Emerging topics in cutaneous wound repair

aptara | NYAS | nyas˙6636-1583006 | Dispatch: 5-31-2012 | CE: AFL
Journal | MSP No. | No. of pages: 9 | PE: Allie/Kelly

Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Environmental Stressors in Biology and Medicine*

Emerging topics in cutaneous wound repair

Giuseppe Valacchi,[1,2] Iacopo Zanardi,[3] Claudia Sticozzi,[1] Velio Bocci,[4] and Valter Travagli[3]

[1]Dipartimento di Biologia ed Evoluzione, Università degli Studi di Ferrara, Italy. [2]Department of Food and Nutrition, Kyung Hee University, Seoul, South Korea. [3]Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Italy. [4]Dipartimento di Fisiologia, Università degli Studi di Siena, Italy

Address for correspondence: Valter Travagli, Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Via Aldo Moro 2-53100 Siena, Italy. valter.travagli@unisi.it.

The intervention strategies in various types of skin wounds include several treatment programs according to the identified disease. Several factors such as aging, defective nutrition, traumatism, atherosclerosis, and diabetes concur to the formation of a wound which has no tendency to heal due to a defective and complicated repair process. The numerous advances on the understanding of the wound healing process in both acute and chronic lesions have been recently described. The purpose of this paper is to describe relatively new approaches as viable alternative to current therapies for the treatment of wound healing. The future challenges for both the best targeting and optimization of these potential treatments are also described.

Introduction

About 1.5 billion people suffer from skin diseases as a consequence of both the progressive aging of the population and the lack of adequate healthcare programs. Among these, skin lesions are of great interest and they can be mainly represented by acute and chronic wounds. Experts debate about the time for closure that defines a chronic nonhealing wound.[1–3]

It has been stated that "acute wounds generally follow trauma or inflammation and usually heal within six weeks."[4] Chronic wounds (in addition to failing to heal after six weeks) have characteristic pathological associations that inhibit or delay the healing process.[5] In particular, in the industrialized world it is estimated that 1–1.5% of the population undergo problems related to the recovery process of the correct function of the skin, with particular reference to the elderly and patients with diabetes and/or arteriosclerosis which can easily suffer ulcers and bedsores. The International Diabetes Federation estimates that 285 million people around the world have diabetes.[6] This total is expected to rise to 438 million within 20 years. Moreover, type II diabetes, most often in connection with obesity, further increases the number of people with chronic wounds.

Throughout the world, beside millions of sick days, the total expenses spent on the impaired healing of chronic wounds is enormous, about 2–4% of health spending, particularly because about 15% of type I diabetes must undergo lower extremity amputations. Certainly not less important are the anguish and the suffering of the affected patients. Moreover, a number of issues directly related to treatment modalities and its correct evaluation are still open,[7] despite the numerous recent advances on the understanding of the "wound healing" process in both acute and chronic lesions.[8,9] Furthermore, since a wound represents a violation of natural defense barriers, infectious complications are very common and should be readily avertible.[10] In this sense, the parenteral and/or topical use of valid antibiotics, antivirals, vaccines, antiparasitic drugs makes a valuable contribution. Unfortunately, with time, pathogen agents may become drug resistant as well as the conventional drugs side effects frequently limit their compliance. Another drawback is represented by their cost, which compromise their use or their availability in poor countries. Moreover, wound patients are suffering not only because they become noncompliant to frequent medications but they are discouraged by observing a lack of healing.

doi: 10.1111/j.1749-6632.2012.06636.x

Cutaneous wound healing is an age-related as well as gender multiphase process,[11,12] but both innate and adoptive immune systems are too often hindered by the chronic infection naturally difficult to overcome. This is also the reason explaining the failure of growth factors in heavily contaminated ulcers. This paper briefly summarizes the sequential phases of physiological wound healing and then analyses the causes complicating the healing in chronic wounds. Moreover, after resuming pros and cons of current therapies, it extensively describes both a relatively new approach, which is not mentioned in recent relevant reviews,[13,14] as well as future concepts.

Physiological aspects of the wound repair process

In humans, and more widely in all mammalian species, the wound healing process can be subdivided in three consecutive and overlapping phases: inflammation, tissue formation, and matrix formation and remodeling.[15] The transition from one phase to another depends on the maturation and differentiation of the main cell populations involved, among which the keratinocytes, the fibroblasts, the neutrophils and the macrophages play the main role.[15–18]

Recent observations show that stem cells have an unclear but likely major role in response to cutaneous injury,[19] as well as the evidence for the roles of M1 and M2 macrophage, and that of T cells.[20,21] The first event occurring after injury is the formation of a blood clot and several cells are involved in the blood plug such as platelet, red, and white blood cells. Due to the action of the fibrin fibers, the clot is stabilized and then will be "invaded" by several infiltrating cells such as neutrophils, macrophages, mastocytes, platelets, and possibly of bacteria and toxins counteracted by the generated H_2O_2. Neutrophils massively infiltrate the wound during the first 24 hours postinjury as are attracted by the numerous inflammatory cytokines produced by the activated platelets, endothelial cells, as well as by the degradation products from pathogens. Macrophages massively infiltrate the wound two days postinjury and exacerbate at this stage an intense phagocytic activity.[22]

