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Copper's Role in Wound Healing

Review of Literature

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Wound healing – Overview

Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct, but overlapping phases: Hemostasis, Inflammation, Proliferation and Remodeling [1;2]. The normal healing response begins the moment the tissue is injured. As the blood components spill into the site of injury, the platelets come into contact with exposed collagen and other elements of the extracellular matrix (Fig 1).

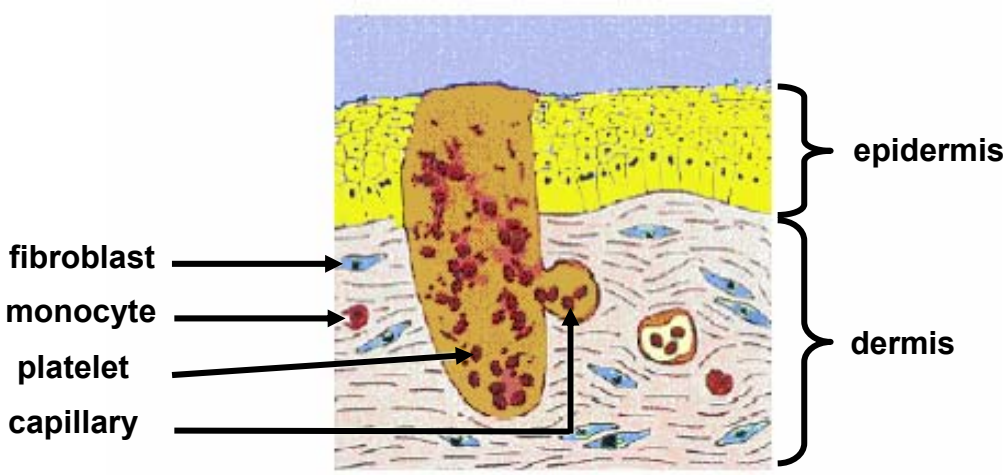

Figure 1. At the time of injury, the tissue is disrupted and the platelets adhere to the exposed collagen and to each other.

This contact triggers the platelets to release clotting factors as well as essential growth factors and cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-ß). Following Hemostasis, the neutrophils enter the wound site and begin the critical task of removing foreign materials, bacteria and damaged tissue (Fig 2).

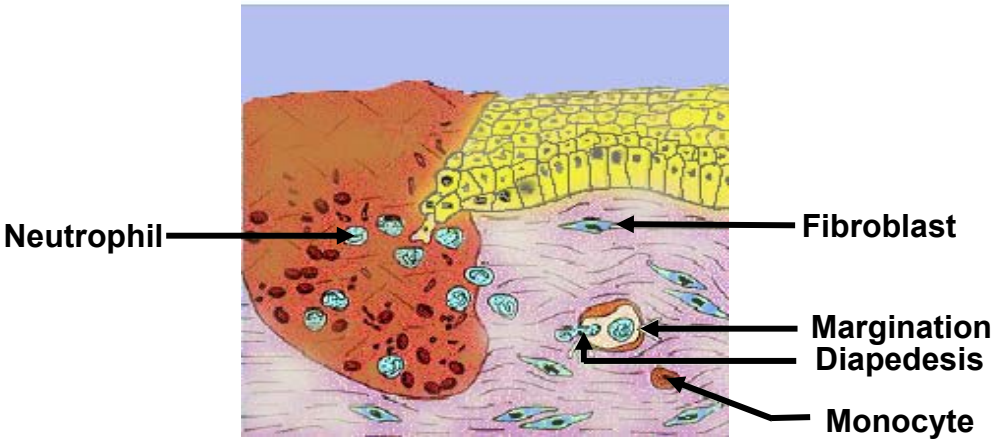

Figure 2. Neutrophils attach to endothelial cells in the vessel walls surrounding the wound (margination), then change shape to move through the cell junctions (diapedesis) and migrate to the wound site (chemotaxis). This is the beginning of the Inflammatory Phase.

