Molecular mechanisms of enhanced wound healing by copper oxide-impregnated dressings

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Manuscript received: June 1, 2009
Accepted in final form: January 4, 2010

DOI:10.1111/j.1524-475X.2010.00573.x

ABSTRACT

Copper plays a key role in angiogenesis and in the synthesis and stabilization of extracellular matrix skin proteins, which are critical processes of skin formation. We hypothesized that introducing copper into wound dressings would enhance wound repair. Application of wound dressings containing copper oxide to wounds inflicted in genetically engineered diabetic mice (C57BL/KsOlaHsd-Lepr^{db}) resulted in increased gene and in situ up-regulation of proangiogenic factors (e.g., placental growth factor, hypoxia-inducible factor-1 alpha, and vascular endothelial growth factor), increased blood vessel formation ($p < 0.05$), and enhanced wound closure ($p < 0.01$) as compared with control dressings (without copper) or commercial wound dressings containing silver. This study proves the capacity of copper oxide-containing wound dressings to enhance wound healing and sheds light onto the molecular mechanisms by which copper oxide-impregnated dressings stimulate wound healing.

Copper is postulated to be an essential element in many wound-healing-related processes.[1] It has been demonstrated to (a) enhance angiogenesis by inducing generation of vascular endothelial growth factor (VEGF)[2]; (b) increase the expression of integrin[3]; (c) increase the stabilization of fibrinogen and collagen[4,5]; and (d) up-regulate the activity of copper-dependent enzymes and polysaccharides, important for matrix remodeling, cell proliferation, and reepithelization.[6] Copper's importance and benefit in wound healing have been further demonstrated by its systemic administration in cases of severe burn trauma in children[7] and by its local administration in the management of phosphorus burns.[8] Topical application of a tripeptide with high affinity for copper ions that was isolated from human plasma resulted in accelerated wound healing of diabetic ulcers.[9]

The risk of adverse skin reactions due to copper exposure is extremely low.[10] Copper is considered safe to humans, as demonstrated by the widespread and prolonged use of copper intrauterine devices. Copper is an essential metal needed for normal metabolic processes.[11] The ancient Greeks discovered the sanitizing power of copper thousands of years ago. Since then, copper has been used by inhabitants of America, Africa, and Asia for treating sores and skin diseases.[12]

Venous and diabetic foot ulcers are among the most often occurring chronic wounds, affecting millions of people annually worldwide, and frequently leading to impaired mobility and amputation.[13] Aggressive wound care, consisting of infection control, sharp debridement, weight bearing restriction, and other basic approaches, often results in wound closure. However, many chronic wounds fail to heal and novel treatments are needed. We have recently reasoned that the incapacity of wounds to heal in patients with compromised circulation, particularly those

Efna1	Ephrin A1
EGFR	Epidermal growth factor receptor
eNOS	Endothelial NO synthase
FGFR	Fibroblast growth factor receptor
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Hif-1α	Hypoxia-inducible factor-1alpha
hsp90	Heat shock protein-90alpha
iNOS	Inducible NO synthase
Kdr	a receptor of VEGF
LEPR	Leptin receptor
Mmp	Matrix metalloproteinase
Npr1	Natriuretic peptide receptor 1
Nrp1	Neuropilin
PDGF	Platelet derived growth factor
PLGF	Placental growth factor
SEM	Scanning electron microscopy
Stab1	Stabilin 1
TGF	Transforming growth factor
Thbs	Thrombospondins
Tnfaip2	TNF alpha-induced protein 2
VEGF	Vascular endothelial growth factor
VEGFR-1	VEGF receptor-1

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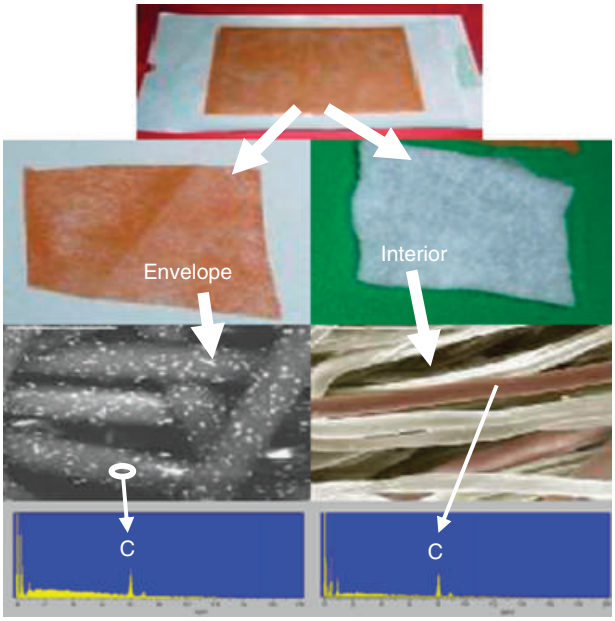

Figure 1. Scanning electron microscopy (SEM) and X-ray photoelectron spectrum of the copper oxide-containing wound dressing. The copper oxide wound dressing consists of two layers. A nonstick spun-bond polypropylene envelope fabric containing 2.3% copper oxide (w/w) impregnated in the fibers (left panel) and an interior highly absorbent needle punch fabric made of viscose/rayon fibers containing 3% (w/w) cellulose fibers plated with copper oxide (right panel). SEM pictures and X-ray photoelectron spectrum analysis of each layer are shown.

with diabetic ulcers, may be partially due to low copper levels in the wound site resulting from decreased blood supply.[1] We thus hypothesized[1] that the slow release of copper ions from wound dressings would enhance wound repair by inducing angiogenesis and up-regulating specific elements that are critical for the healing process.

