

ORIGINAL CONTRIBUTION

Increased pro-collagen 1, elastin, and TGF- β 1 expression by copper ions in an ex-vivo human skin model

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Funding information

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Abstract

Background: Clinical studies demonstrated that continued exposure to copper oxide-embedded textiles, such as pillowcases, significantly reduces depth of facial wrinkles and skin sagging and enhances skin elasticity.

Objective: Study the mechanisms by which the exposure to copper ions improve the well-being of the skin.

Methods: Human skin explants, cultured ex-vivo, were exposed topically to saline alone or saline containing 0.02 or 1 μ mol/L copper ions. The skin explants viability, histology and secretion of elastin, pro-collagen 1, and TGF- β 1 to the culture medium were determined at various time intervals.

Results: Exposure to saline containing 0.02 or 1 μ mol/L copper ions did not affect the viability or morphological profile of the explants as compared to control explants treated with saline only. Notably, exposure of the skin grafts to 0.02 or to 1 μ mol/L of copper ions resulted in ~100% and ~20% increases in elastin and pro-collagen 1 concentrations, respectively, in the culture supernatants already after 1 day of incubation, which remained statistically significantly elevated also after 6 days on incubation, as compared to the control explants. In addition, ~2- and ~4-fold increases in TGF- β 1 levels in the culture supernatants of explants exposed to the copper ions were detected after 4 and 6 days of culture, as compared to the explants exposed to saline alone.

Conclusion: This study substantiated the anti-aging effect that copper ions have on the skin and gave insights into the mechanisms by which exposure of the skin to copper ions improves the skin well-being.

KEYWORDS

copper oxide, cuprous oxide, elastin, ex-vivo skin model, pro-collagen, TGF- β 1

1 | INTRODUCTION

The dermal layer of the skin is composed mainly of extracellular matrix (ECM) structures and fibroblasts. The ECM structures are composed of proteins, mostly of collagens I, III, and IV, elastin and fibrillin, and glycosaminoglycan-rich proteoglycans. The dermal fibroblasts are primarily

responsible for the synthesis and secretion of the ECM protein precursors. After being secreted, these protein precursors undergo structural changes that allow for the cross-linking between the mature proteins, providing the skin with strength, extensibility, and elasticity.

Lysyl oxidase (LOX), an extracellular copper-dependent enzyme,¹ oxidizes lysine and hydroxylysine residues in elastin and collagen

precursors, enabling the cross-linking between elastin and collagen.¹ This cross-linking is essential for the stabilization of collagen fibrils and for the integrity and elasticity of mature elastin. Transforming growth factor beta (TGF- β), especially TGF- β 1, is a key cytokine regulator of the production and secretion of elastin and collagen.² Interestingly, LOX also binds and regulates the signaling of TGF- β 1.³

With aging, there is a reduction in the expression of TGF- β , decreased LOX activity, fewer numbers of dermal fibroblasts, reduced elastin and collagen production by the remaining dermal fibroblasts, and breakdown of the collagen/elastin fibers forming the ECM by matrix metalloproteinases (MMP).¹⁻⁴ These events are accelerated by inflammation and oxidative stress in photoaged skin.⁴ All the above leads to the decline of the skin elasticity and recoil, and in increase of wrinkles and skin sagging.

Copper is an essential trace element involved in many cellular, metabolic, and physiological processes in almost all body tissues.⁵ Copper is found in meat, vegetables, grains, and many other food sources and the recommended daily intake of copper for adults is ~1 mg. Copper is extremely well metabolized. In the skin, copper, in addition of serving as a cofactor of LOX, stimulates dermal fibroblast proliferation,⁶ enhances production and secretion of different collagen and elastin types by fibroblasts,⁷ and stabilizes the skin ECM once formed.⁸ Copper serves as a cofactor of superoxide dismutase, an antioxidant enzyme present in the skin that is important for protection against free radicals, and inhibits cellular oxidative effects, such as membrane damage and lipid peroxidation.⁸ Copper is also a cofactor of tyrosinase, a melanin biosynthesis essential enzyme, responsible for skin and hair pigmentation.

