



# Dermal excisional wound healing in pigs following treatment with topically applied pure oxygen

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Received 11 February 2005; received in revised form 18 February 2005; accepted 18 February 2005  
Available online 18 August 2005

## Abstract

Hypoxia, caused by disrupted vasculature and peripheral vasculopathies, is a key factor that limits dermal wound healing. Factors that can increase oxygen delivery to the regional tissue, such as supplemental oxygen, warmth, and sympathetic blockade, can accelerate healing. Clinical experience with adjunctive hyperbaric oxygen therapy (HBOT) in the treatment of chronic wounds have shown that wound hyperoxia may increase granulation tissue formation and accelerate wound contraction and secondary closure. However, HBOT is not applicable to all wound patients and may pose the risk of oxygen toxicity. Thus, the efficacy of topical oxygen treatment in an experimental setting using the pre-clinical model involving excisional dermal wound in pigs was assessed. Exposure of open dermal wounds to topical oxygen treatment increased tissue  $pO_2$  of superficial wound tissue. Repeated treatment accelerated wound closure. Histological studies revealed that the wounds benefited from the treatment. The oxygen treated wounds showed signs of improved angiogenesis and tissue oxygenation. Topically applied pure oxygen has the potential of benefiting some wound types. Further studies testing the potential of topical oxygen in pre-clinical and clinical settings are warranted.

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*Keywords:* Pre-clinical; Therapy; Angiogenesis; Swine

## 1. Introduction

Hypoxia, caused by disrupted vasculature and peripheral vasculopathies, is a key factor that limits der-

mal wound healing [1,2]. The  $pO_2$  of dermal wounds ranges from 0 to 10 mmHg centrally to 60 mmHg at the periphery, while the  $pO_2$  in the arterial blood is approximately 100 mmHg. Oxygen delivery is a critical element for the healing of wounds [3–5]. Factors that can increase oxygen delivery to the regional tissue, such as supplemental oxygen, warmth, and sympathetic blockade, can accelerate healing [6,7]. The clinical use of

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oxygen to promote wound healing began in the 1960s with administration of systemic hyperbaric O<sub>2</sub> (HBOT) to treat wounds [8]. Clinical experience with adjunctive HBOT in the treatment of chronic wounds [9] have shown that wound hyperoxia increases wound granulation tissue formation and accelerates wound contraction and secondary closure [10,11]. The application of topical oxygen gas on exposed dermal wounds is also used clinically to oxygenate the wound tissue [2,12–19]. This therapeutic modality remains poorly studied.

While the conditions (e.g., pressure, O<sub>2</sub> concentration, frequency and duration of administration) for systemic hyperbaric O<sub>2</sub> therapy (HBOT) have not been optimized on the basis of randomized clinical trials, HBOT is an FDA-approved therapeutic modality used in wound clinics with variable success. HBOT delivers 100% O<sub>2</sub> at 2–3 atmospheres (atm) of pressure and patients typically receive 10–30 treatments, depending upon the diagnosis. These treatments are usually 60–120 min long, given 5 days a week and performed in specialized chambers at facilities with physician supervision. HBOT is capable of elevating arterial pO<sub>2</sub> as high as 1200 mmHg [2]. This brings with it the clear risk of oxygen toxicity. Like many other risk factors including cigarette smoking, HBOT does not typically result in immediate manifestation of clinical abnormalities. This line of evidence cannot be accepted as proof of safety unless detailed biochemical and molecular investigation is conducted to test markers of oxidative damage in the blood and urine of treated subjects. It is general knowledge that exposure of biological cells and tissues to pure O<sub>2</sub> may result in oxidative stress and genotoxicity [20]. There is no question that exposure to pure O<sub>2</sub> presents risk and that it is prudent to avoid unnecessary exposure to a risk factor. HBOT is contraindicated in a number of clinical conditions. Moreover, some patients opt against HBOT because of claustrophobia as the chambers used to administer HBOT are relatively small.

Favorable outcome in studies using sub-pure O<sub>2</sub> under normobaric conditions [21] lead to question the use of pure O<sub>2</sub> under pressure for wound therapy. Furthermore, encouraging outcome obtained from the use of topical O<sub>2</sub> alone [19] warranted a more detailed investigation testing the efficacy of topical O<sub>2</sub> treatment under controlled conditions. Such fine-tuning of conditions for O<sub>2</sub> therapy should result in a more cost-

effective and efficient care minimizing barotraumas and other risks associated with use of pressurized pure O<sub>2</sub>. If proven to be efficient, topical O<sub>2</sub> therapy has the added advantage of caring for much larger potential patient population especially under conditions of public disaster and in a field-setting where HBOT may not be applicable. In response to favorable outcomes of the clinical case series study conducted by surgeons at the Ohio State University, we sought to test the efficacy of topical oxygen treatment in an experimental setting using the pre-clinical model [22,23] involving excisional dermal wound in pigs.