Copper oxide is a nonsoluble form of copper, which slowly releases copper ions in the presence of moisture.[14] Our study was designed to evaluate the effect of a continuous dermal application of copper oxide-containing wound dressings (Figure 1) on the wound-healing processes by studying full-thickness skin wounds inflicted in genetically engineered diabetic mice.

MATERIALS AND METHODS

Test dressings

Based on recently developed technologies that introduce copper oxide into fibers,[15] a unique copper oxide-containing nonstick wound dressing was designed (hereafter referred to as copper dressing). The copper dressing includes an internal, highly absorbent (165 μL/cm^2=165 μL/10 mg) needle punch fabric made of viscose/rayon (Galaxy®, Kelheim Fibres GmbH, Kelheim, Germany) fibers of 1.4 denier and 38 mm in length and 3% of cellulose copper oxide-plated fibers. The internal layer is enveloped with an external hydrophobic spun bond layer made of polypropylene fibers impregnated with 2.3% weight/weight (w/w) copper oxide particles (Figure 1). The fibers were examined using a Jeol JMS 5410 LV scanning electron microscope (JEOL, Tokyo, Japan), and the presence of copper was determined using a Link IV, ISIS X-ray photoelectron spectrum analyzer (Oxford Instruments, Oxfordshire, UK), as shown in Figure 1.

Two additional wound dressings were tested. One, hereafter referred to as control dressing, is identical to the pad described above, but without any copper. The second pad is a commercially used absorbent wound dressing containing silver particles (Acticoat Absorbent Antimicrobial Dressing; batch # 051115; Smith & Nephew, Largo, FL), hereafter referred to as silver dressing.

Animal experiments

The potential wound-healing effects of copper dressings were assessed by using db/db diabetic mice (C57BL/KsOlaHsd-Lepr^{db}; Harlan Sprague–Dawley, Indianapolis, IN) 7–8 weeks of age at study initiation, following review of the protocol by the Committee for Ethical Conduct in the Care and Use of Laboratory Animals of the Hebrew University, Jerusalem. The db/db mice have a phenotype similar to diabetes type 2, which is achieved via a homozygous point mutation on the leptin receptor (LEPR) gene in the hypothalamus.[16] These mice show significant wound-healing impairment compared with wild-type mice,[16] reaching complete closure of dorsal full-thickness skin wounds 7–10 days later than wild-type mice.[17] The mice were acclimatized to laboratory conditions for 5 days before wounding. They were provided ad libitum with a Harlan Teklad *2018S* Global 18% Protein Rodent Diet (Harlan, Rehovot, Israel). A temperature of 20–24 °C and a 12:12 hours light:dark cycle were maintained throughout the study. Animals were randomly assigned to experimental groups.

An hour before the surgical wounding, all animals were subjected to an opioid analgesic injection (Buprenorphine, 0.075 mg/kg, subcutaneously). The wounding was carried out under general anesthesia by Isoflurane inhalation and under sterile conditions. The fur covering the entire dorsum surface of the trunk was clipped, using an electric clipper. Care was taken to avoid abrading the skin. The clipped area was scrubbed with 4% w/v chlorhexidine gluconate (Septal Scrub®, Teva Medical, Ashdod, Israel) and wiped with 70% ethanol. A circular full-thickness single skin wound was created on the dorsum of each animal with a disposable 6-mm skin biopsy punch instrument (Acuderm, Inc., Fort Lauderdale, FL). The entire wound site was then directly and continuously covered by 20× 20 mm copper dressing, control dressing, or silver dressing. The dressings were held in contact with the skin with a nonirritating tape, which was then bandaged by a semiocclusive dressing to continuously retain the pad/dressing on the animal's trunk. All the animals were subjected to subcutaneous injections of Buprenorphine at a dose level of 0.075 mg/kg throughout the 48-hour period postwounding.

Experiment 1 lasted 17 days and included two groups of mice (n=15/group). One group was treated with the control dressings and one group with the copper dressing. One, 5, 10, 14, and 17 days postwounding, three animals

per group were euthanized (CO_2 asphyxiation). Before each respective scheduled termination, the dressings were carefully removed from the animals assigned to the specific scheduled termination time point. Concomitantly (excluding the first day postwounding), the dressings were carefully removed from all the animals for the purpose of wound area measurements (detailed below). Following the respective measurement, a fresh dressing was reapplied and retained using the method described above. Removal of dressings, wound measurement, and reapplication of the test pads were carried out under general anesthesia, as described above. Experiment 2, which also lasted 17 days, was similar to Experiment 1, but included a third group of mice (n=15/group) treated with silver dressings. In this experiment, measurements were performed at 6, 12, and 17 days postwounding. Six and 12 days after wounding, three mice per group were euthanized, with the remaining nine mice euthanized 17 days after wounding.

The wound area (defined as open skin surface or skin surface covered with granulation tissue, but not epithelialized skin surface) of each animal was measured immediately postwounding (i.e., baseline wound area, measured before the application of the test pad) and thereafter at various days postwounding as detailed above. Measurements of the wound areas were carried out by outlining the wound boundaries onto a transparency sheet, placed directly over the wound. Measurements were performed in duplicate and the outlined wound areas in each animal (mm^2) were determined using a millimeter-grid paper.