After two to three days, the second phase lasts about two weeks and it is characterized by the neoangiogenesis and granulation. During the reepithelialisation process keratinocytes from the wound edges migrate over the wound bed at the interface between the wound dermis and the fibrin clot. This migration is facilitated by the production of specific proteases such as the collagenase by the epidermal cells to degrade the extracellular matrix. Activated fibroblasts also migrate to the wound bed and form, with the macrophages, the granulation tissue. A massive angiogenesis allowing the supply of oxygen and nutrients necessary for the healing process also occurs within this tissue. Both growth factors and reactive oxygen species (ROS) produced by the granulation tissue will favor proliferation and differentiation of epithelial cells restoring the epithelial barrier integrity. The last stage of the wound healing process consists in a gradual involution of the granulation tissue and dermal regeneration. This step is associated with the apoptosis of myofibroblasts, endothelial cells and macrophages. The remaining tissue is therefore composed mostly of extracellular matrix proteins, essentially collagen type III that will be remodeled by the metalloproteinase produced by the epidermal cells, endothelial cells, fibroblasts, and the macrophages remaining in the scar and be replaced by collagen type I.[8]

The chronic wound healing is mainly sustained by a chronic inflammation which without an appropriate therapy tends to worsen. The basic reasons are not necessarily old age but rather hypertension, atherosclerosis leading to ischemia, diabetes, and venous insufficiency. Common pathogenetic causes are the local tissue hypoxia, edema, abundant bacterial colonization, and possibly repeated ischemia-reperfusion injuries. The surface area of a nonhealing wound tends to widen and shows fibrin deposition, necrotic areas, and a few islands of granulation tissue.

Current therapies

It is clear that delayed wound healing is due to the deficiency of essential parameters as a normal vascular bed, a physiological pO2, an active local immune system, and the normal sequential release of growth factors able to, step by step, heal the wound.[23] A number of therapies have been evaluated and they may vary in relation to the causes of the wound: diabetes, peripheral arterial obstructive disease (PAOD), trauma, and venous insufficiency.[24,25] Moreover, infection complications and severity may vary from mild when infection is limited to the skin

surrounding the wound, to moderate, when infection is spread on the nearby tissues and severe, with systemic toxicity, high fever and metabolic problems. Antibiotic therapy to be effective should be dictated by repeated culture data indicating the optimal antibiotics and it should be continued for at least two to three weeks.[26] However, such a subject cannot be considered as an emergent topic, except than for the use of topical doxycycline to enhance healing of chronic wounds because of its enzyme inhibitors properties.[27] On the other hand, both the type of culture and the role of contamination have been long debated, together with the role of biofilm bacteria.[28,29] Several approaches useful for wound repair will be reviewed below.

Wound cleansing and debridement

Particular attention has been placed on measures of wound cleansing as part of wound bed preparation that gently and continuously removes debris and exudate to prepare the wound bed for closure.[30,31] It is increasingly well recognized that clearing a wound bed of nonviable tissue is an important step that may facilitate the healing process for a variety of wound types. In fact, removal of necrotic tissue is essential for the treatment of chronic wounds because devitalized tissue poses a major impediment to wound healing. A number of different modalities exist for wound debridement. The four most common debridement methods are surgical, autolytic, enzymatic, and mechanical.[32]

Wound care[33]

In order to prevent tissue dehydration and cell death and to enhance neo-angiogenesis and reepithelialization the use of a clean, moist wound-healing environment appears to be very useful.[34] Recent advancements in technology and in the understanding of human physiology have led to the commercial development of dressings that offer material improvements on these same ancient fundamental principles.[35]

Revascularization

Whenever possible, the treatment of peripheral arterial obstructive disease (PAOD) consists in performing endovascular or open surgery using either angioplasty, or endoarterectomy. Catheter-based options for revascularization currently play an important role. Available technology will continue to improve and innovation will be rapid. These innovations include biodegradable stents, drug eluting balloons, and new stent platforms.[36–38] Moreover, pharmaceutical therapy with statins, platelet-antiaggregants together with dieting with a low-fat diet, and daily physical exercise are helpful.

Pressure off-loading

The evidence for the role of offloading in the prevention and treatment of plantar ulcers in the diabetic foot has been assessed, even if it has been pointed out that there is a gap between evidence-based guidelines and current practice.[39] A highly evaluated offloading technique is represented by a contact casting, half shoe, and felted foam dressings to be changed every one to two weeks. Soft-tissue infections and osteomyelitis are contraindications to total-contact casting. Pressure offloading with the total-contact cast is the gold standard of care for chronic neuropathic noninfected, nonischemic plantar foot ulcers in individuals with diabetes mellitus. The recent trials of removable cast walkers rendered irremovable suggest that this alternate approach may be preferable given that less technical expertise for fitting is required.[40]

Negative-pressure wound therapy

Negative-pressure wound therapy (NPWT) is a fairly popular method for providing a subathmospheric pressure (about −125 mmHg) to dressed wounds connected to a vacuum pump and the area sealed with an adhesive film. There is no doubt that the negative pressure reduces the perilesional edema, the hypoxia and stimulates cellular proliferation. This procedure has been found effective and safe in advanced diabetic foot ulcer,[41,42] even if recently the FDA issued a Preliminary Public Health Notification and Advice for patients on serious complications, especially bleeding and infection, from the use of NPWT systems.[43] Although rare, these complications can occur wherever NPWT systems are used, including hospitals, long-term healthcare facilities, and at home.[44]