Bacteria give off chemical signals, which attract the neutrophils. The bacteria are ingested by the neutrophils by the process of phagocytosis. Neutrophils will engorge themselves until they are filled with bacteria and constitute what is called "pus". It has been postulated that neutrophils

also secrete sterilizing oxygen radicals. Mast cells release granules filled with enzymes, histamines and other active amines and these mediators are responsible for the characteristic signs of inflammation around the wound site. The active amines released from the mast cell, causes surrounding vessels to become leaky and thus allow the speedy passage of T lymphocytes and mononuclear cells (macrophages) into the injury area (Fig. 3).

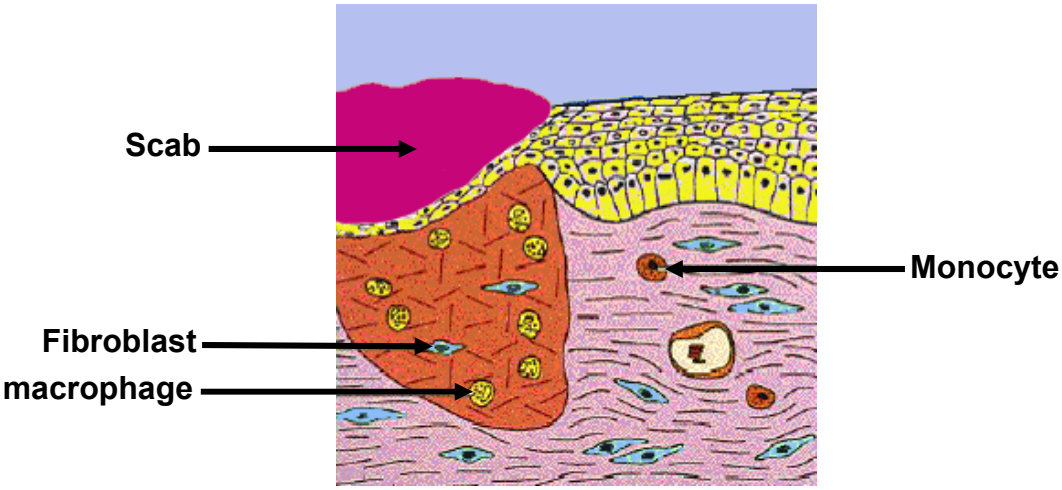

Figure 3. The Inflammatory Phase continues as fixed tissue macrophages become active and move into the site of injury and transform into very active wound macrophages.

The highly phagocytic macrophages are responsible for removing nonfunctional host cells, bacteria-filled neutrophils, damaged matrix, foreign debris and any remaining bacteria from the wound site. The macrophages continue releasing more PDGF and TGF-ß to recruit fibroblasts. Once the wound site is cleaned out, the migrated fibroblasts begin the Proliferative Phase by depositing new extracellular matrix. As the Proliferative Phase progresses, the TGF-ß released by the platelets, macrophages and T lymphocytes becomes a critical signal. TGF-ß is considered to be a master control signal that regulates a host of fibroblast functions. TGF-ß has a three-branched effect on extracellular matrix deposition. First, it increases transcription of the genes for collagen, proteoglycans and fibronectin, thus increasing the overall production of matrix proteins. At the same time TGF-ß decreases the secretion of proteases responsible for the breakdown of the matrix and it also stimulates the protease inhibitor, tissue inhibitor of metallo-protease (TIMP). Other cytokines considered to be important are interleukins, fibroblast growth factors and tumor necrosis factor-alpha. As healing progresses several other important biological responses are activated. The process of epithelization is stimulated by the presence of EGF (epidermal growth factor) and TGFα (transforming growth factor alpha) that are produced by activated wound macrophages, platelets and keratinocytes (Fig. 4).