The entire wound site and the surrounding $\sim$0.5 cm skin area were surgically removed as soon as possible following the respective scheduled termination time point. Each excised wound site was cut into two fragments: one fragment was placed onto a cardboard and fixed in 10% neutral-buffered formalin and paraffin-embedded. Four μm sections of this fragment were stained with hematoxylin and eosin and routine histopathological analysis was performed. The other fragment (of $<$ 0.5 cm in any one dimension) was individually collected into five volumes of RNA Save Stabilization Solution (Biological Industries, Beit Haemek, Israel) for RNA extraction. The samples collected into the RNA Save solution were stored at $-70\,^{\circ}C$ to $-80\,^{\circ}C$ until analyzed for mRNA expression.

A third experiment was performed for the purpose of performing immunohistochemical analysis of biopsy samples obtained shortly after wounding. It included two groups of mice (n=6/group); one group was treated with the control dressings and the second group was treated with the copper dressings. Three mice per group were euthanized 1 and 5 days postwounding, respectively. In this experiment, the whole excised wound site was used for immunohistochemical analysis.

Analysis of mRNA expression

Total RNA from pooled wound biopsies (three samples) taken at various time points after wounding was extracted by Trizol (Invitrogen, Carlsbad, CA). Total RNA samples containing 2 μg RNA were analyzed for expression of genes using Real-Time PCR Array (Mouse Angiogenesis Array—APMM-024A, Superarray, Frederick, MD) according to the manufacturer's instructions. Gene expression levels were analyzed by software provided by Superarray. The up-regulation of several genes (VEGF, Leptin and fibroblast growth factor receptor 3 [FGFR3]) detected in PCR Array analysis was confirmed by conducting standard RT-PCR analysis using the following primers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene: VEGF-f 5′-cctccgaaaccatga actttc-3′; VEGF-r 5′-cggggtactcctggaagatgtc-3′; Leptin-f 5′-cggttcctgtggctttggtc-3′; Leptin-r 5′-ctcagaatggggtgaagcc-3′; FGFR3-f 5′-gttgagaacaagtttggcagc-3′; FGFR3-r 5′-cac tggatgtgtggctgtg-3′; GAPDH-f 5′-acagaccccagagacccttt-3′ and GAPDH-r 5′-ggcttctgaggaagcaagtg-3′.

Immunohistochemical analysis

For immunohistochemical analysis, antigen unmasking was performed by pressure cooking in 0.6 M citrate buffer (pH 6.0) for 18 minutes. Slides were quenched in 0.3% hydrogen peroxide in methanol for 10 minutes. The following primary antibodies, final dilutions, time, and incubation temperatures were used: Rabbit polyclonal to Von Willebrand factor (vWF) (ab6994, Abcam, Cambridge, UK) at 1 : 200, overnight at 4 °C; rabbit polyclonal to placental growth factor (PLGF) (ab9542; Abcam) at 1 : 500, overnight at 4 °C; mouse mAb to Vascular Endothelial growth factor (VEGF) (ab1316; Abcam) at 1 : 200, 1 hour at room temperature; and mouse mAb to hypoxia-inducible factor-1 alpha (Hif-1α; ab1; Abcam) at 1 : 50, 1 hour at room temperature. Detection of primary antibodies was performed using the SuperPicTure™ Polymer Detection Kit (Zymed Laboratories, Invitrogen immunodetection, San Francisco, CA) and 3, 3-diaminobenzidine (DAB) as a chromogen. Twenty-four slides were carefully examined using high-power microscopy (Olympus BX51 Microscope, Tokyo, Japan) and photography (Olympus ColorView3U, Tokyo, Japan) analysis. The wounded areas were distinguished from the adjacent skin by an expert pathologist (M.H.) based on the presence of reparative processes, such as granulation tissue formation and ulceration. Blood vessels in the wounded areas were detected by immunohistochemical staining for vWf. Endothelial cells expressing vWf showed brown staining of the cytoplasm. Vascularity in the wounds was determined by light microscopy counting the number of vWf positively stained endothelium lined vascular structures per high-power field.

Statistical analysis

Data of wound size and number of blood vessels were analyzed using SigmaStat Version 2.0 (Systat Sowftware, Richmond, CA) and GraphPad Instat® Version 3.02 (GraphPad Software, La Jolla, CA) and expressed as the mean ± SD. Significant differences between treatments were evaluated using unpaired t-tests, two-tailed p value test, one-way ANOVA, and Dunnett multiple comparisons test.

RESULTS

Wound area measurements

As shown in Table 1 and Figure 2A, the mean size of the wound area in the first experiment was significantly

Table 1. Mean group wound area (mm^2) following full thickness surgical wounding and continuous dermal application of wound dressings

Experiment	Dressings	Day after wounding	Number of mice (mean ± SD)	Wound area	*p* values[1]
1	Control	0	15	33.7 ± 1.24	
		1	3	34.3 ± 0.58	
		5	12	28.7 ± 3.6	
		10	9	21.78 ± 6.1	
		14	6	14.5 ± 5.75	
		17	3	10.2 ± 3.33	
	Copper	0	15	33.6 ± 1.51	
		1	3	34.3 ± 2.02	
		5	12	26.0 ± 3.67	
		10	9	14.1 ± 6.1	< 0.05[2]
		14	6	6.33 ± 4.41	< 0.05[2]
		17	3	4.7 ± 2.93	
2	Control	0	15	32.7 ± 0.98	
		6	15	28.87 ± 2.26	
		12	12	20.83 ± 4.88	
		17	9	11.17 ± 3.32	
	Silver	0	15	32.6 ± 0.85	
		6	15	27.47 ± 4.02	
		12	12	20.63 ± 4.7	
		17	9	12.11 ± 4.2	
	Copper	0	15	32.7 ± 1.88	
		6	15	24.3 ± 4.65	< 0.01[3]
		12	12	15.9 ± 2.47	< 0.01[2]; < 0.05[3]; < 0.01[4]
		17	9	7.78 ± 2.88	< 0.05[2]; < 0.05[4]