Wound management agents

Recently, many agents have been used in the management of wounds. In detail, becaplermin, the recombinant human platelet-derived growth factor, subunit B is a potent mitogen for cells of mesenchymal origin. Binding of this growth factor to its receptor elicits a variety of cellular responses. It is released by platelets upon wounding and plays an

important role in stimulating adjacent cells to grow and thereby heals the wound.[45–47] For completeness sake, results of clinical trials conducted in developed Western countries cannot be directly extrapolated and applied to populations living in developing countries. In fact, a prospective comparative study, conducted on 613 consecutive patients with diabetic foot lesions, documented the regional differences in risk factors and clinical presentation of diabetic foot lesions.[48]

Finally, other types of treatment options such as the potential use of stem cells have elicited interest but at this stage, if they are not autologous, they may be rejected.[49] Autologous cells may be used in some patients but not in diabetics because defective. Gene therapy programmed in cells able to release growth factors is promising but again release of growth factor in infected wounds is useless.[50] Moreover, there is little evidence to justify the routine use of bioengineered skin substitutes, extracellular matrix proteins surface or dressing compared with standard care.[51,52]

A plethora of natural products and derivatives like terpenoids have been more or less anecdotically used as wound healing agents.[53,54] Among these, alkannins, and shikonins—chiral-pairs of naturally occurring isohexenylnaphthazarins found in the external layer of the roots of at least 150 species of the Boraginaceae family—show significant and promising wound healing properties.[55]

As for nonnatural compounds as wound healing agents, benzazepines, phenytoin, raxofelast, molsidomine, and *S*-nitrosothiols, together with nitric oxide in its gaseous form are very important for a better understanding of the wound healing process and they represent lead compounds for the design of new efficient drugs.[54]

Proteolytic enzymes

The properties and functions of the extracellular and cell surface proteases involved in wound healing are well known. They involve matrix protein synthesis and degradation; release of cryptic bioactive fragments; regulation of growth factors and other cytokines; and the control of cell adhesion, migration, apoptosis, and signaling.[56] Moreover, it has been confirmed that collagenase ointment is a safe and effective choice for debridement of cutaneous ulcers and burn wounds.[57]

Maggot therapy

It represents the application of disinfected fly larvae to chronic wounds. Maggots are gradually finding their way into a more acceptable system of wound management and they have been used for treating diabetic foot ulcers unresponsive to conventional therapy.[58,59] Maggot secretions and excretions possess antibacterial activity against a wide range of pathogens and in their wound healing capabilities in biosurgery.[60]

Hyperbaric oxygen therapy

Hyperbaric oxygen therapy (HBOT) delivers 100% O_2 at 2–3 bar for a period of—one to two hours usually five days a week in a suitable chamber. The leading idea is to enhance the low pO_2 values of hypoxic tissues and to display some bactericidal effect. Normally, 10–30 treatments are necessary if the patient tolerates them. On the whole, HBOT improves the chance of healing and it reduces the risk of amputation.[61–63] However even if to day there is practically no risk of chamber explosion, it is a costly therapy,[64] both tympanic membrane rupture and pneumotorax are common side effects,[65] and presently, there are conflicting data regarding the efficacy of this therapy.[63,66,67] It may be in conjunction with topical oxygen therapy localized to the wound area using a portable inflatable device. This method lowers the risk of oxygen toxicity, improves the oxygenation of the lesion, has a low cost, and it has the advantage of home treatment. Kalliainen *et al.* evaluated this topical therapy and found it was beneficial in improving wound.[68]

Having summarily described the current main types of treatments for cutaneous wound healing, there are emerging topics in cutaneous wound repair involving the use of substances correlated with the ROS, with particular emphasis on the use of ozone and its derivatives, relating to cellular and humoral responses to cutaneous injuries. Such topics will be following discussed.

Role of ROS in wound repair

As mentioned before, the wound healing process is regulated by a large variety of different growth factors, such as cytokines and hormones[69] but also by ROS. Such factors, among which the superoxide anion ($^{-}O_2$) is central because it may be converted into other physiologically relevant ROS by enzymatic or nonenzymatic reactions, are required

for the defense against invading pathogens,[70] and low levels of ROS are also essential mediators of intracellular signaling.[71] It has been shown that low doses of H_2O_2 can improve wound healing. On the other hand, excessive amounts of ROS are deleterious due to their high reactivity.[72,73] Due to the short half-life of ROS, their concentrations *in vivo* are difficult to determine, although the levels of H_2O_2 have been recently measured in wound fluid from acute murine excisional wounds.[74] These studies revealed that concentrations of H_2O_2 ranging from 100 μM to 250 μM are present at the wound site and these levels are even higher in the first stage of wound healing.

