, Fibrillins (1, 2), TGF-β 1, and VEGF Protein Levels

The protein levels in the media or cell lysates of fibroblasts that had been dosed with 0, 0.05, 0.3, and 0.5 nM copper ions were determined using ELISA (Kirkguaard and Perry Laboratories Inc., Millipore, Stressgen, R&D Systems) [1-5]. One hundred microliter of aliquots of media or cell lysates from each sample, and respective standards were added to independent wells of 96 well plates for 24 hr at 4° C. The wells were blocked with bovine serum albumin and then incubated with respective antibodies [(Millipore—type I collagen: CC050, AB745, AB758B; type III collagen: CC054, AB747; type V collagen: CC077, AB763P), (KPL Lab. Inc.—protein detector ELISA kit, 55-81-10), (Elastin Products Co.—Elastin: GH421, PR 533, PR 938; Fibrillin-1: PR 217; Fibrillin-2: PR 225), (Stressgen—HSP-47: SPA-40), (R&D Systems—TGF-β : DY240; VEGF: DY293B)] for 1 hr at room temperature. The plates were washed with wash buffer, incubated with respective secondary antibodies linked to peroxidase or biotin for 1 hr at room temperature, washed, and subsequently

incubated with peroxidase substrate until color development, which was measured spectrophotometrically at 405 nm. The absorbance data were quantitated through respective standards [1-5]. The types I and III collagens are coordinately regulated; their standards are 90% pure, <10% cross-contaminated; and with <1% of other collagens or non-collagen proteins (Millipore). The type V collagen standard is of 95% purity, and with <2% contaminants of each of the collagens or 0.5% of the non-collagen proteins (Millipore). There was no significant cross-reactivity of the respective antibodies for each collagen type with the standards of other collagen or non-collagen proteins in our assays.

Type I Collagen, and Elastin Promoter Activity

Fibroblasts were co-transfected with COL1α 1 promoter-firefly luciferase or elastin promoter-firefly luciferase (pGL4 vector) (gifts from Dr. Joel Rosenbloom, School of Dental Medicine, University of Pennsylvania, PA, USA) and thymidine kinase (TK) promoter-hRenilla luciferase plasmids (Promega) (for normalization of transfection efficiency) using Escort (Sigma) for 24 hr prior to dosing with or without copper for 24 hr [1,2]. The cells were measured for luminescence sequentially, in each sample, from firefly (collagen or elastin promoters) and renilla (control TK promoter) luciferase activities, normalized for luciferase values, and quantitated using recombinant luciferase as a standard (Promega: Dual luciferase reporter assay, E1910) [1,2].

Membrane Damage

The membrane damage was determined by testing the media of fibroblasts dosed with 0-0.5 nM copper ions for lactate dehydrogenase (LDH), indicative of membrane damage (Sigma). An LDH reaction mixture (substrate, enzyme, and a tetraxolium dye) was added to aliquots of media at 1:2 ratio, incubated at room temperature for 30 min, and the LDH-mediated conversion of the tetraxolium dye to a colored product was measured spectrophotometrically at 490 nm. The absorbance data were converted to percentage of control.

Lipid Peroxidation

Lipid hydroperoxides, indicative of lipid peroxidation, were measured using the K-Assay (Kamiya Biomedical Company). Reagent 1 (ascorbic oxidase and lipoprotein lipase) was added to media or cell lysates at 1:1 ratio and incubated for 10 min at 30° C. Equal volume aliquots of reagent 2 (methylene blue derivative and hemoglobin) were subsequently added, the color change due to the formation of methylene blue by the lipid hydroperoxides determined spectrophotometrically at 660 nm, and the data converted to percentage of control.

Data Analysis

The significant effects of copper were analyzed relative to control (without copper) by ANOVA and student *t*-tests at 95% confidence interval [1-5].

RESULTS

Copper Stimulates Expression of Fibrillar Collagens and HSP-47

Copper, at each of the concentrations 0.05, 0.3, and 0.5 nM, significantly stimulated the expression of fibrillar collagens (types I, III, V), and HSP-47 in dermal fibroblasts (Figure 1A-1E).