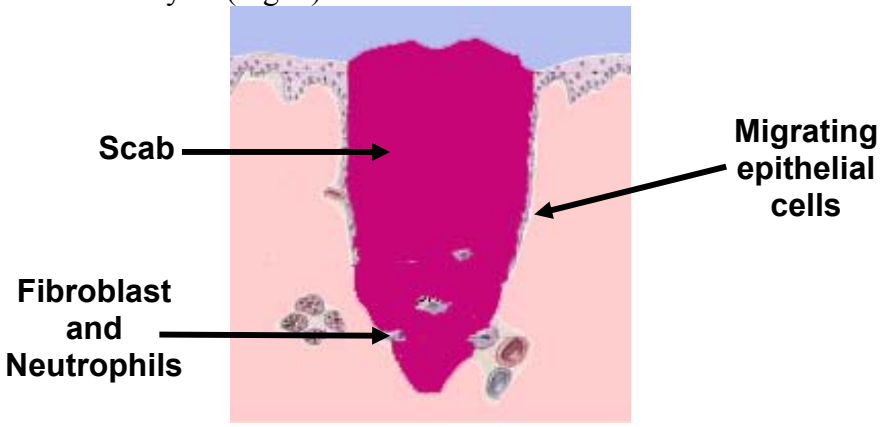

Figure 4. Epithelization

Once the epithelial bridge is complete, enzymes are released to dissolve the attachment at the base of the scab, resulting in its removal. Due to the high metabolic activity at the wound site, there is an increasing demand for oxygen and nutrients. Local factors in the wound microenvironment such as low pH, reduced oxygen tension and increased lactate actually initiate the release of factors needed to bring in a new blood supply. This process is called angiogenesis or neovascularization and is stimulated by vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF) and TGF ß. Epidermal cells, fibroblasts, macrophages and vascular endothelial cells produce these factors. As the Proliferative Phase progresses the predominant cell in the wound site is the fibroblast. This cell is responsible for producing the new matrix needed to restore structure and function to the injured tissue. Fibroblasts attach to the cables of the provisional fibrin matrix and begin to produce collagen. The new collagen matrix then becomes cross-linked, a process facilitated by the enzyme lysyl oxidase. Thus, during the final Remodeling Phase, the collagen forms well organized stable cross-links (Fig. 5).

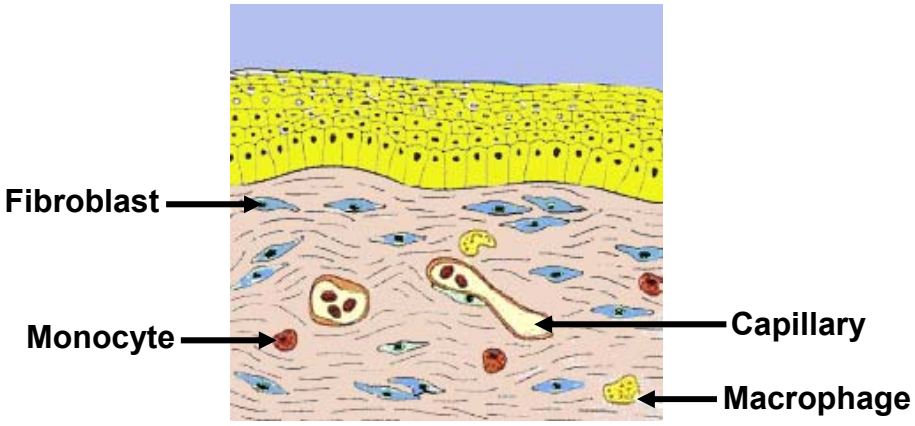

Figure 5. The Remodeling Phase is characterized by continued synthesis and degradation of the extracellular matrix components trying to establish a new equilibrium.

As noted above, in order for this efficient and highly controlled repair process to take place, there are numerous cell-signaling events that are required. In pathologic conditions such as non-healing pressure ulcers, this efficient and orderly process is lost and the ulcers are locked into a state of chronic inflammation characterized by abundant neutrophil infiltration with associated reactive oxygen species and destructive enzymes. Healing proceeds only after the inflammation is controlled. On the opposite end of the spectrum, fibrosis is characterized by excessive matrix deposition and reduced remodeling. Often fibrotic lesions are associated with increased densities of mast cells [2].

The events of early wound healing reflect a finely balanced environment leading to uncomplicated and rapid wound healing. Chronic wounds, for many reasons, have lost this fine balance. All chronic wounds are colonized by bacteria, with low levels of bacteria being beneficial to the wound healing process. The progression from wound colonization to infection depends not only on the bacterial count or the species present, but also on the host immune response, the number of different species present, the virulence of the organisms and synergistic interactions between the different species. There is increasing evidence that bacteria within chronic wounds live within biofilm communities, in which the bacteria are protected from host defences and develop resistance to antibiotic treatment [3].