It has been therefore suggested that the low levels of ROS that are produced in normal wounds are important for the repair process. In other studies the wound levels of ROS have been determined indirectly through analysis of oxidation products of lipids, proteins or DNA.[75] A major product of lipid peroxidation is 4-hydroxy-2-nonenal (4-HNE), which could be detected by immunohistochemistry at the edge of murine excisional wounds. Interestingly, coimmunostaining revealed that 4-HNE mainly colocalizes with neutrophils, suggesting that the respiratory burst of these inflammatory cells results in the production of superoxide, which in turn causes lipid peroxidation.[76]

Topical use of ozone and its derivatives as mediators of ROS

Such an issue represents a neglected but important opportunity for the treatment of chronic wounds. Since its use is increasing in the scientific community, to stimulate the knowledge about this topic it will be discussed more in detail. It is generally understood that, although O_3 is not a radical species per se, the toxic effects of O_3 are mediated through free radical reactions and they are achieved either directly by the oxidation of biomolecules to give classical radical species (hydroxyl radical), H_2O_2 or by driving the radical-dependent production of cytotoxic, nonradical species (aldehydes).[77,78] The first mention of the use of ozone for treating dermatological pathologies dates back to the 19th century when it was identified as a potent antiinfective gas and it was used during World War I for treating German soldiers affected by gaseous gangrene due to *Clostridium* anaerobic infections.[79] Recently, in several countries such as Germany, Italy, Russia, and Cuba the possibility of using ozone under its various forms as antiinfective in veterinary and human medicine has been evaluated.[80,81] In fact, ozone is slowing being appreciated, as a gas, or as ozonated water, or as ozonated natural matrices in a variety of infections, trophic ulcers, burns, cellulitis, abscesses, anal fissures, decubitus in paralytic patients, fungal diseases, gingivitis, peritonitis, and vulvovaginitis.[82,83] It is realized that ozone, under various formulations, can display a cleansing effect and act as a potent disinfectant able to practically kill all pathogens present in the skin and mucosal surfaces also due to its oxidative properties. However, the bactericidal action of ozone, while is rapidly effective in contaminated water, it is markedly reduced when antioxidant compounds are present. In fact, our studies performed with various bacterial suspensions either in pure saline medium or saline addition with human serum has yielded discouraging results: the presence of only 5–10% of serum almost blocks the ozone bactericidal effect. Retrospectively, such an outcome is not surprising because serum shows a potent antioxidant capacity able to neutralize the ozone oxidant effect.[84] On the other hand, the same result appears to be very instructive in the sense that the application of ozonated derivatives, mainly ozonated oils, must be preceded by a careful cleaning and washing of the biological exudates present in wounds and ulcers. Moreover, it is necessary to have a series of ozonated oils graduated in terms of peroxide value to be used whether the wound is highly contaminated with bacteria mixed to dead cells. On the other hand, the decomposition of ozone derivatives has the additional advantage to improve the local metabolism and the proliferation of tissues, essential for the eventual mucosal or/and cutaneous healings.[85]

Today, especially in modestly developed countries, the value of ozone is greatly estimated while in highly technologically advanced countries ozone remains neglected because of prejudice, lack of knowledge, and the wide availability of pharmaceutical products, unfortunately not always effective.

The topical ozone application can be practiced with either: (a) the bagging of the ulcerated lesion with exposure to gaseous oxygen/ozone mixture for about 30 minutes daily, by using ozone concentration from 80 μg/mL down to 5–10 μg/mL in clean wounds; (b) the local treatment of the wound with ozonated water or ozonated saline, again using variable concentrations of ozone. This is considered

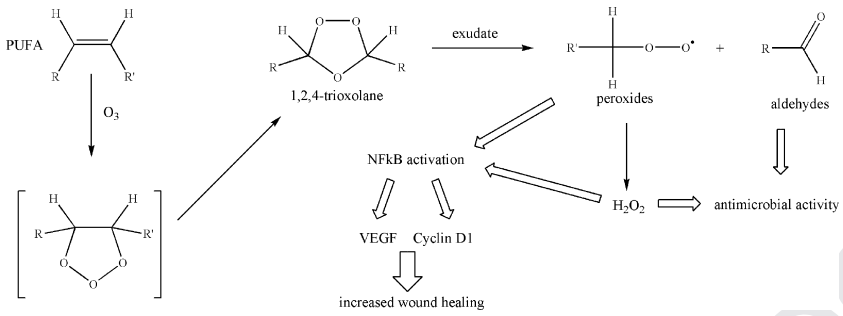

Figure 1. **Schematic of ozonated derivative formation by chemical reaction of ozone with unsaturated fatty acids of triglycerides from oils, and their possible mechanisms. The interaction between ozone and PUFA leads to the formation of aldehydes, peroxides, and H_2O_2 that can affect wound healing process by activating redox sensitive transcription factors (NF-κB) that is responsible for the expression of proangiogenic (VEGF) and proliferative (Cyclin D1) genes involved in the wound healing processes.**

a chemical, most effective debridement where pus, deposits of fibrin, and necrotic tissue are removed with consequent activation and oxygenation of the wound, followed by (c) the application of ozonated oil for the night which maintains the wound sterile and activated by the release of ozonated products and oxygen. In addition, ozonated oils with a reliable peroxide value can also be used for wound healing purposes.[85] How ozonated oils act remains an open question (Fig. 1). Probably, when the stable triozonide comes into contact with the warm exudate of the wound, it slowly decomposes into different peroxides, which readily dissolves in water, probably generating hydrogen peroxide that can explain the prolonged disinfectant and stimulatory activity. Consequently, it should be used titrated preparations with high, medium, or low ozonide concentrations during the inflammatory septic phase I, regenerating phase II, or remodeling phase III, respectively. These phases have been related to the rapidly changing cell types and to the release of cytokines and growth factors that modulate the complex healing process.