The stimulation of type I collagen by copper was at the protein and promoter levels. Copper at 0.05, 0.3, and 0.5 nM stimulated type I collagen protein levels to 200% (p = 0.005), 214% (p = 0.003), and 228% (p = 0.001) of control (100%: 0.7 μg/ml); and type I collagen promoter activity to 169% (p = 0.011), 247% (p = 0.016), and 225% (p = 0.003) of control (100%: 750 μg/ml) (Figure 1A and >1B). Copper at 0.05, 0.3, and 0.5 nM stimulated type III collagen protein levels to 167% (p = 0.012), 200% (p = 0.006), and 200% (p = 0.007) of control (100%: 0.3 μg/ml); and type V collagen protein levels to 200% (p = 0.016), 150% (p = 0.019), and 150% (p = 0.04) of control (100%: 0.2 μg/ml) (Figure 1C and >1D).

Copper stimulated HSP-47 protein at 0.3 nM, and 0.5 nM to 220% (p = 0.001), and 160% (p = 0.04) of control (100%: 5 μg/ml) (Figure 1E).

Copper Stimulates Expression of Elastin, Fibrillin-1, and Fibrillin-2

Copper significantly stimulated the expression of elastin protein and promoter activity from 0.05 to 0.5 nM; and fibrillin-1 and fibrillin-2 proteins at 0.3 and 0.5 nM (Figure 2).

Copper stimulated elastin protein levels at 0.05, 0.3, and 0.5 nM to 230% (p = 0.004), 207% (p = 0.021), and 207% (p = 0.004) of control (100%: 70 μg/ml); and elastin promoter activity to 191% (p = 0.003), 195% (p = 0.014), and 186% (p = 0.001) of control (100%: 400 μg/ml) (Figure 2A and 2B). The stimulation of fibrillin-1/fibrillin-2 by copper at 0.3 and 0.5 nM was to 180% (p = 0.02)/206% (p = 0.01) and 360% (p = 0.01)/251% (p = 0.01) of respective controls (100%: 0.5 μg/ml, fibrillin-1; 100%: 35 μg/ml, fibrillin-2) (Figure 2C and 2D).

Copper Stimulates Expression of TGF-β and VEGF

The protein levels of TGF-β 1 and VEGF were significantly stimulated by copper at 0.3 and 0.5 nM (Figure 3).

Copper significantly stimulated TGF-β 1 protein levels to 147% (p = 0.03) and 216% (p = 0.04) of control (100%: 1000 pg/ml); and VEGF protein levels in dermal fibroblasts at 0.3 and 0.5 nM to 199% (p = 0.04) and 143% (p = 0.02) of control (100%: 750 pg/ml) (Figure 3A and 3B).