Angiogenesis and copper

Angiogenesis plays a central role in wound healing. Among many known growth factors, vascular endothelial growth factor (VEGF) is believed to be the most prevalent, efficacious, and long-term signal that is known to stimulate angiogenesis in wounds. Whereas the direct role of copper in facilitating angiogenesis has been evident for two decades [4-6], the specific targets of copper action remained unclear. In a recent study, categorical effort was made to identify the cytokine(s) responsible for mediating the angiogenic effects of copper [7]. The study reported that copper-induced proliferation of endothelial cells is not inhibited by serum deprivation or by the presence of antibodies against a variety of angiogenic, growth, and chemotactic factors, including angiogenin, fibroblast growth factors, epidermal growth factor, platelet-derived growth factor, tumor necrosis factor-α, transforming growth factor-β, macrophage/monocyte chemotactic and activating factor, and macrophage inflammatory protein-1α. Ironically, VEGF was not studied [7]. In both *in vitro in vivo* models, $CuSO_4$ clearly promotes angiogenic responses. These observations have led to the development of anti-copper-based, anti-angiogenic strategies for the treatment of cancer [8]. (However, it was only recently shown that inducible VEGF expression is sensitive to copper and that the angiogenic potential of copper may be harnessed to accelerate dermal wound healing [9].) At physiologically relevant concentrations, copper sulfate induces VEGF expression in primary as well as transformed human keratinocytes. The effect of Cu^{2+} on inducible VEGF expression is not mediated by H_2O_2 but is dependent on the thiol-redox state of the cells. Topical copper sulfate accelerated the closure of excisional murine dermal wounds. Histological analysis of wound-edge tissue substantiated that $CuSO_4$ treatment did not only accelerate wound closure but that the quality of regenerating tissue was distinctly different. $CuSO_4$ treatment was associated with more hyperproliferative epithelial tissue, and the density of cells in the granulation layer of copper-treated wounds was clearly higher. Immunohistochemical studies show that wound edges of copper-treated wounds have more prominent VEGF expression [9]. Thus, inducible VEGF expression is sensitive to copper and the angiogenic potential of copper may be harnessed to accelerate dermal wound contraction and closure. Furthermore, copper chelation represses the vascular response to injury [10]. Taken together, copper-based therapeutics therefore represent a feasible approach to promote dermal wound healing.

Fibronectin and Copper

Fibronectin, a large extracellular matrix cell adhesion glycoprotein, has diverse functions in wound repair including organization of matrix deposition and promotion of angiogenesis. Purified plasma fibronectin can be made into three-dimensional, fibrous materials, termed fibronectin mats (Fn-mat) [11]. Such cables of fibronectin have a potential application in tissue engineering. It was found that the stability of Fn-mats may be increased by treating them with micromolar concentrations of copper ions, allowing for improvement of wound healing. Dissolution of protein from the Fn-mat showed that treatment with the lowest concentration of copper used (1 μM) increased the stability of mats by 3-4 times at room temperature relative to control mats and twofold at 37 degrees C. Copper mediated increase in stability was dose dependent. Orientation of the Fn-fibres (within mats), monitored by scanning electron microscopy, was retained with 1 μM copper but disappeared with higher concentrations. Schwann cells grew in culture with mats stabilized by 1 μM copper treatment without reduction in cell number but growth was inhibited at 10-200 μM Cu. All types of fibroblasts were unaffected by copper treatment up to 200 μM. Thus, Fn-mats can be successfully stabilized by this technique producing longer survival *in vitro* [11]. Low concentrations of copper (1μM) not only caused significant fibronectin stabilisation but the greatest amount of cell ingrowth was observed for copper treated cables [12].