Future challenges

In recent years, research in the pharmaceutical field has turned a growing commitment in the development of new technologies to optimize the application, increase the bioavailability while minimizing potential side effects of substances applied onto both the skin and mucosae. In that regard, a great interest in new techniques that enhance the permeation of drugs at the different membrane levels has been developed. In particular, the topical application of drugs through lipid colloidal carriers, such as vesicular systems (traditional liposomes, deformable and ethosomes) and solid lipid nanoparticles (SLN) has stirred much interest.[86,87] These carriers are proposed for dermal and topical application of useful substances for a number of advantages: they enhance the penetration of both lipophilic and hydrophilic substances by incorporation; they have high affinity and similarity with epidermal barrier offering the possibility to improve the absorption of drugs across the skin barrier, thus ensuring a greater local concentration; they are natural (bio-compatible, nontoxic, nonimmunogenic) and they have a natural skin-moisture action because the small size give them their adhesive properties to form a lipid film on the skin surface. Topical application allows for reconstruction of the lipid layer and alters the skin barrier damaged by various diseases. Eventually, they can control the rate of supply of therapeutic agents. At the time of either the carrier-to-skin or carrier-to-mucosa contact, the first is absorbed from the surface and slowly releases its biologically active content.

Moreover, of great interest is the potential application of micro-and nano-bubbles for the targeted release of efficient drugs, among which ozone and its derivatives. Bubbles may be used in a number of ways to aid drug delivery. Drugs may be encapsulated within the bubbles, they may be incorporated into the shell or incorporated into it in some way, for example by ligands embedded into the lipid membrane.[88,89] It is also possible to construct microbubbles with a multilayered shell containing drug. The spatial localization relies on the ability to confine the ultrasound beam to the required volume.[90] If these bubbles can be accumulated within the target

volume, ultrasound can destroy them locally, releasing the efficient therapeutic agents able to influence neuronal, stromal, vascular, and circulatory system cells by chemical, physical, or biological stimuli.[91]