[1]only *p* values below 0.05 are shown.
[2]vs. control dressings (unpaired *t*-test, two-tailed *p* value test).
[3]vs. control dressings (one-way ANOVA, Dunnett multiple comparisons test).
[4]vs. silver dressings (unpaired *t*-test, two-tailed *p* value test).

smaller on days 10 and 14 after wounding in the group of mice treated with copper dressings compared with the mice treated with control dressings. In the second experiment, the mean size of the wound area was significantly smaller on days 6, 12, and 17 after wounding in mice treated with copper dressings than with control dressings (Table 1 and Figure 2B). In contrast, no significant differences were found in the wound area of mice treated with control dressings as compared with those treated with silver dressings (Table 1 and Figure 2B). The mean wound area of the copper dressing-treated group was also significantly lower on days 12 and 17 as compared with the silver dressing-treated group (Table 1).

Histopathological analysis

Histopathological analysis of skin specimens taken from the wounded diabetic mice that were treated with control dressings or copper dressings showed a similar normal wound-healing process in both treated groups. This included epidermal regeneration and granulation tissue formation, with numerous new blood vessels, chronic inflammatory infiltrate, generation of new hair follicles and sebaceous glands, and fibroplasia (Figure 3). There were no signs of adverse reactions, precancerous changes, or atypia of any kind in both groups of mice.

Gene expression analysis

The mRNA levels in the wound areas of 84 genes associated with angiogenesis and wound repair were determined using real-time PCR gene-array analysis. The percent of genes that were significantly up-regulated or down-regulated in the copper dressing-treated group compared with the control dressing group was 3.6%, 58%, 40%, 65%, and 52% on days 1, 5, 10, 14, and 17, respectively (a difference of more than fivefold between both groups was considered significant). On the first day following wounding, only integrin-alpha 5, integrin-beta 3, and PLGF were significantly up-regulated in the copper dressing group compared with the control dressing group (by 5.3, 7.57, and 21.7 fold, respectively). Figure 4 and Table 2 demonstrate the quantitative differences in gene expression between the copper dressing and the control dressing-treated

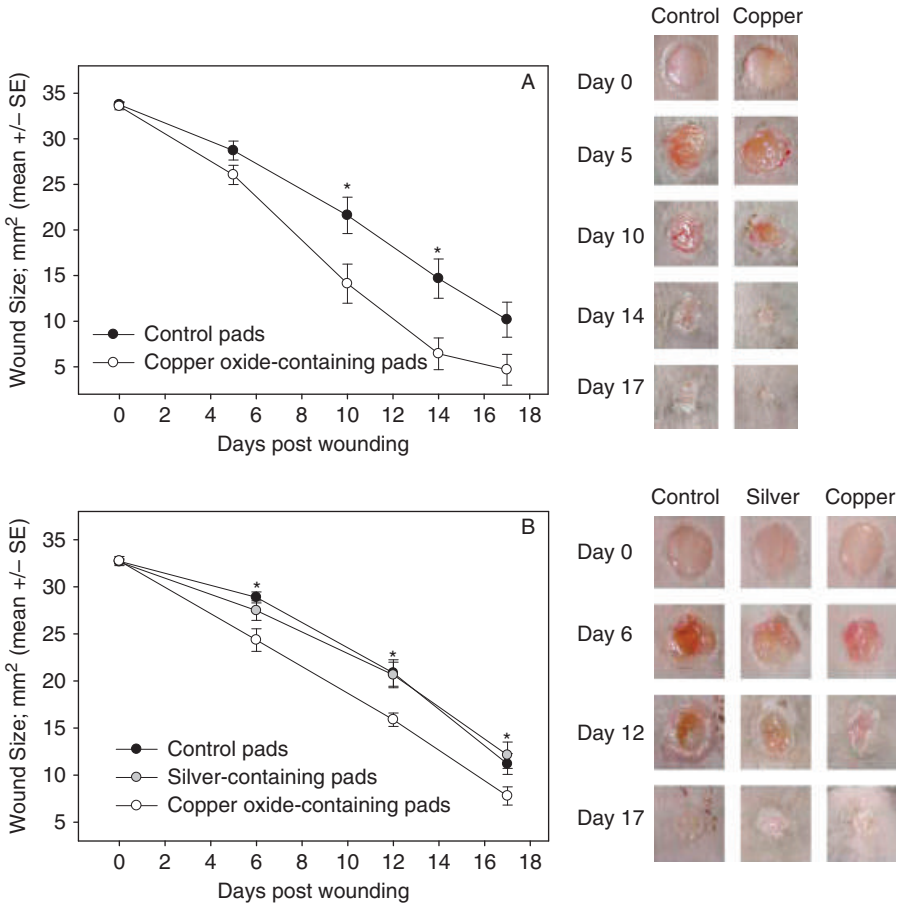

Figure 2. Copper oxide-containing wound dressings enhance wound healing in diabetic mice. Following circular full-thickness single skin wounds surgically inflicted in the dorsum of the mice, the wounds were directly and continuously covered with control or copper oxide-containing wound dressings (Experiment 1, A) or silver-containing wound dressings (Experiment 2, B). The mean wound area $\pm$SE at several time points following wounding is depicted. *Statistical difference (p value < 0.05) between the mean wound areas in the control dressing and the copper dressing-treated groups. Representative pictures of the dorsal wounds after several days of wounding are shown.

groups. The up-regulation of VEGF, leptin, and FGFR3 in the copper dressing-treated group was confirmed by RT-PCR analysis (data not shown).