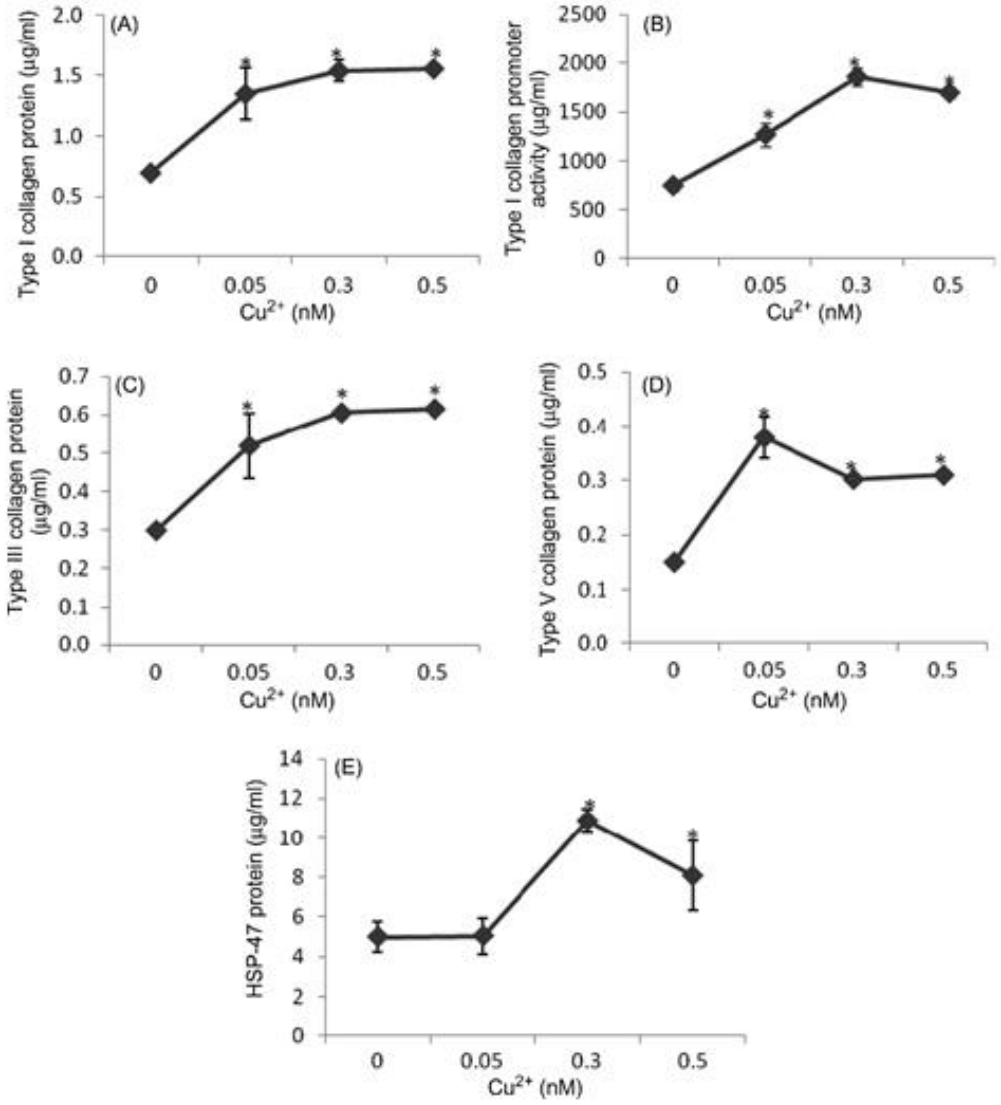

Figure 1. Stimulation of fibrillar collagens and HSP-47 by copper. Fibroblasts were dosed with 0, 0.05, 0.3, or 0.5 nM copper for 24 hr and examined for type I collagen protein level (A), type I collagen promoter activity (B), type III collagen protein level (C), type V collagen protein level (D), and HSP 47 protein level (E); * $p < 0.05$ relative to control; Error bars represent standard deviation, $n = 4$.

Copper Inhibits Reactive Oxygen Species, Membrane Damage, and Lipid Peroxidation

Copper significantly inhibited cellular membrane damage, and lipid peroxidation (Figure 4A and 4B).

Copper at 0.05, 0.3, and 0.5 nM inhibited cellular membrane damage to 56% ($p = 0.001$), 59% ($p = 0.004$), and 50% ($p = 0.008$) of control (Figure 4A). Copper at 0.3 and 0.5 nM slightly, though significantly, inhibited cellular lipid peroxidation: the lipid hydroperoxides in media/cells were 90% ($p = 0.01$)/92% ($p = 0.02$) and 91% ($p = 0.01$)/88% ($p = 0.01$) of respective controls (100%: 10 nmol/ml, media; 100%: 20 nmol/ml, cells) (Figure 4B).

DISCUSSION

This research associates the anti-skin aging potential of lower concentrations of copper through the stimulation of fibrillar collagens, HSP-47, elastin fiber components, TGF-β 1, VEGF, and inhibition of cellular membrane damage and lipid peroxidation. However, the in vivo validity of these concentrations of copper on reaching the dermal fibroblasts through the epidermis would need to be investigated.

Copper stimulated the expression of types I, III, and V collagens at 0.05-0.5 nM, and HSP-47 at 0.3 and 0.5 nM in dermal fibroblasts. The stimulation of type I collagen protein levels and promoter activity by copper was similar, suggesting transcriptional regulation. We have previously reported that the expression of matrixmetalloproteinase-1 (MMP-1), which degrades fibrillar collagens, is not altered by these lower concentrations of copper, suggesting a net beneficial effect of copper in nM concentrations on ECM structure [4]. The stimulation of collagen synthesis or total collagenous proteins by GHK-Cu has been reported to be between 1 pM and 1 nM and by copper chloride in oral fibroblasts between 0.1 and 50 µ M [15,19]. The