Copper oxide particles can be permanently introduced into textile products, where they serve as a source of slow release of copper ions.^{9,10} In four double blind placebo controlled trials, in all groups of individuals who slept for a month on pillowcases embedded with copper oxide particles, there were statistically significant reductions in the depth of the facial wrinkles and overall improvement of the well-being of the skin, as compared to the groups of individuals using pillowcases without copper oxide.^{11,12} Sleeping for four weeks on pillowcases impregnated with copper oxide particles also resulted in statistically significant skin lifting on the cheek and eye areas as compared to baseline and as compared to the control group.¹³ Likewise, the use of copper oxide-impregnated socks resulted in increased elasticity in the group of healthy volunteers as compared to the group of individuals using placebo socks.¹⁴ All these studies indicated that the exposure of the skin to copper ions had a positive effect on the skin. We hypothesized that the copper oxide-impregnated fabrics continuously release copper ions that are absorbed through the skin, which results in the upregulation of the production of extracellular skin proteins and the stabilization of the ECM, improving the skin's well-being.¹⁵ The capacity of copper to cross the skin epithelial barrier has been demonstrated.¹⁶

In the current study, we aimed to substantiate our hypothesis by studying the effect of copper ions obtained from copper oxide-impregnated fabric on intact skin explants by using a human ex-vivo model of normal skin tissue culture.¹⁷

2 | METHODS

2.1 | Ex-vivo model

Skin tissues were obtained from 35- to 65-year-old healthy women undergoing esthetic abdomen surgery, after signing an informed consent form. All experiments were conducted with approval of the IRB (Helsinki Committee) of Soroka Medical Center, Be'er Sheva, Israel. The study was initiated in the day of surgery. Skin culturing and treatment were performed under sterile conditions. After the removal of the hypodermis and fat, the full-thickness skin graft was harvested and cut into 0.8 cm² pieces by costume made apparatus. The skin samples were placed dermis down and cultured at the air-liquid interface at 37°C with 5% CO₂ in Dulbecco's Modified Eagle's Medium (Biological Industries), 10% fetal calf serum (Biological Industries), and penicillin/streptomycin (100 IU/mL penicillin, 100 mg/mL streptomycin; Biological Industries). Culture medium was refreshed twice a week. Each individual experiment was performed with skin explants obtained from the same individual, and the experiments were repeated at least three times, each time in triplicates. Figure 1 shows a sketch of the model.

2.2 | Copper source and application on the skin explants

As a source of copper ions, commercially available copper-impregnated anti-aging pillowcases (<https://cupron.com/applications/consumer-products/>), impregnated with ~1% weight/weight cuprous oxide particles, were used. 3.6 grams of the cuprous

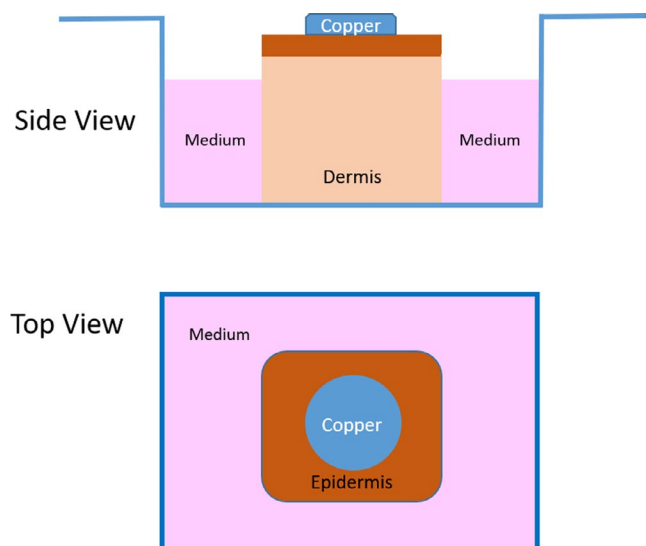


FIGURE 1 Side and top view of the ex-vivo explant model. The skin explants are placed dermis down. Culture media were added to the bottom of the well, without reaching the epidermal layer, which is exposed to the air. Three μ L of saline only or containing different concentrations of the copper ions was carefully added on top of the epidermis making sure they do not reach the culture media in the bottom of the well