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Dealey, C. 2005. *The Care of Wounds: A Guide for Nurses.* 3rd ed. Blackwell Publishing Ltd. Oxford, UK.
2. Whitney, J.D. 2005. Overview: acute and chronic wounds. *Nurs. Clin. North Am.* **40:** 191–205.
3. Bryant, R.A. & D. Nix. 2011. *Acute and Chronic Wounds, Current Management Concepts*. 4th ed. Mosby Elsevier. Missouri, USA.
4. Kumar, S. & D.J. Leaper. 2008. Classification and management of acute wounds. *Surgery* **26:** 43–47.
5. Jones, K.R., K. Fennie & A. Lenihan. 2007. Evidence-based management of chronic wounds. *Adv. Skin Wound Care* **20:** 591–600.
6. Understand diabetes, take control. Available at URL: http://www.idf.org/worlddiabetesday/2009-2013/booklet (last accessed May 15, 2012).
7. Gottrup, F. & T. Karlsmark. 2009. Current management of wound healing. *G. Ital. Dermatol. Venereol.* **144:** 217–228.
8. Singer, A.J. & R.A. Clark. 1999. Cutaneous wound healing. *N. Engl. J. Med.* **341:** 738–746.
9. Singer, A.J. & A.B. Dagum. 2008. Current management of acute cutaneous wounds. *N. Engl. J. Med.* **359:** 1037–1046.
10. Bowler, P.G., B.I. Duerden & D.G. Armstrong. 2001. Wound microbiology and associated approaches to wound management. *Clin. Microbiol. Rev.* **14:** 244–269.
11. Swift, M.E., A.L. Burns, K.L. Gray & L.A. Di Pietro. 2001. Age-related alterations in the inflammatory response to dermal injury. *J. Invest. Dermatol.* **117:** 1027–1035.
12. Gilliver, S.C., J.J. Ashworth, S.J. Mills, M.J. Hardman & G.S. Ashcroft. 2006. Androgens modulate the inflammatory response during acute wound healing. *J. Cell Sci.* **119:** 722–732.
13. Schreml, S., R.M. Szeimies, L. Prantl, S. Karrer, M. Landthaler & P. Babilas. 2010. Oxygen in acute and chronic wound healing. *Br. J. Dermatol.* **163:** 257–268.
14. Tecilazich, F., T. Dinh & A. Veves. 2011. Treating diabetic ulcers. *Expert Opin. Pharmacother.* **12:** 593–606.
15. Rodero, M.P. & K. Khosrotehrani. 2010. Skin wound healing modulation by macrophages. *Int. J. Clin. Exp. Pathol.* **3:** 643–653.
16. Leibovich, S.J. & R. Ross. 1975. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am. J. Pathol.* **78:** 71–100.
17. Deonarine, K., M.C. Panelli, M.E. Stashower, *et al*. 2007. Gene expression profiling of cutaneous wound healing. *J. Transl. Med.* **5:** 11.
18. Becker, D.L., C. Thrasivoulou, A.R. Phillips. 2011. Connexins in wound healing; perspectives in diabetic patients. *Biochim. Biophys Acta*, in press. DOI: 10.1016/j.bbamem.2011.11.017 **Q1**
19. Lanza R. 2004. *Handbook of Stem Cells*. Academic Press. Boston, MA. pp. 731–736.
20. Gilliver, S.C., E. Emmerson, J. Bernhagen & M.J. Hardman. 2011. MIF: a key player in cutaneous biology and wound healing. *Exp. Dermatol.* **20:** 1–6.
21. Sindrilaru, A., T. Peters, S. Wieschalka, *et al*. 2011. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *J. Clin. Invest.* **121:** 985–997. doi: 10.1172/JCI44490. Epub 2011 Feb 7.
22. Mosser, D.M. & J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nature Rev. Immunol.* **8:** 958–969.
23. Braiman-Wiksman, L., I. Solomonik, R. Spira & T. Tennenbaum. 2007. Novel insights into wound healing sequence of events. *Toxicol. Pathol.* **35:** 767–779.
24. Grey J.E. & G.K. Harding. 2006. *ABC of Wound Healing*. Wiley-Blackwell. New York.
25. Springer, M.L. 2006. A balancing act: therapeutic approaches for the modulation of angiogenesis. *Curr. Opin. Investig. Drugs* **7:** 243–250.
26. Eaglstein, W.H., P.M. Mertz & O.M. Alvarez. 1984. Effect of topically applied agents on healing wounds. *Clin. Dermatol.* **2:** 112–115.
27. Stechmiller, J., L. Cowan & G. Schultz. 2010. The role of doxycycline as a matrix metalloproteinase inhibitor for the treatment of chronic wounds. *Biol. Res. Nurs.* **11:** 336–344.
28. James, G.A., E. Swogger, R. Wolcott, *et al*. 2008. Biofilms in chronic wounds. *Wound Repair Regen.* **16:** 37–44.
29. Fazli, M., T. Bjarnsholt, K. Kirketerp-Møller, *et al*. 2011. Quantitative analysis of the cellular inflammatory response against biofilm bacteria in chronic wounds. *Wound Repair Regen.* **19:** 387–391.
30. Andriessen, A.E. 2010. Assessment of a wound cleansing solution in the treatment of problem wounds. *Wounds* **20:** 171–175.
31. National Pressure Ulcer Advisory Panel & European Pressure Ulcer Advisory Panel. 2009. Pressure ulcer treatment recommendations. In: *Prevention and Treatment of Pressure Ulcers: Clinical Practice Guideline*. National Pressure Ulcer Advisory Panel. Washington (DC). pp. 51–120.
32. Shi, L., R. Ermis, K. Lam, *et al*. 2009. Study on the debridement efficacy of formulated enzymatic wound debriding agents by in vitro assessment using artificial wound eschar and by an in vivo pig model. *Wound Repair Regen.* **17:** 853–862.
33. Sussman C & B. Bates-Jensen. 2007 *Wound Care: A Collaborative Practice Manual*. Lippincott Williams & Wilkins. Baltimore.
34. Cowan, L.J. & J. Stechmiller. 2009. Prevalence of wet-to-dry dressings in wound care. *Adv. Skin Wound Care* **22:** 567–573.
35. Lee, J.C., S. Kandula & N.S. Sherber. 2009. Beyond wet-to-dry: a rational approach to treating chronic wounds. *Eplasty* **9:** 131–137.
36. Tautenhahn, J., R. Lobmann, B. Koenig, *et al*. 2008. The influence of polymorbidity, revascularization, and wound therapy on the healing of arterial ulceration. *Vasc. Health Risk Manag.* **4:** 683–689.