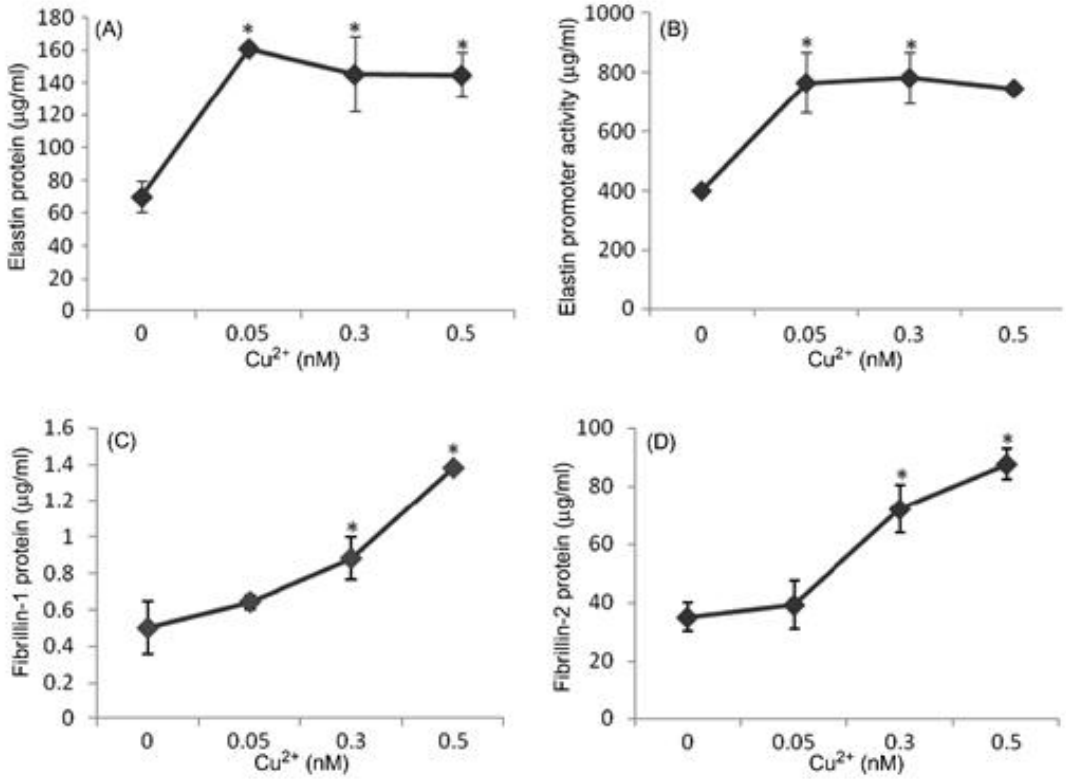

Figure 2. Stimulation of elastin and fibrillins by copper. Fibroblasts were dosed with 0, 0.05, 0.3, or 0.5 nM copper for 24 hr and examined for elastin protein level (A), elastin promoter activity (B), fibrillin-1 protein level, and (C) fibrillin-2 protein level (D); * $p < 0.05$ relative to control; Error bars represent standard deviation; $n = 4$.

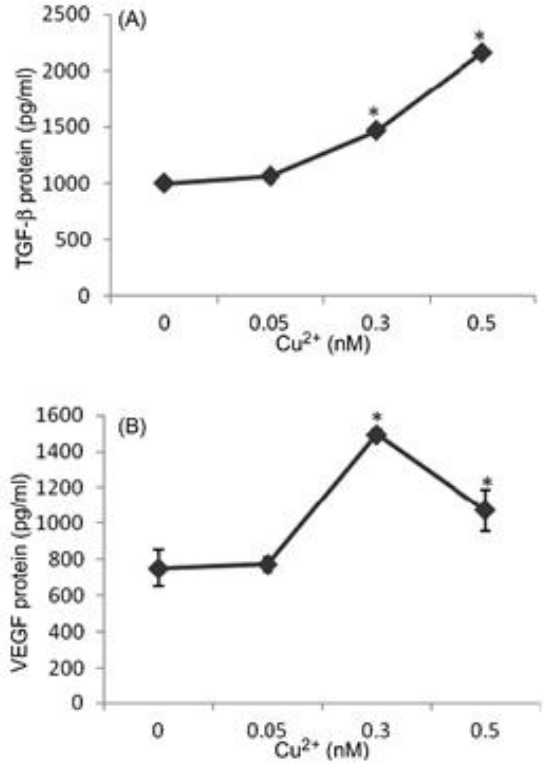

Figure 3. Stimulation of growth factors by copper. Fibroblasts were dosed with 0, 0.05, 0.3, or 0.5 copper for 24 hr and examined for TGF-β protein (A) and VEGF protein (B); * $p < 0.05$ relative to control; Error bars represent standard deviation; $n = 4$.

co-stimulation of HSP-47 with fibrillar collagens by copper, which has not been previously reported, suggests improved collagen fibril formation in the presence of copper.