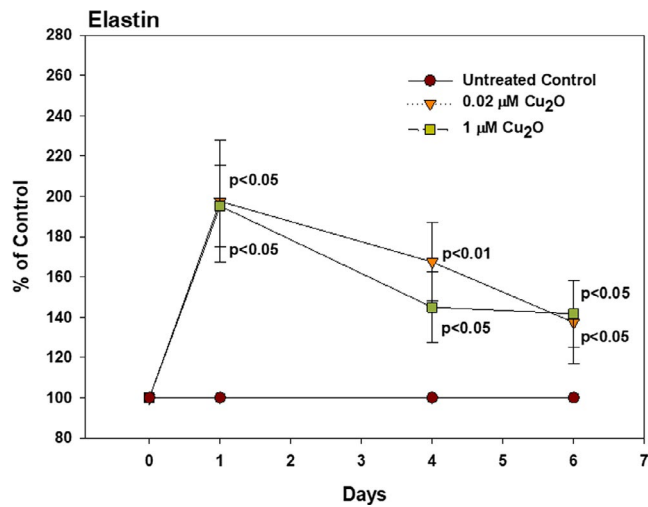


FIGURE 2 Increase in elastin levels in the explants exposed to copper. The concentration of elastin in the media collected from the bottom of the wells was determined by the Fastin quantitative method. Results shown are mean \pm standard deviation of triplicates. The *P* values as compared to the untreated control are depicted

oxide-impregnated fabrics were immersed in 25 mL of 0.9% saline overnight at 37°C for each experiment. The resulting concentrations of copper ions in saline were determined by using Aquachek™ copper ions test strips (Hach Company), and the solutions served as copper ions stock solutions. From the stock solutions, solutions of 0.02 or 1 μmol/L of copper ions were prepared in saline. Three μL of saline only, 0.02 or 1 μmol/L copper solutions were then added on Day 0 and then after every two days onto the skin explants on top of the epidermis, while making sure they do not reach the medium in the chamber but stay on the air interface on top of the skin. Each control and treatment were performed in at least three replicate explants. In a separate experiment, sterile copper oxide-impregnated fabric swatches were placed directly on top of the explants for 6 days and the explants were used for histological analysis.

2.3 | Measurement of viability

To assess the cell viability throughout the culture period, on days 0, 1, 4, and 6 after exposing the skin grafts to copper ions or fabric, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as follows: Skin explants were incubated for 60 seconds in prewarmed PBS (56°C). Then the epidermis section of each replicate was separated from dermis, transferred to 96 well-plate that contained 150 μmol/L MTT (Sigma, 0.5 mg/mL) and incubated for 1 hour at 37°C and reduced light conditions. Then, the epidermis pieces were transferred to additional 96 well-plate containing 150 μmol/L of Isopropanol. Plates were placed for 5 minutes, 200 rpm in a plate shaker (MRC). Following incubation, epidermis sections were removed, and absorbance was measured at 570 nm in Tecan plate reader (Tecan Group Ltd.). MTT results were normalized to the respective naive control group at each time point.

2.4 | ELISA and Elastin Measurement

During harvesting time points, the spent medium from all test groups was collected and centrifuged at 1500 \times g for 5 minutes to remove particulates. Media were kept at -80°C until use. The secretion levels of pro-collagen 1, hyaluronic acid (HA) and TGF-β1 were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer instructions (R&D systems for pro-collagen 1 and HA; Biologend for TGF-β1). Briefly, plates were coated for 24 hours prior to assay with a specific anti-human capture antibody. At the day of the assay, coated plates were incubated with samples, followed by washes for unbound molecules. Then additional detection antibody was added and detected by Avidin-HRP solution. Finally, wells were incubated with a substrate solution, while absorbance was measured at 570 nm. Elastin levels were measured by the Fastin™ Elastin Assay kit (Biocolor Ltd.) according to the manufacturer protocol.

2.5 | Histology

Following treatments, skin explants were fixed with 4% formaldehyde for 1 hour at room temperature. Then, the samples were washed twice with PBS and kept at 70% ethanol until use. Following dehydration in gradual increasing concentrations of ethanol and embedment, paraffin sections (8 μm) were prepared, pasted, on slides and stained with hematoxylin and masson trichrome, according to the manufacturer specification (Sigma-Aldrich). Elastin fibers were stained using the Elastic Stain Kit (Verhoeff-Van Gieson Stain, Abcam). Slides were mounted, and pictures were captured by using the Zeiss Inverted microscope Axio Observer 7 and the Moticam 5 + Camera.

2.6 | Statistical analysis

Values are presented as average of three replicates, and standard errors of the mean (SEM) are provided. Significant differences between values were analyzed using the unpaired *t* test, while significant results are for *P* < .05.