37. Kirksey, L. & M. Troiano. 2011. The evolving paradigm of revascularization for wound healing: which intervention for which wound? *Wounds* **23:** 49–52.
38. Neville, R.F. 2011. Open surgical revascularization for wound healing: Past performance and future directions. *Plast. Reconstr. Surg.* **127:** 154S–162S.
39. Cavanagh, P.R. & S.A. Bus. 2010. Off-loading the diabetic foot for ulcer prevention and healing. *J. Vasc. Surg.* **52:** 37S–43S.
40. Hunt, D.L. 2011. Diabetes: foot ulcers and amputations. *Clin. Evid. (Online).* **2011**: pii 0602.
41. Armstrong, D.G., L.A. Lavery &Diabetic Foot Study Consortium. 2005. Negative pressure wound therapy after partial diabetic foot amputation: a multicentre, randomised controlled trial. *Lancet* **366:** 1704–1710.
42. Xie, X., M. McGregor & N. Dendukuri. 2010. The clinical effectiveness of negative pressure wound therapy: a systematic review. *J. Wound Care* **19:** 490–495.
43. FDA safety communication: UPDATE on serious complications associated with negative pressure wound therapy systems. Date Issued February 24, 2011. Available at URL: http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/ucm244211.htm (last accessed May 15, 2012)
44. Gregor, S., M. Maegele, S. Sauerland, *et al*. 2008. Negative pressure wound therapy. A vacuum of evidence? *Arch. Surg.* **143:** 189–196.
45. Smiell, J.M., T.J. Wieman, D.L. Steed, *et al*. 1999. Efficacy and safety of becaplermin (recombinant human platelet-derived growth factor-BB) in patients with nonhealing, lower extremity diabetic ulcers: a combined analysis of four randomized studies. *Wound Repair Regen.* **7:** 335–346.
46. Papanas, N. & E. Maltezos. 2010. Benefit-risk assessment of becaplermin in the treatment of diabetic foot ulcers. *Drug Safety* **33:** 455–461.
47. Buchberger, B., M. Follmann, D. Freyer, *et al.* 2011. The evidence for the use of growth factors and active skin substitutes for the treatment of non-infected diabetic foot ulcers (DFU): a health technology assessment (HTA). *J. Exp. Clin. Endocrinol. Diabetes* **119:** 472–479.
48. Hardikar, J.V., Y. Chiranjeev Reddy, D.D. Bung, *et al*. 2005. Efficacy of recombinant human platelet-derived growth factor (rhPDGF) based gel in diabetic foot ulcers: a randomized, multicentre, double-blind, placebo-controlled study in India. *Wounds* **17:** 14–152.
49. Cha, J. & V. Falanga. 2007. Stem cells in cutaneous wound healing. *Clin. Dermatol.* **25:** 73–78.
50. Hirsch, T., M. Spielmann, F. Yao & E. Eriksson. 2007. Gene therapy in cutaneous wound healing. *Front. Biosci.* **12:** 2507–2518.
51. Reddy, M., S.S. Gill, S.R. Kalkar, *et al.* 2008. Treatment of pressure ulcers: a systematic review. *JAMA* **300:** 2647–2662.
52. Dumville, J.C., S. O'Meara, S. Deshpande & K. Speak. 2011. Hydrogel dressings for healing diabetic foot ulcers. *Cochrane Database Syst. Rev.* **9:** CD009101.
53. Kumar, B., M. Vijayakumar, R. Govindarajan & P. Pushpangadan. 2007. Ethnopharmacological approaches to wound healing-exploring medicinal plants of India. *J. Ethnopharmacol.* **114:** 103–113.
54. de Fátima, A., L.V. Modolo, A.C. Sanches & R.R. Porto. 2008. Wound healing agents: the role of natural and non-natural products in drug development. *Mini Rev. Med. Chem.* **8:** 879–888.
55. Papageorgiou, V.P., A.N. Assimopoulou & A.C. Ballis. 2008. Alkannins and shikonins: a new class of wound healing agents. *Curr. Med. Chem.* **15:** 3248–3267.
56. Moali, C. & D.J. Hulmes. 2009. Extracellular and cell surface proteases in wound healing: new players are still emerging. *Eur. J. Dermatol.* **19:** 552–564.
57. Ramundo, J. & M. Gray. 2009. Collagenase for enzymatic debridement: a systematic review. *J. Wound Ostomy Continence Nurs.* **36:** S4–S11.
58. Sherman, R.A. 2003. Maggot therapy for treating diabetic foot ulcers unresponsive to conventional therapy. *Diabetes Care* **26:** 446–451.
59. Gupta, A. 2008. A review of the use of maggots in wound therapy. *Ann. Plast. Surg.* **60:** 224–227.
60. Arora, S., C. Baptista & C.S. Lim. 2011. Maggot metabolites and their combinatory effects with antibiotic on Staphylococcus aureus. *Ann. Clin. Microbiol. Antimicrob.* **10:** 6.
61. Smith, B.M., L.D. Desvigne, J.B. Slade, *et al*. 1996. Transcutaneous oxygen measurements predict healing of leg wounds with hyperbaric therapy. *Wound Repair Regen.* **4:** 224–229.
62. Fife, C.E., C. Buyukcakir, G.H. Otto, *et al*. 2002. The predictive value of transcutaneous oxygen tension measurement in diabetic lower extremity ulcers treated with hyperbaric oxygen therapy: a retrospective analysis of 1,144 patients. *Wound Repair Regen.* **10:** 198–207.
63. Kranke, P., M. Bennett, I. Roeckl-Wiedmann & S. Debus. 2004. Hyperbaric oxygen therapy for chronic wounds. *Cochrane Database Syst. Rev.* **2:** CD004123.
64. Flegg, J.A., D.L. McElwain, H.M. Byrne & I.W. Turner. 2009. A three species model to simulate application of Hyperbaric Oxygen Therapy to chronic wounds. *PLoS Comput. Biol.* **5:** e1000451.
65. Kaur, S., M. Pawar, N. Banerjee & R. Garg. 2012. Evaluation of the efficacy of hyperbaric oxygen therapy in the management of chronic nonhealing ulcer and role of perimwound transcutaneous oximetry as a predictor of wound healing response: a randomized prospective controlled trial. *J. Anaesthesiol. Clin. Pharmacol.* **28:** 70–75.
66. Kranke, P., M. Bennett, I. Roeckl-Wiedmann & S. Debus. 2004. Hyperbaric oxygen therapy for chronic wounds. *Cochrane Database Syst. Rev.* **4:** CD004123.
67. Berendt, A.R. 2006. Counterpoint: hyperbaric oxygen for diabetic foot wounds is not effective. *Clin. Infect. Dis.* **43:** 193–198.
68. Kalliainen, L.K., G.M. Gordillo, R. Schlanger & C.K. Sen. 2003. Topical oxygen as an adjunct to wound healing: a clinical case series. *Pathophysiology* **9:** 81–87.
69. Werner, S. & R. Grose. 2003. Regulation of wound healing by growth factors and cytokines. *Physiol. Rev.* **83:** 835–870.
70. Arul, V., J.G. Masilamoni, E.P. Jesudason, *et al*. 2011. Glucose oxidase incorporated collagen matrices for dermal wound repair in diabetic rat models: a biochemical study. *J. Biomater. Appl.* in press. DOI: 10.1177/0885328210390402. **Q2**