## 2. Materials and methods

Telazol was obtained from Fort Dodge Animal Health, Fort Dodge, Iowa. Telazol (tiletamine HCl and zolazepam HCl) is supplied in individual vials and when this is reconstituted produces a solution containing equivalent of 50 mg tiletamine base, 50 mg zolazepam base and 57.7 mg manitol/ml. Duragesic was obtained from Janssen Pharmaceutica Products, L.P. Titusville, NJ. Duragesic (fentanyl transdermal system; *N*-phenyl-(1-2 phenyl ethyl-4-piperidyl) propanamide) is a transdermal system providing continuous systemic delivery of fentanyl, a potent opioid analgesic, for 72 h. Tegaderm bandage was obtained from 3M Health Care, St. Paul, MN. Elastikon (4 in.) bandage wrap material was purchased from Johnson and Johnson, Indianapolis, IN. Punch biopsies were taken using 3 mm dermal punch biopsy supplied by Miltex Inc. York, PA. Topical oxygen devices were provided by GWR Medical, Chadds Ford, PA.

### 2.1. Experimental model, wounding and treatment protocol

Four female specific pathogen free domestic pigs weighing 80 pound were used. For wounding, the animals were initially sedated using Telazol (tiletamine and zolazepam, 6 mg/kg body weight). During wounding and treatment, animals were kept anesthetized with isoflurane via a face mask. The wound sites over the dorsal trunk area were shaved using a size 40 clipper blade. The area was cleaned using alcohol and Beta-dine scrub. Excisional dermal wounds ( $n = 10$ ; two sets of 5) were created on the back of each pig using a

size 10 scalpel. A total of 40 wounds in four pigs were studied. Full-thickness sections of skin (1 × 1 in.) were removed during the wounding process. Duragesic (fentanyl transdermal system) patches were placed on the pinna to alleviate pain in response to wounding. All wounds were dressed with a Tegaderm (3M Health Care, St. Paul, MN) patch. The patches were held in place by a Elasticon bandage wrap (Johnson and Johnson, Indianapolis, IN). After trying several types of bandage material, Elasticon was found to stay adhered to the skin yet it could be easily removed for treatments without irritating the underlying skin. In order to keep the bandages clean, the animals were housed in elevated vinyl-coated wire floored runs. Sterile techniques were utilized when doing bandage changes to minimize introduction of pathogens to the wound site. Finally, the psychological well-being of the pigs was addressed by providing them with conspecific visual interaction, various toys, and hand-fed treats under professional supervision. These forms of enrichment serve to lower the distress that may otherwise be experienced and potentially confound the experimental results.

The Tegaderm dressed wounds were allowed to heal by secondary intention. Half of the wounds were subjected to topical oxygen treatment whereas the other half of the wounds in the same pig was left exposed to room air. Out of five wounds in each treatment group, two were designated for biopsy collection. Punch biopsies (3 mm) were collected from the wound edge at specified time intervals. Animals were provided with standard laboratory diet and water ad lib. Individual housing (70 ± 4 °F; 40–70% humidity) and care for animals were in accordance with the guidelines of the Institutional Lab Animal Care and Use Committee (ILACUC) of the Ohio State University.

For topical treatment with pure oxygen, a plastic device that is routinely used to treat patients was employed [17,19]. The device has a triangular textile base containing skin adhesive. Medical grade oxygen was used to inflate the device and then the flow rate was set to 3–6 l/min. The treatment was performed for 3 h daily for the first 7 days (day 0–6) from the day of wounding.

## 2.2. Wound area assessment

All wounds were digitally photographed in the presence of a standard reference ruler. Wound area

was computed using the WoundMatrix™ software as described previously [24,25].

## 2.3. Wound-bed $pO_2$ measurements

Real-time wound-bed  $pO_2$  was performed non-invasively using Oxy-Lite (Oxford-Optronix, Oxford, UK) as described by us previously [17,26]. An  $O_2$  electrode, specially designed for our application purposes by the vendor, was placed at 2 mm depth in the center of the wound bed.

## 2.4. Histology

Formalin-fixed wound-edges embedded in paraffin were sectioned. The sections (8–10 μm) were deparaffinized and stained with hematoxylin and eosin (H&E) as well as for Masson Trichrome staining for histological analysis using standard procedures [17,26]. Furthermore, the sections were immunostained with the following primary antibodies: Keratin 14 (1:500; Covance, Berkeley, CA), hVEGF (1:50 dilution; R&D Systems, MN) or anti-smooth muscle actin (1:1000; Sigma, St. Louis, MO). To enable fluorescence detection, sections were incubated with appropriate Alexa Fluor® 488 (Molecular probes, Eugene, OR) conjugated secondary antibody (1:250 dilution). In some cases, the sections were stained with DAPI (Molecular probe, Eugene, OR) to visualize the nuclei. Images were collected using a Zeiss Axiovert 200M motorized microscope supported by an AxioCam digital camera, Axiovision software and Apotome.