3 | RESULTS

The exposure of the skin explants to 0.02 or 1 μmol/L of copper ions obtained from the copper oxide-impregnated fabrics, or to the copper oxide-impregnated fabrics, did not significant affect the metabolic activity, as determined by the MTT assay, and as compared to the untreated control skin explants exposed to saline only on Days 1, 4, and 6 of incubation (data not shown).

As depicted in Figure 2, the exposure of the skin grafts to 0.02 or to 1 μmol/L of copper ions resulted in a dramatic increase in Elastin secreted levels already after 1 day of incubation, as compared to the controls exposed to the saline vehicle only. The increase in Elastin levels was statistically significantly higher and almost doubled that

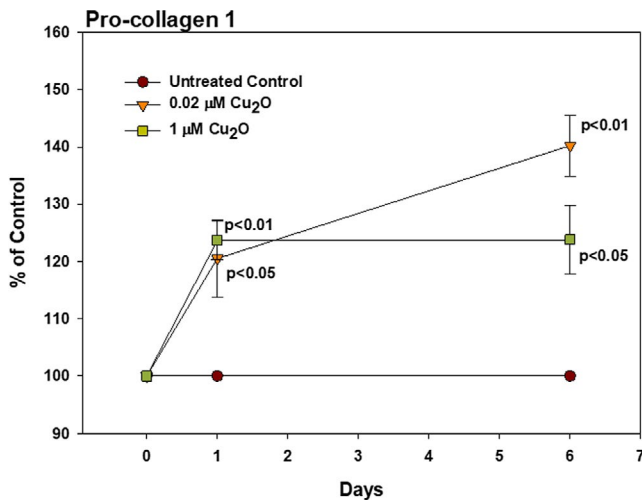


FIGURE 3 Increase in pro-collagen 1 levels in the explants exposed to copper. The concentration of pro-collagen 1 in the media collected from the bottom of the wells was determined by ELISA. The data shown are the mean ± standard deviation of triplicates. The P values as compared to the untreated control are depicted

of the controls and remained significantly elevated also after 4 and 6 days of culture.

Similarly, after 1 day of incubation, there was a statistically significant increase, although to a lesser extent (~20%), in pro-collagen 1 levels in the culture medium of skin explants exposed to 0.02 or to 1 μmol/L of copper ions (Figure 3) as compared to the control explants exposed to saline only. At the lower copper ion concentration (0.02 μmol/L), the levels of pro-collagen 1 were even higher after 6 days of culture.

In order to determine potential glycosaminoglycan (GAG) induction, we measured hyaluronic acid levels in the media collected, but did not find any significant changes at 4 and 6 days following the treatments as compared to the saline alone treatment (data not shown).

In addition to the above increase in the levels of both ECM proteins, elastin, and pro-collagen-1, the levels of TGF-β1 increased dramatically in the culture medium of skin extracts exposed to either 0.02 or to 1 μmol/L of copper ions after 4 and 6 days of culture (~2.5 and ~ 4 folds, respectively) as compared to the explants exposed to saline only (Figure 4).

Figure 5 shows representative pictures of skin explants exposed to saline alone or saline containing 0.02 or to 1 μmol/L of copper ions or to the copper oxide-impregnated fabric only for 6 days. All skin explants showed normal histology, intact epithelial, and dermal layers. We could not detect significant alterations in the thickness of the epidermis upon copper treatment based on the histology analyses. Noteworthy, the skin explants exposed to fabric alone, but especially to the 1 μmol/L of copper ions, had a very high expression of collagen deposits. Furthermore, as can be seen in Figure 6 in a representative example, there was a very significant increase in elastin fibers in the mid-dermal layer of the explants treated with the copper ions, in accordance with the increased secretion of elastin detected in the medium (Figure 2).