Integrins and Copper

Some integrins expressed by basal layer keratinocytes play an essential part in wound healing, notably alpha2beta1, alpha3beta1, alpha6beta4 and alphaVbeta5, whose expression and distribution in epidermis are modified during the re-epithelialization phase. The expression of these integrins is modulated *in vitro* by trace elements. In a study in which integrin expression was analysed in proliferating keratinocytes in monolayer cultures and in reconstituted skin that included a differentiation state, it was shown that zinc, copper and manganese induce integrin expression. The inductive effect of zinc was particularly notable on integrins affecting cellular mobility in the Proliferation Phase of wound healing (alpha3, alpha6, alphaV) and that of copper on integrins expressed by suprabasally differentiated keratinocytes during the final healing phase (alpha2, beta1 and alpha6), while manganese had a mixed effect [13].

S100A13 and copper

The export of FGF1 and IL-1alpha, two pro-angiogenic polypeptides, is based on the Cu2+-dependent formation of multiprotein complexes containing the S100A13 protein. The formation of a such a multiprotein aggregate enables the release of FGF1 in response to stress [14]. Since Cu2+ chelation represses the release of FGF1, the ability of Cu2+ chelators to potentially serve as effective clinical anti-cancer agents may be related to their ability to limit the export of these proinflammatory and angiogenic signal peptide-less polypeptides into the extracellular compartment [15].

Copper-dependent enzymes required for cell proliferation and matrix remodeling

Several copper-dependent enzymes, mainly amine oxidases, are known to be increased during wound healing [16-20]. These copper-dependent enzymes are important in the remodeling and healing of the wounds. For example, lysyl oxidase catalyses the formation of aldehyde cross-links and acts primarily on collagen and elastin during wound healing [20].

Upregulation of Metallothioneins

Metallothioneins are a family of low molecular weight proteins containing approximately 30% cysteine. Expression of the metallothionein gene is up-regulated in the skin following topical application of zinc and copper, and in wound margins particularly in regions of high mitotic activity. This induction of metallothionein in the wound margin may reflect its role in promoting cell proliferation and re-epithelization [21]. The action of metallothioneins in these processes may result from the large number of zinc-dependent and copper-dependent enzymes required for cell proliferation and matrix remodeling.

Tripeptide-copper complex

Glycyl-L-histidyl-L-lysine (GHK) is a tripeptide with affinity for copper (II) ions that was isolated from human plasma [22-25]. This tripeptide-copper complex appears to play a physiological role in wound healing, since it was shown to be an activator of wound healing [26-30] in several animal and *in vitro* studies. It appears that GHK-Cu(2+) has two main functions: (1) first as a potent tissue protective, anti-inflammatory agent that limits oxidative damage after tissue injury,

and (2) as a signal that activates tissue remodeling, that is, the processes for removal of damaged protein and scar tissue and their replacement by normal tissue. A localized generation of GHK-Cu(2+) after tissue damage causes an influx of macrophages which, in turn, release families of growth factor proteins appropriate to the repair of the damaged tissue, allowing for the initiation of skin repair mechanisms. It is postulated, by those who isolated the tripeptide, that the decrease in the blood concentrations of GHK-Cu(2+) during human aging may be a factor in the decreased tissue repair and subsequent increased organ failure that occur during aging [31].

The postulated sequence of events of GHK-Cu(2+) induced wound healing effects are as follows [31-33]:

1. Initially after tissue damage, the first stage of wound healing processes is activated. As discussed in the overview, these processes include localized blood coagulation, an early neutrophil invasion that secretes sterilizing oxygen radicals, and later an induction by growth factors, such as TGF-ß-1, of copious amounts of scar-forming collagen to form a protective covering over the injury.

2. A second stage of healing begins to be activated as disrupted cells release proteases that generate a population of peptides that include Gly-His-Lys, which has a very high affinity for copper (+2) ion.

3. The Gly-His-Lys begins to accumulate copper (+2) ion from albumin and form GHK-Cu(2+).

4. The accumulation of peptide-bound copper ion produces multiple anti-inflammatory effects that help to stop the actions of sterilizing oxygen radicals and permit the initiation of healing events. GHK-Cu(2+) blocks ferritin channels and the release of free (oxidative) iron, thus blocking iron catalyzed lipid peroxidation that occurs after injury [34]. GHK-Cu(2+) also blocks interleukin-1 damage to tissue cells.

5. GHK-Cu(2+) released into the blood stream raises the body's production of and circulating blood concentration of wound macrophages that enhance repair.