Immunohistochemical analysis

No differences in the number of blood vessels and PLGF, VEGF, Hif-1α, and vWF were noted between mice treated with copper dressings or control dressings on day 1 after wounding. However, a significant increase in blood vessel number, as determined by staining with vWF (which stains capillaries), was noted on day 5 in the copper dressing-treated mice (19 ± 2.65 vs. 12 ± 3.5; $p < 0.05$) that paralleled a significant increase in staining for PLGF, Hif-1α, and VEGF, as compared with the control-treated mice (Figure 5). PLGF was only detected in newly formed blood vessels (already existent and well-developed blood capillaries were not stained) and in hair follicles. VEGF was noted in all capillaries, both on the endothelial cells and extracellularly. Hif-1α was expressed in the dermal layer, apparently in macrophages, as determined by morphological assessment (Figure 5).

DISCUSSION

Silver-containing wound dressings are widely used in wound treatment to reduce the risk of wound and wound-dressing contamination. However, their usefulness in promoting wound healing is questionable.[18,19] Copper also has potent biocidal properties,[20] but in contrast to silver, copper is an essential trace element[11] that plays a key role in skin generation and angiogenesis.[1–6] We thus hypothesized that the inability of wounds to heal in individuals with compromised peripheral blood supply (e.g., diabetes) is partially due to low levels of copper in the wound site.[1] We suggested that by introducing copper oxide particles (known to slowly release copper ions[14]) into wound dressings, the constant release of copper ions directly into the wounds would enhance angiogenesis, skin regeneration, and wound healing.

Our previous studies demonstrated that copper-impregnated dressings, while possessing potent biocidal properties, did not cause any skin irritation in rabbits and no local damage to open wounds or systemic pathological alterations in a porcine full-thickness wound model (unpublished data).

The current study extended these findings by using a murine diabetic model considered to be the most suitable in diabetic wound-healing studies.[16] Wounding and subsequent treatments were performed under sterile conditions, so that the possible effects of the copper dressings would not be related to the biocidal properties of copper,[20] such as reduction of bacterial contamination. This study demonstrates that in wounds inflicted in diabetic (db/db) mice

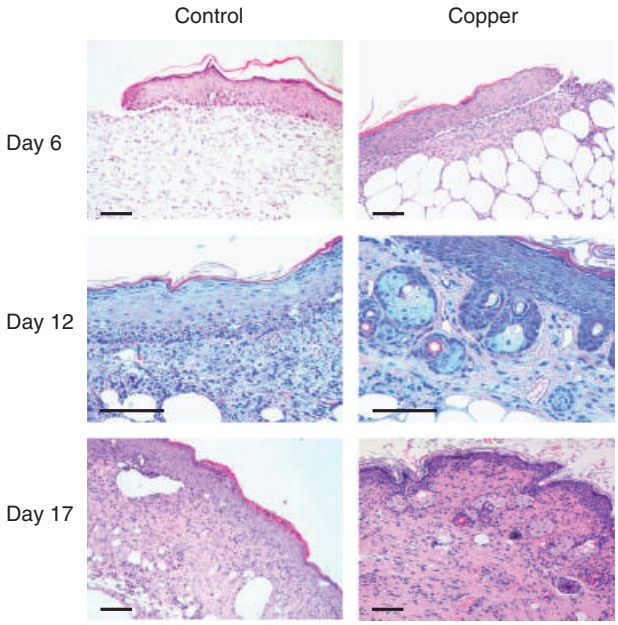

Figure 3. Representative hematoxylin and eosin staining of wound sites taken at days 6, 12, and 17 from mice treated with the control and copper dressings. Scale bars = 100 μm.

under sterile conditions and kept covered throughout the study with sterile copper-impregnated dressings, there is a statistically significant enhancement of wound closure. As early as 6 days postwounding, the wounds were statistically significantly smaller in the copper dressing-treated mice than in the control group. It should be pointed out that the elimination of three animals at each scheduled termination day (in order to collect biopsies) in the first experiment allowed only limited statistical evaluation of the test results on day 17 in this experiment. However, in the second experiment, nine animals per group were evaluated on day 17 and the difference between the wound size in the copper-treated and the control-treated groups was statistically significant. Enhanced wound healing was comparable to what had been noted in wild-type mice, where similar full-thickness dorsal skin wounds reach complete closure 7–10 days earlier than in db/db mice.[17] In contrast, commercially used silver-containing wound dressings did not accelerate wound healing in this model.

To better understand the mechanisms by which copper induces enhanced wound healing, we analyzed the expression of 84 genes known to be involved in angiogenesis and/or wound healing at the wound sites at various days after wounding. Based on the gene analysis and known roles and interactions of the various factors, Figure 6 describes the possible mechanisms by which copper may accelerate wound healing.