71. Valacchi, G., V. Fortino & V. Bocci. 2005. The dual action of ozone on the skin. *Br. J. Dermatol.* **153:** 1096–1100.
72. auf dem Keller, U., A. Kümin, S. Braun & S. Werner. 2006. Reactive oxygen species and their detoxification in healing skin wounds. *J. Investig. Dermatol. Symp. Proc.* **11:** 106–111.
73. Vermeij, W.P. & C. Backendorf. 2010. Skin cornification proteins provide global link between ROS detoxification and cell migration during wound healing. *PLoS One.* **5:** e11957.
74. Roy, S., S. Khanna, K. Nallu, T.K. Hunt & C.K. Sen. 2006. Dermal wound healing is subject to redox control. *Mol. Ther.* **13:** 211–220.
75. Schäfer, M. & S. Werner. 2008. Oxidative stress in normal and impaired wound repair. *Pharmacol. Res.* **58:** 165–171.
76. Ojha, N., S. Roy, G. He, *et al*. 2008. Assessment of wound-site redox environment and the significance of rac2 in cutaneous healing. *Free Radic. Biol. Med.* **44:** 682–691.
77. Pryor, W.A. 1994. Mechanisms of radical formation from reactions of ozone with target molecules in the lung. *Free Radic. Biol. Med.* **17:** 451–465.
78. Sathishkumar, K., M. Haque, T.E. Perumal, *et al*. 2005. A major ozonation product of cholesterol, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al, induces apoptosis in H9c2 cardiomyoblasts. *FEBS Lett.* **579:** 6444–6450.
79. Stoker, G. 1916. The surgical uses of ozone. *Lancet* **188:** 712.
80. Bocci, V., E. Borrelli, V. Travagli & I. Zanardi. 2009. The ozone paradox: ozone is a strong oxidant as well as a medical drug. *Med. Res. Rev.* **29:** 646–682.
81. Bocci, V. 2011. *Ozone. A New Medical Drug*. Springer. Dordrecht, The Netherlands.
82. Travagli, V., I. Zanardi & V. Bocci. 2009. Topical applications of ozone and ozonated oils as anti-infective agents: an insight into the patent claims. *Recent Pat. Antiinfect. Drug Discov.* **4:** 130–142.
83. Travagli, V., I. Zanardi, G. Valacchi & V. Bocci. 2010. Ozone and ozonated oils in skin diseases: a review. *Mediators Inflamm.* **2010:** 610418.
84. Burgassi, S., I. Zanardi, V. Travagli, *et al*. 2009. How much ozone bactericidal activity is compromised by plasma components? *J. Appl. Microbiol.* **106:** 1715–1721.
85. Valacchi, G., Y. Lim, G. Belmonte, *et al*. 2011. Ozonated sesame oil enhances cutaneous wound healing in SKH1 mice. *Wound Repair Regen.* **19:** 107–115.
86. Cosco, D., C. Celia, F. Cilurzo, *et al*. 2008. Colloidal carriers for the enhanced delivery through the skin. *Expert Opin. Drug Deliv.* **5:** 737–755.
87. Jeschke, M.G., G. Sandmann, C.C. Finnerty, *et al*. 2005. The structure and composition of liposomes can affect skin regeneration, morphology and growth factor expression in acute wounds. *Gene Ther.* **12:** 1718–1724.
88. Cavalli, R., A. Bisazza, P. Giustetto, *et al*. 2009. Preparation and characterization of dextran nanobubbles for oxygen delivery. *Int. J. Pharm.* **381:** 160–165.
89. Yoon, C.S., H.S. Jung, M.J. Kwon, *et al*. 2009. Sonoporation of the minicircle-VEGF(165) for wound healing of diabetic mice. *Pharm. Res.* **26:** 794–801.
90. Johnson S. 2003. Low-frequency ultrasound to manage chronic venous leg ulcers. *Br. J. Nurs.* **12:** S14–S24.
91. ter Haar, G. 2007. Therapeutic applications of ultrasound. *Prog. Biophys. Mol. Biol.* **93:** 111–129.

Queries

Q1 Author: Please update reference 18.

Q2 Author: Please update reference 70.