6. GHK-Cu(2+) suppresses the synthesis of scar development by repressing fibroblast production of TGF-ß-1.

7. GHK-Cu(2+) also chemoattracts wound macrophages to the wound area. These macrophages act directly to stimulate healing by removing cellular debris and secreting a family of approximately 20 growth factor proteins.

8. GHK-Cu(2+) acts directly on fibroblasts to stimulate m-RNAs for collagen, elastin, proteoglycans, metalloproteinases, and TIMP-1 and TIMP-2. This in turn raises the levels of these proteins. This results in a condition whereby protein synthesis and deposition occurrs concomitantly with protein breakdown that removes scar tissue and cellular debris remaining from the tissue disruption. Thus, GHK-Cu(2+) links scar reduction and the rebuilding of tissues.

9. GHK-Cu(2+) induces angiogenesis by serving as a chemoattractant to direct new blood capillaries to the wound area and by inducing the production of several protein essential for angiogenesis.

10. GHK-Cu(2+) induces neuronal outgrowth and re-innervation of the damaged tissues.

11. This mechanism of copper-peptide induced tissue repair appears to function for skin, hair follicles, the stomach lining, the intestinal lining, bone tissue, animal hooves and fingernails.

The presence of a GHK triplet in the alpha 2(I) chain of type I collagen suggests that the tripeptide might be liberated by proteases at the site of a wound and exert *in situ* healing effects [26]. Interestingly, the increase in metalloproteinase-2 levels in conditioned media of cultured fibroblasts GHK-Cu(2+), could be reproduced by copper ions alone but not by the tripeptide GHK alone [35], indicating the crucial role of copper in the remodeling of extracellular matrix, a central step in wound repair.

The first generation products designed around GHK-Cu(2+) performed well in many controlled tests, but failed in FDA clinical trials on the healing of very difficult-to-heal human wounds (e.g. Ref. 36; reviewed in Ref. 31). The fragility and rapid breakdown of GHK and similar peptides is the major problem in developing products for clinical and cosmetic use. In the human body, the GHK-Cu complex can be constantly generated. However, when used as a single dose therapy, its fragility leads to rapid breakdown, clearance from the dermis, and a loss of effectiveness.

In 1994, Skin Biology developed more effective copper peptides with tissue regenerative actions. Several hundred copper-peptide complexes were evaluated but none were significantly better than GHK-Cu(2+). Some complexes, such as f-Met-Leu-Phe-Cu(2+) actually produced more scar formation. The principal defects of single peptides were a lack of stability and poor adhesion to the skin's surface. In veterinary studies, creams made from these new copper complexes produced rapid and scar-free healing in dogs after spaying operations, and in young horses after leg-straightening operations. This allowed the dogs to be returned to their owners in four days instead of the usual five, while the foals were returned in five days instead of seven [31].

Copper related wound healing treatments

Alcoholic extracts of bakers' yeast (*Saccharomyces cerevisiae*) have been used for over 60 years in over-the-counter medications for the treatment of hemorrhoids, burns, and wounds. It has been suggested that mall peptides are responsible for the medical observations. A peptide fraction, containing 4 polypeptides was isolated, which is 600 times more active than the initial extract in enhancing wound closure in both diabetic and non-diabetic littermates. The peptides are active in nanomolar amounts. One of the peptides is a low molecular weight stress-associated protein: copper, zinc superoxide-dismutase [37].

A great many Brazilian medicinal plants are used in wound healing. They are usually applied directly to wounds, some in natura as poultices, some as dried powders, and others as water extracts (teas) for bathing. A correlation between the healing effect of 16 plants analysed and their content of silicon, manganese, iron, copper, and zinc was reported [38].

Tolmetin is a nonsteroidal anti-inflammatory agent. This medicine is used for the treatment of pain and inflammation, rheumatoid arthritis and osteoarthritis, and juvenile forms of arthritis, since it blocks production and release of chemicals that cause pain and inflammation. Interestingly, in male albino rats bearing either sutured incision, dead space or excision wounds, Tolmetin suppresses wound contraction and epithelization. However, this effect is totally reversed by copper present when Tolmetin is complexed with copper [39].