Copper stimulated the elastin protein and promoter activity similarly at 0.05-0.5 nM, indicating transcriptional regulation; and fibrillin-1 and fibrillin-2 at 0.3-0.5 nM; suggesting formation of proper elastin fibrils by copper. While the in vitro promotion of the formation of cross-linked elastin matrices by copper ions released from copper oxide particles has been demonstrated, the regulation of elastin or fibrillin in dermal fibroblasts has not been previously reported [20]. Elastin is reduced with intrinsic aging and is associated with loss of skin firmness and resiliency [1]. In addition, the reduction in fibrillins that form microfibrils in the epidermal-dermal junction as well as elastin fibers with elastin are reduced with skin aging [1,27]. The research indicates dual strengthening of the ECM integrity through collagen and elastin fibrils by copper at its lower concentrations.

Copper stimulated the expression of TGF-β 1 in dermal fibroblasts at 0.3 and 0.5 nM. While GKK-Cu does not stimulate TGF-β , copper oxide-incorporated dressings stimulate TGF-β in diabetic wounds [16,24]. The regulation of TGF-β 1 by copper in dermal fibroblasts has not been previously reported. TGF-β is the predominant regulator of collagen and elastin

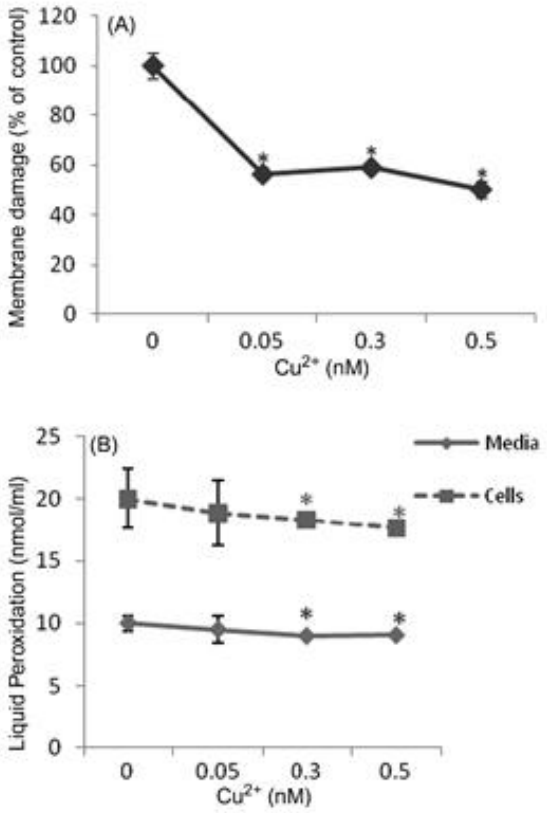

Figure 4. Inhibition of membrane damage and lipid peroxidation by copper. Cellular inhibition of membrane damage (A) and lipid hydroperoxides in media (straight blue line) or cells (dash red line) (B) by copper at 0, 0.05, 0.3, or 0.5 nM; * $p < 0.05$ relative to control; Error bars represent standard deviation; $n = 4$.

in dermal fibroblasts [2,28]. Hence, the mechanism of copper's induction of the fibrillar collagens and elastin fiber components may include the stimulation of TGF-β 1.

Copper induced the expression of VEGF in dermal fibroblasts. Copper has been associated with angiogenesis through VEGF and interleukin-8 in tumors and vascular tissue as well as in epidermal keratinocytes and diabetic wounds [22,29-31]. The induction of VEGF has been associated with oxidative stress and induction of oxidative associated pathways and genes [24,31]. However, the induction of VEGF in this research may be through the induction of TGF-β 1 and not through the generation of reactive oxygen species for the copper ion-exhibited antioxidant properties [31].

Copper, including its increased ratio to zinc, has been associated with oxidative stress, increased lipid peroxides, and degenerative diseases [32]. Conversely, copper at the lower concentrations exhibited antioxidant property as inferred from the cellular inhibition of membrane damage and lipid peroxidation. The direct antioxidant potential could be from the redox of cuprous to cupric ions. The cellular antioxidant properties may be from copper's role as cofactor to superoxide dismutase or cytochrome c oxidase [33,34].

CONCLUSION

Copper at lower concentrations (0.05-0.5 nM) stimulated types I, III, and V collagens, HSP-47, elastin, fibrillin-1 and fibrillin-2, TGF-β , and VEGF, while inhibiting cellular membrane damage and lipid peroxidation in dermal fibroblasts. Conversely, skin aging is associated with the reduced expression of these predominant structural ECM proteins and growth factors, and increased oxidative stress effects. Hence this research supports the use of copper in low concentrations in cosmetics, and further research to validate its biosafety as well as its regulation of ECM components in anti-skin aging and wound healing pathways.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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