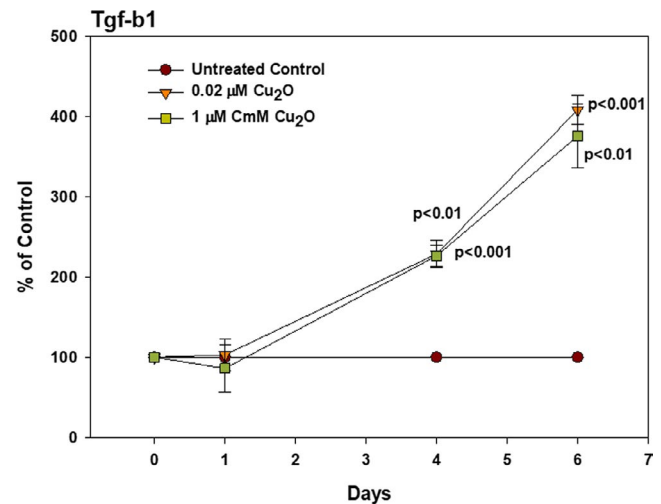


FIGURE 4 Increase in TGF-β1 levels in the explants exposed to copper. The concentration of TGF-β1 in the media collected from the bottom of the wells was determined by ELISA. The data are presented as mean ± standard deviation of triplicates. The P values as compared to the untreated control are depicted

4 | DISCUSSION

Several double blind clinical trials and other observations have demonstrated that continuous contact with the skin of copper oxide-impregnated textiles has a positive effect on the skin.^{11-15,18-20} Copper can be absorbed through skin.¹⁶ Accordingly, some copper-containing ointments are used, for example, for treatment of rheumatic diseases, reduction in swelling associated with trauma, and improvement of renal function.²¹ Currently, many different textiles impregnated with copper oxide particles are widely used, for example hospital linens for reduction in bioburden and nosocomial infections.^{22,23} Some of these copper oxide-containing textiles, such as pillowcases, gloves, and face masks, are used for cosmetic purposes, that is for the reduction in wrinkles and skin sagging and overall improvement of the skin.¹⁵ Copper oxide-impregnated consumer and medical products have been tested in more than a dozen clinical trials and in many nonclinical studies and have been found to be nonirritating, nonsensitizing, and safe to use, with not even one adverse reaction recorded, both when in contact with intact and broken skin.²⁴

We hypothesized that the continuous contact of copper oxide-impregnated textiles with human skin may improve the skin well-being based on the following rationale: the copper oxide-impregnated textiles serve as a continuous source of copper ions that are released in the presence of skin moisture. Some of these ions cross the epithelial layer and reach the dermis.¹⁶ Once in the dermis, the copper ions stimulate fibroblast proliferation⁶ and increase formation of elastin, fibrillin, and several collagens by the dermal fibroblasts.⁷ They also stimulate heat shock protein-47 (HSP-47), a collagen-specific chaperone that is essential for the formation of the collagen triple helical structure, as well as its maturation and secretion.⁷ Furthermore, the copper ions also serve as essential

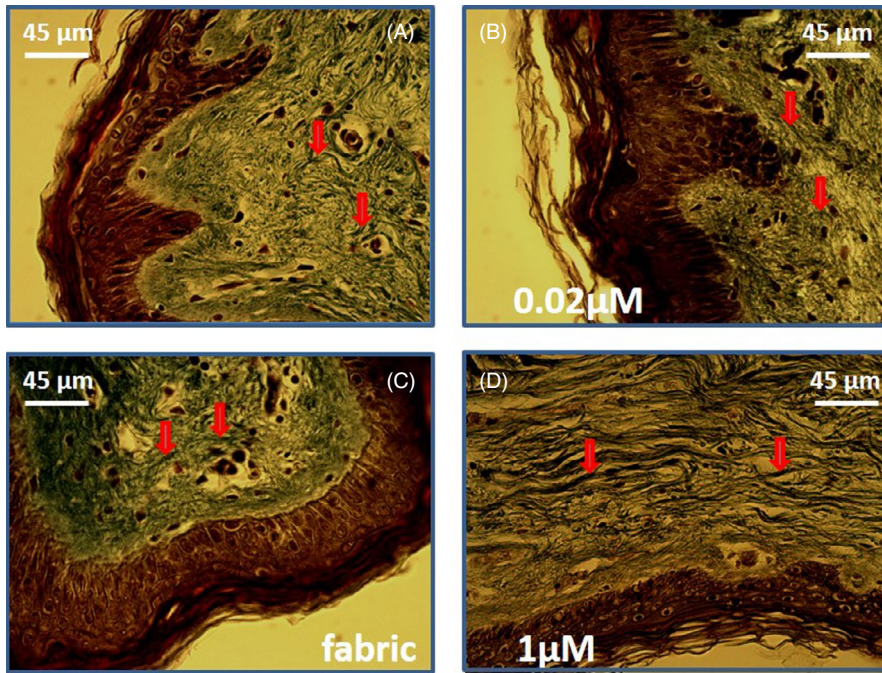


FIGURE 5 Normal histology of skin explants exposed to copper ions. A, Section of skin explant treated with saline only; B, section of skin explant treated with 0.02 μmol/L copper ions; C, section of skin explant covered with a swatch of a 1% w/w copper oxide-impregnated fabric; and (D) section of skin explant treated with 1 μmol/L copper ions. The red arrows point to representative collagen fibers