Oral administration of copper for wound healing in children

Ceruloplasmin (CP), the primary Cu-transport protein, responds as an acute-phase reactive protein after trauma. However, for severe burn trauma, this response is absent in the early catabolic phase despite Cu provision. The reduction in CP reflects burn severity. Increasing Cu oral supplementation to improve CP is the general pediatric guideline. Administration of 20 μg/kg/day is safe and reasonable for severely burned children [40].

Lasers, wound healing and copper

The reduction of lateral thermal damage during cutaneous incisional laser procedures should decrease the time in wound healing. Recently novel heat-conducting templates were developed that reduce laser lateral thermal damage [41]. Interestingly, a comparison between copper,

aluminum, glass, plexiglass heat-conducting templates and no template (air) showed that only copper and aluminum templates reduced thermal damage caused by cutaneous incisional laser procedures. More specifically, in one study utilizing a free-electron laser at several wavelengths to produce 1.0 cm incisions on *in vitro* lightly pigmented human skin, using the copper template reduced lateral thermal damage by an average of 67% with no apparent wavelength dependence. The aluminum template reduced thermal damage by an average of 54% with no apparent wavelength dependence. The glass and Plexiglas templates did not reduce the lateral thermal damage [41]. In another study, only copper and aluminum templates significantly reduced lateral thermal damage with a continous wave laser (50% and 39%, respectively), while with a pulsed laser, only the copper template significantly reduced thermal damage (by 39%) [42].

The management of white phosphorus burns and copper

Phosphorus burns are a rarely encountered chemical burn, typically occurring in battle, industrial accidents, or from fireworks. Death may result even with minimal burn areas. Early recognition of affected areas and adequate resuscitation is crucial. A typical treatment protocol comprises 1% copper sulfate solution for neutralization and identification of phosphorus particles, copious normal saline irrigation, keeping wounds moist with saline-soaked thick pads even during transportation, prompt debridement of affected areas, porcine skin coverage or skin grafts for acute wound management, as well as intensive monitoring of electrolytes and cardiac function [43].

Ascorbic acid, pantothenic acid, wound healing and copper

A study aimed at testing human skin wound healing improvement by a 21-day supplementation of ascorbic acid (AA) and pantothenic acid (PA) was conducted in 49 patients undergoing surgery for tattoos. It was shown that in skin (day 8) Fe increased and Mn decreased; in scars (day 21), Cu and Mn decreased and Mg increased; and that the mechanical properties of scars were significantly correlated to their contents in Fe, Cu and Zn. Although no major improvement of the wound healing process could be documented in this study [44], the results suggest that the benefit of AA and PA supplementation could be due to the variations of the trace elements, as they are correlated to mechanical properties of the scars.

Summary

Based on the review of the literature, it is clear that copper is involved and may be a key player in many of the complicated processes that together comprise the wound repair mechanism. The function of many factors involved in the wound healing signaling cascade are dependant on their interaction with copper. Some obvious example include: (i) GHK, the tripeptide with high affinity for copper (II) ions, that was isolated from human plasma, which potently reduces tissue oxidative damage after injury and activates tissue remodeling; (ii) the stimulation of angiogenesis, facilitated by the Cu2+-dependent formation of multiprotein complexes containing the S100A13 protein, and by induction of vascular endothelial growth factor (VEGF); (iii) expression of integrin; (iv) stabilization of fibrinogen; and (v) up-regulation of copper-dependant enzymes, such as lysyl oxidase, important for matrix remodeling. Thus, copper ions play an important role especially, but not only, during the Proliferation and Remodeling Phases. It is therfore not surprising that many over-the-counter treatments for wound healing contain copper, and as a matter of fact, it may well be that copper is the key ingredient in these remedies. The importance of copper in wound healing is further demonstrated by the positive effect of its administration in cases of severe burn trauma in children and in the management of phosphorus burns.

Taken together the very low risk of adverse skin reactions associated with copper [45], the biocidal activities of copper [46], and its roles in the wound healing process, strongly support the notion that the addition or application of copper or copper containing products, such as bandaids and gauze containing copper, to wounds may significantly enhance the wound healing process.

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