## 2.5. Statistics

Data shown as bar graphs are mean ± S.D. Student's paired *t*-test was used to test significance of difference between means.  $p < 0.05$  was interpreted as significant difference between means.

## 3. Results

A clinical topical oxygen device (Fig. 1) was used on wounds without dressing. The presence of any petroleum based dressings prevents oxygen penetration into the wound. These are single use disposable devices



Fig. 1. Topical oxygen device affixed on pig dermal wounds. Photograph showing four wounds treated in a pig that was used to standardize the oxygen application approach used in the current study.

that come as sacral devices. They have an adhesive strip for fixation of the device to the skin. The device is connected to an oxygen gas cylinder. Initially, the bag is fully insufflated at high pressure. Subsequently, flow is initiated at 3–6 l/min. Each device has a release valve to prevent excessive pressure build-up within the bag. Although topical oxygen therapy for wounds has been used clinically in numerous wound care centers, the literature contains no direct report testing the effect of topical oxygen application on wound tissue  $pO_2$ . Exposure of open dermal wounds to topical oxygen treatment did not influence deep tissue  $pO_2$  acutely. However, using a probe, specially designed to measure superficial  $pO_2$  at 2 mm depth, topical application of pure oxygen slowly elevated wound bed  $pO_2$  (Fig. 2). Note that this  $pO_2$  reading reflects superficial wound tissue oxygen tension at the center of the wound bed and is not comparable to the routine clinical transcutaneous oxygen measurement (TCOM).

Repeated treatment of the excisional dermal wounds in pigs clearly accelerated wound closure in the early post-wound phase. This early advantage was maintained during the subsequent phase resulting in a significant acceleration of wound closure (Fig. 3). To test the quality of the regenerated tissue, we performed Masson-Trichrome and Hematoxylin-Eosin (H&E) staining of the wound-edge tissue on day 22 post-wounding. A broad region of hyperproliferative epithelium is a hallmark of the dermal wound edge.

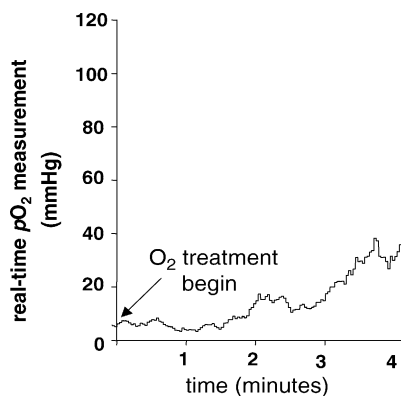


Fig. 2. Wound-bed  $pO_2$  measurements in pigs treated or not with topical oxygen. The dermal wound model is described in this figure.  $pO_2$  measurement was performed non-invasively using Oxy-Lite (Oxford-Optronix). An  $O_2$  electrode was specially designed for our application ( $pO_2$  assay at 2 mm depth) by the vendor. A real-time measurement of  $pO_2$  in response to topical oxygen application is shown. The arrow indicates the time of initiation of topical  $O_2$  treatment.

As the healing matures, this region narrows until it is reduced to a very thin margin typically observed in the intact skin. Both H&E as well as trichrome staining consistently revealed that the wounds treated with topical oxygen were in a more advanced stage of healing. The section of the regenerated tissue from wound treated with oxygen had a narrower hyperproliferative epithelium region compared to that in the tissue from the wound of the room air exposed wounds (Fig. 4). The expression of distinct keratin pairs during epidermal differentiation is assumed to fulfill specific and essential cytoskeletal functions. Keratin 14 plays a key role in epidermal remodeling. The intact skin stains positive for a thin epithelial band of keratin 14. Incomplete healing is associated with a broader distribution of keratin 14 in the healing skin along the hyperproliferative epithelium. As the healing matures and the hyperproliferative epithelium region narrows, the keratin 14 positive band becomes narrower and is pressed against the epidermis. Our results from keratin-14 staining of the regenerated tissue confirmed that indeed the wounds treated with oxygen presented histological signs of a higher maturity in healing compared to the tissues studied from the edge of the room-air treated wounds (Fig. 5). Immunohistochemical studies revealed a stronger presence of VEGF in the tissue from oxygen treated wounds compared to the