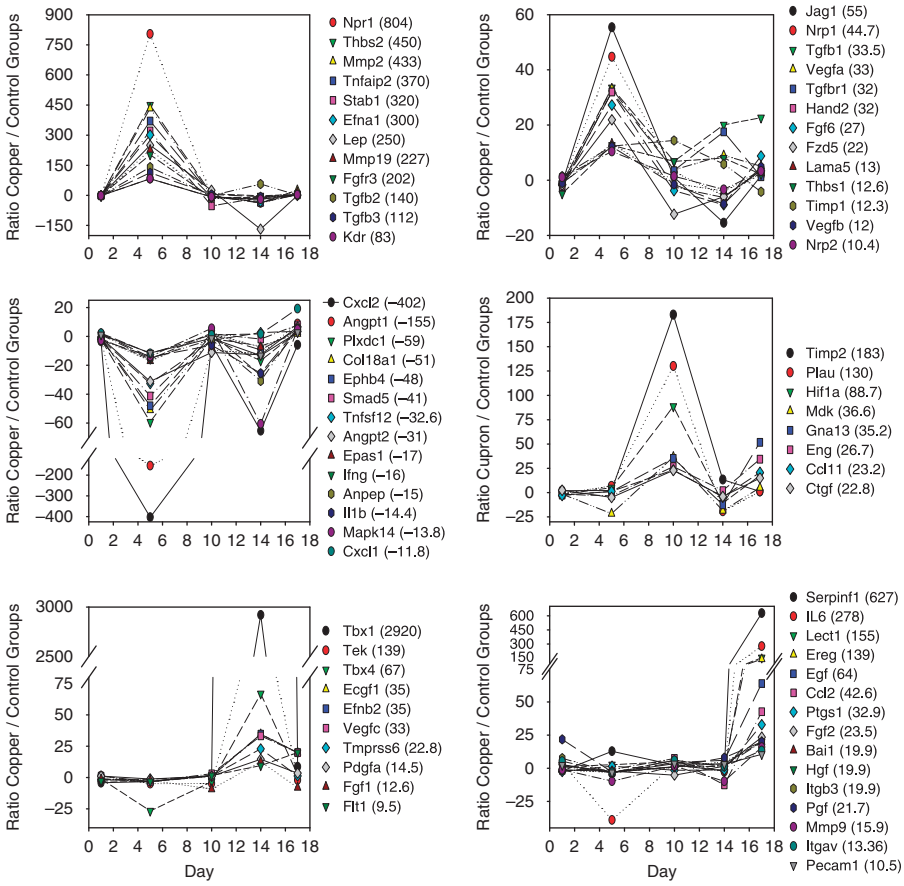

Figure 4. Expression of 84 genes at the wound site at various days following wounding. The expression of 84 genes is depicted as the ratio between their mean expression in the wounds of the mice treated with the copper oxide-containing dressings and their expression in the wounds of the mice treated with the control dressings. For ease of analysis, the genes are shown in six separate panels and the exact ratio per gene is given in parentheses next to the name of each gene.

Table 2. Gene expression at the wound site of selected analyzed genes[1]

Name of protein	Abbreviation	Day after wounding				
		1	5	10	14	17
Ephrin A1	Efna1	−1.89[2]	300.73	−8.72	−30.98	2.95
Epidermal growth factor	Egf	2.79	−3.19	4.25	6.03	64.00
Fibroblast growth factor 6	Fgf6	−1.56	27.22	−3.80	−8.91	8.76
Fibroblast growth factor receptor 3	Fgfr3	−2.04	201.99	−11.50	−10.11	3.68
Hypoxia inducible factor 1, alpha subunit	Hif1a	−1.76	5.05	88.67	−10.74	17.55
Kinase insert domain protein receptor	Kdr	−1.38	82.74	−7.35	−19.54	2.69
Leptin	Lep	−1.64	249.55	24.89	−168.69	−1.56
Matrix metallopeptidase 19	Mmp19	−2.71	227.18	−16.35	−14.20	14.08
Matrix metallopeptidase 2	Mmp2	−3.17	433.19	−1.72	−38.75	26.02
Natriuretic peptide receptor 1	Npr1	−1.84	804.12	−4.30	−37.65	5.03
Neuropilin 1	Nrp1	−1.05	44.74	3.03	−5.70	2.47
Placental growth factor	Plgf	21.76	−2.87	1.04	7.96	19.87
Stabilin 1	Stab1	−1.44	320.62	−52.54	−11.75	3.00
Transforming growth factor, beta 1	Tgfb1	−1.15	33.49	6.84	20.06	22.61
Transforming growth factor, beta 2	Tgfb2	−2.05	140.56	−1.62	56.53	1.57
Transforming growth factor, beta 3	Tgfb3	−1.74	112.48	4.14	−7.08	2.71
Transforming growth factor, beta receptor I	Tgfbr1	−2.38	32.03	3.47	17.54	1.26
Thrombospondin 1	Thbs1	−4.92	12.65	6.81	8.02	2.17
Thrombospondin 2	Thbs2	−4.24	450.14	8.11	−2.37	4.62
Tumor necrosis factor, α-induced protein 2	Tnfaip2	−2.54	370.62	−2.841	−35.57	2.43
VEGF A	Vegfa	−1.08	33.17	3.75	9.00	5.03

[1]Please see Supporting Information, Table S1 in order to see all genes analyzed.
[2]Gene expression is expressed as the ratio of the mean expression in mice treated with the copper oxide-containing wound dressings and to that expressed in mice treated with control wound dressings.