cofactors of LOX, a key enzyme needed for efficient ECM protein cross-linking.¹

In the current study, we corroborated the above hypothesis by using a human explant model. We used intact swatches of the ~1% copper oxide-impregnated pillowcase in order to unequivocally substantiate our assumptions made that the pillowcases favorably affect the skin properties when in contact with the skin. However, by using the fabric alone, it was hard to measure the amount of copper ions that are in contact with the skin. We thus incubated the fabric in saline in order to allow copper ions to migrate into the saline solution, then determined the copper concentration in the solution, and used those solutions to have a copper quantifiable and repetitive assay. As can be clearly seen in Figure 1, this experimental model allowed us to demonstrate that while the copper ions are in contact only with the epidermal layer (as is the case with the cosmetic pillowcases), they affect also the internal skin layers. In addition to histological analyses, this was demonstrated by determinations of alterations in secretion of precursors of dermal ECM proteins to the media collected from the bottom of the wells, though the dermis layer was not in contact with the fabric swatches or the copper solutions.

We found that the exogenous exposure of intact skin to copper ions resulted in the increased levels of elastin, collagen, and TGF-β1 in the culture media, most probably due to increased secretion of these factors by the dermal fibroblast. The secretion profile we observe, in which TGF-β1 levels increase following the elevation in ECM proteins, may indicate a secretion kinetics which is yet to be studied. This increased secretion is in accordance with our previous observations that exposure of dermal fibroblast cultured in vitro to copper ions results in a dose dependant increased secretion of these factors.⁷ Elastin confers elasticity to the skin, collagen confers firmness, and TGF-β1 is key cytokine involved in extracellular matrix deposition.²⁵ Our findings are also in agreement with the observations that individuals using copper oxide-impregnated socks had increase skin elasticity¹⁴ and that those sleeping on copper oxide-impregnated pillowcases had lower wrinkles, reduced sagging and improved overall skin.¹²⁻¹⁴

Our findings, using a human explant skin model, support the emerging new approach to skin care—cosmetotextiles.²⁶ This approach combines textile materials with cosmetic active substances, transforming daily ordinary textile products, such as pillowcases or

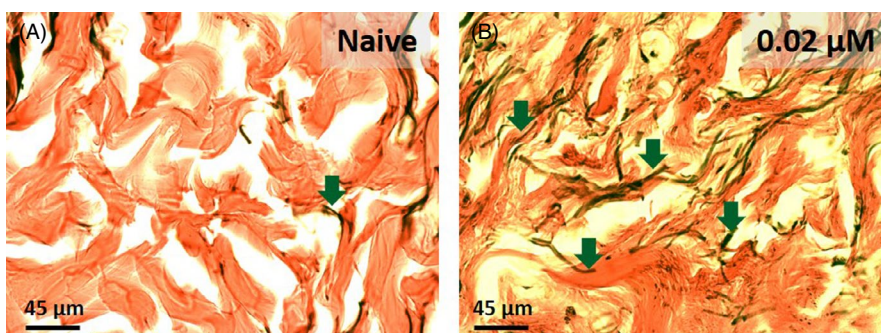


FIGURE 6 Increase in elastin fibers in skin explants exposed to copper ions. A, Section of dermal layer of a skin explant treated with saline only for 6 d; and (B) section of the dermal layer of a skin explant treated with 0.02 μmol/L copper ions for 6 d; The green arrows point to representative elastin fibers

socks, which are used in any case, into cosmetically active products, diminishing the need to actively apply cosmetic substances.

ACKNOWLEDGMENTS

The author acknowledges the fund from Cupron Inc Richmond, Virginia, USA.

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How to cite this article: Ogen-Shtern N, Chumin K, Cohen G, Borkow G. Increased pro-collagen 1, elastin, and TGF- β 1 expression by copper ions in an ex-vivo human skin model. *J Cosmet Dermatol*. 2020;19:1522-1527. <https://doi.org/10.1111/jocd.13186>