One day postwounding, a five to sevenfold up-regulation of integrins and a 22-fold up-regulation of PLGF in the copper dressing-treated group (Figure 4 and Table 2) was found. Integrins, transmembrane receptor proteins that bind extracellular matrix proteins, transduce signals pivotal for cell processes in wound healing.[21] Up-regulation of integrin by copper has been described in cultured keratinocytes and in reconstituted skin.[3] PLGF, expressed during cutaneous wound healing, improves wound closure by enhancing angiogenesis[22] needed to sustain the metabolic demand for skin regeneration and for recruitment of inflammatory cells. Interestingly, PLGF induction has been previously shown to be markedly reduced in diabetic wounds,[23] and treatment of diabetic wounds with PLGF significantly improved granulation tissue formation, maturation, vascularization, and recruitment of monocytes/macrophages.[24] Thus, our data, which clearly detect PLGF in newly formed capillaries and hair follicles in mice treated with the copper dressings (Figure 5), and almost none in mice treated with control dressings, confirm the critical role of this copper-dependent factor. PLGF binds and activates the VEGF receptor-1 (VEGFR-1) and potentiates VEGF activities.[22] VEGFs are, in turn, key angiogenic stimulants and their role in stimulating wound healing was demonstrated.[25] PLGF may thus be a key initial factor directly (Figure 6) or indirectly affected by copper, responsible for the onset of subsequent processes in wound healing. Further studies are needed to substantiate this postulation.

A very distinct pattern of gene expression was noted between control dressing and copper dressing-treated mice at 5 days and onward (Figure 4 and Table 2). Hif-1α was significantly up-regulated following wounding (by 5- and 88-fold at 5 and 10 days, accordingly) in the mice treated with copper dressings (Table 2 and Figure 5). Hif-1α is the regulatory subunit of Hif-1 transcription factor, a protein induced under various types of stress conditions, including oxidative stress and presence of reactive oxygen species.[26] Importantly, redox between CuO and Cu_2O results in the production of H_2O_2 and hydroxyl radicals,[20] explaining the observed up-regulation of Hif-1α. Copper-dependent activation of Hif-1α has been demonstrated.[27] Notably, Hif-1α protein levels are dramatically reduced in wounds of db/db mice.[28] Hif-1α plays a central role in activating many genes involved in wound angiogenesis and wound healing.[28] Macrophages play a key role in inflammation, angiogenesis, granulation tissue formation, and wound healing. HIF-1α protein was shown to be abundantly expressed by macrophages in inflamed rheumatoid synovia while being absent in healthy synovia.[29] It was also demonstrated that HIF-1α expression is strongly increased in inflammatory cells from wounds.[30] In addition, conditional knockout of HIF-1α in macrophages and other myeloid lineage cells leads to impaired chronic cutaneous

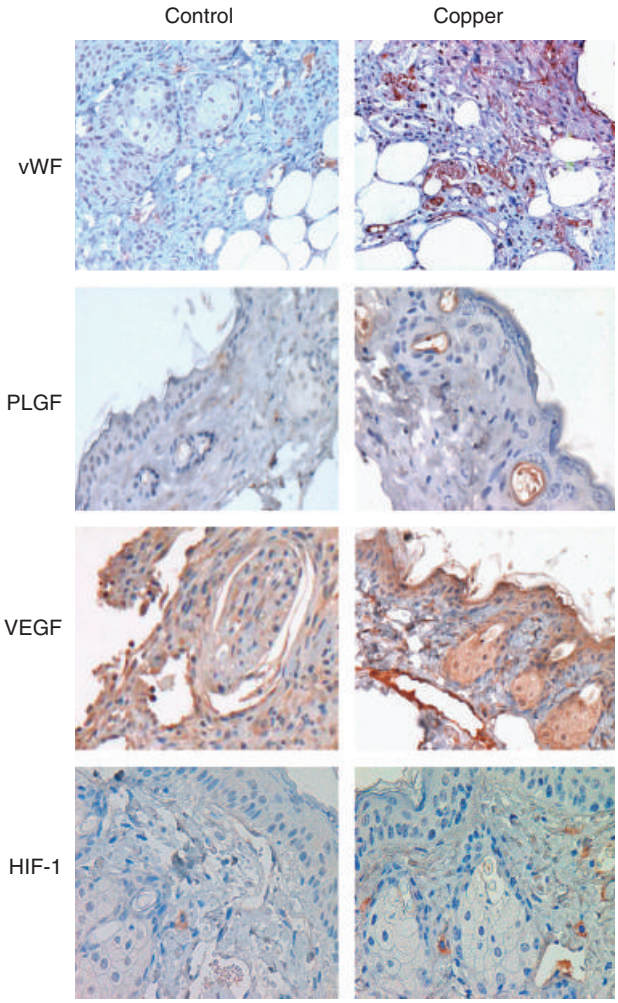

Figure 5. Immunohistological analysis of biopsies taken from the wound area of mice treated with control and copper dressings. Positive staining for Von Willebrand factor (vWF), placental growth factor (PLGF), vascular endothelial growth factor (VEGF), and Hypoxia-inducible factor-1alpha (Hif-1α) resulted in brown coloration.

inflammation.[31] After myocardial infarction, macrophages are particularly densely stained for HIF-1α protein at the border of the infracted area.[32] Taken together, these studies implicate HIF-1α as an important mediator of inflammatory responses in macrophages. HIF-1α protein was recently demonstrated to be expressed in keratinocytes in healing wounds.[33] Interestingly, in our hands, Hif-1α protein staining was positive only in macrophages. This may possibly be attributed to the intensity of protein expression in macrophages as compared with the rest of the tissue.

Several factors that were significantly up-regulated in the copper dressing-treated mice on day 5 have been shown to be up-regulated by Hif-1α in other studies. These include: Ephrin A1 (Efna1) (300-fold increase), which stimulates angiogenesis;[34] VEGF (A, B and C) (33-fold increase)—a major angiogenesis stimulant in wounds (importantly, the expression of VEGF was shown to be reduced in diabetic wounds[25]); matrix metalloproteinase 2 (Mmp2) (433-fold increase)—important in angiogenesis and cutaneous wound healing[35]; and transforming growth factor (TGFb), including TGFb1, TGFb2, and TGFb3 and TGFb1 receptor (33-, 140-, 112-, and 32-fold increase, accordingly)—all of which were shown to be important in angiogenesis and extracellular matrix synthesis.[36]

Possible additional wound-healing-enhancing factors, not analyzed by us but shown by others to be up-regulated by Hif-1α, are (i) epidermal growth factor receptor (EGFR) (copper activation of EGFR downstream signaling has been shown[37]); (ii) Inducible NO synthase (iNOS) and endothelial NO synthase (eNOS); (iii) platelet-derived growth factor (PDGF)-BB; (iv) interleukin 8; and (v) heat shock protein-90alpha (hsp90). Other factors important in wound healing, such as the EGFR receptor ligand amphiregulin, are up-regulated under hypoxia in a Hif-1α-independent manner.[38]

Other growth and angiogenic factors or receptors and factors essential in healing wounds, which were significantly up-regulated on day 5 in the copper dressing-treated mice (Table 2 and Figure 4), were Natriuretic peptide receptor 1 (Npr1, by 804-fold); FGFR3 (by 202-fold); FGF6 (by 27-fold); Kdr (by 83-fold)—a receptor of VEGF expressed in endothelial cells; Leptin (Lep, by 250-fold), which increases early incisional wound angiogenesis; MMP19 (by 227-fold); Neuropilin 1 (Nrp1, by 45-fold), a receptor of VEGFA; Stabilin 1 (Stab1, by 320-fold), expressed by macrophages during tissue remodeling; thrombospondins (Thbs1 and Thbs2, by 13- and 450-fold, respectively); and tumor necrosis factor alpha-induced protein 2 (Tnfaip2, by 370-fold). In contrast, several factors were significantly down-regulated on day 5 in the copper dressing-treated mice. The down-regulation of several of these factors, such as CXCL2 and angiopoietin (Supporting Information, Table S1), and the fluctuating values observed for some genes, are not clear to us and deserve further research. However, the down-regulation of other factors, such as of interleukin 1 (by 14-fold), a cytokine known to induce further tissue damage after injuries, is in accordance with improved wound healing and with previous reports.[39]

In addition, copper may play a role in remodeling of the extracellular matrix because it increases fibronectin synthesis in vitro and is a cofactor for proteins involved in extracellular matrix formation. Copper also induces the assembly of a multiprotein aggregate implicated in the release of FGF1 in response to stress.[40]

Additional studies are needed to further elucidate the mechanisms by which copper stimulates wound healing. It is clear, however, that copper directly or indirectly stimulates many factors, some of which are impaired in diabetics and are important for keratinocyte and fibroblast proliferation, epithelialization, collagen synthesis, extracellular matrix remodeling, and angiogenesis, resulting in accelerated wound healing. As established in this study, the positive effect of the copper oxide-containing dressings is not related to its potent biocidal properties, which may also be important in wound healing, but rather to the direct stimulation of wound repair. Copper dressings appear to hold significant promise in the clinician's ongoing struggle to heal both acute and chronic wounds, and, as such,

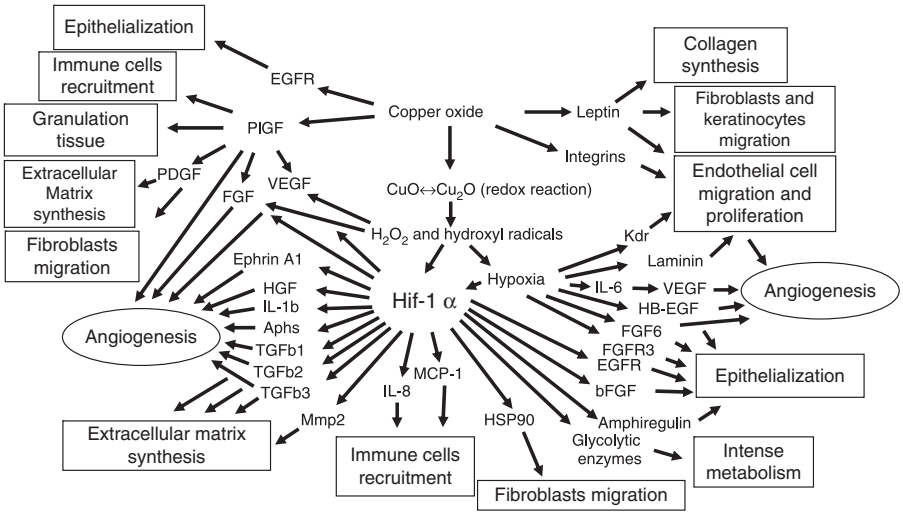

Figure 6. Proposed mechanisms by which copper oxide stimulates angiogenesis and wound healing.

prospective randomized, blinded studies should be conducted to further validate the efficacy of topically applied copper-impregnated dressings.

ACKNOWLEDGMENTS

This study was funded by Cupron Inc. Gadi Borkow is the Chief Medical Scientist of Cupron. Jeffrey Gabbay is the Chief Executive Officer of Cupron. Arthur I. Eidelman is Cupron's Medical Advisor. Moshe Marikovsky and Rima Dardik served as consultants to this study.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Gene expression at the wound site. The ratio between the gene expression in mice treated with the copper oxide-containing wound dressings and those treated with control wound dressings is shown.

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