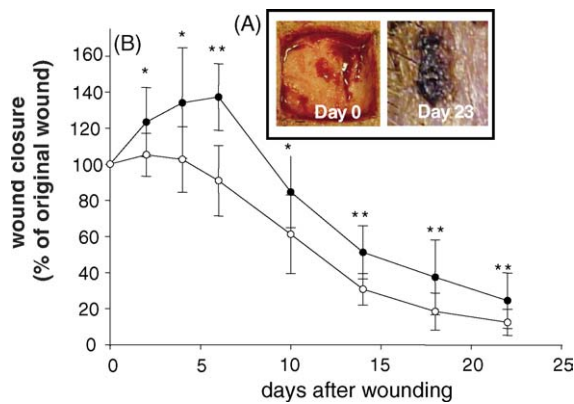


Fig. 3. Full-thickness dermal wound closure in response to topical oxygen administration in pigs. Ten (two clusters of five; on the back) secondary-intention full-thickness excisional dermal wounds ( $1 \times 1$  in.) were inflicted. Digital images of a typical wound on days 0 and 23 after wounding are shown in the inset. Five of ten wounds in each pig were treated with pure oxygen (open circles) for 3 h using a topical oxygen treatment device at a flow rate of 3–6 l/min. This treatment was performed every day for the first 7 days (day 0–6) from the day of wounding. Five of the control wounds (solid circles) were exposed to room air for the similar period. After treatment, wounds were dressed with moist Tegaderm dressing firmly held in place by Elasticon tape wrapped around the body. Digital imaging of wound was performed on days of oxygen treatment and every 4 days (during changes of wound dressing) following the treatment phase. One of the five wound in the treatment and placebo group was used for collection of biopsy. Images were analyzed using WoundMatrix® software. Mean  $\pm$  S.D. \* $p < 0.05$ ; \*\* $p < 0.005$ . Significantly smaller compared to corresponding control wounds.

room air exposed controls (Fig. 6A). Smooth muscle actin represents an integral component of blood vessel wall. Dual fluorescence staining of the tissue sections for smooth muscle actin and cell nuclei (DAPI, red) showed that the edge of oxygen treated wounds had a higher density of blood vessels than that in the edge of the room air exposed control wounds (Fig. 6B). While occurrence of blood vessels is indicative of angiogenesis, it is not a functional measure of vascularization. Tissue oxygen tension was chosen as a functional marker of the extent of vascularization. A well vascularized tissue is expected to have higher oxygen tension compared to a tissue with limited vasculature. Wound site  $pO_2$  was assessed in both oxygen treated and room air exposed wounds. The analysis was carried out under resting conditions when oxygen treatment was not in progress. Results from such analyses showed that the oxygen treated wounds have better

vascularization than room air treated control wounds (Fig. 6C).

#### 4. Discussion

Wound healing is a multi-factorial process. Impairment of this process can be caused by the inadequacy of or lack of synchrony between multiple critical factors. It is widely acknowledged that limited oxygenation of the wound site is one key factor that results in wound chronicity. Angiogenesis is a rate-limiting factor in wound healing [27]. Oxygen and its reactive derivative hydrogen peroxide are known to induce angiogenic responses such as the induction of VEGF expression [24,25,28]. While hypoxia can initiate neovascularization by inducing angiogenic factor expression, it cannot sustain it. Acutely, hypoxia facilitates the angiogenic process [29] while chronic hypoxia impairs wound angiogenesis [30]. Sustained hypoxia causes death and dysfunction of tissue. Supplemental  $O_2$  administration accelerates vessel growth [31]. VEGF is a major long-term angiogenic stimulus at the wound site.  $O_2$  treatment induces VEGF mRNA levels in endothelial cells and macrophages [32–34] and increases VEGF protein expression in wounds in vivo [35]. Recently, it has been observed that  $O_2$  may trigger the differentiation of fibroblasts to myofibroblasts [26], cells responsible for wound contraction.

Collagen deposition is a fundamental step in wound healing that provides the matrix for angiogenesis and tissue remodeling. There are several post-translational steps in collagen synthesis that are directly  $O_2$  dependent. The enzymes prolyl hydroxylase, lysyl hydroxylase and lysyl oxidase all require molecular  $O_2$  as a cofactor. Prolyl hydroxylase is required to convert proline residues to hydroxyproline, which allows the procollagen peptide chains to assume their triple helix configuration. Without this triple helix configuration, the synthesized procollagen chains accumulate in the rough endoplasmic reticulum and are eventually excreted as non-functional gelatinous protein [36]. Once the procollagen has assumed the triple helix conformation and has been excreted, the individual collagen fibers are arranged into linear fibrils via cross-linking of lysyl hydroxylase and finally cross-linking between large fibrils is performed by lysyl oxidase. These extracellular cross-linkages are ultimately



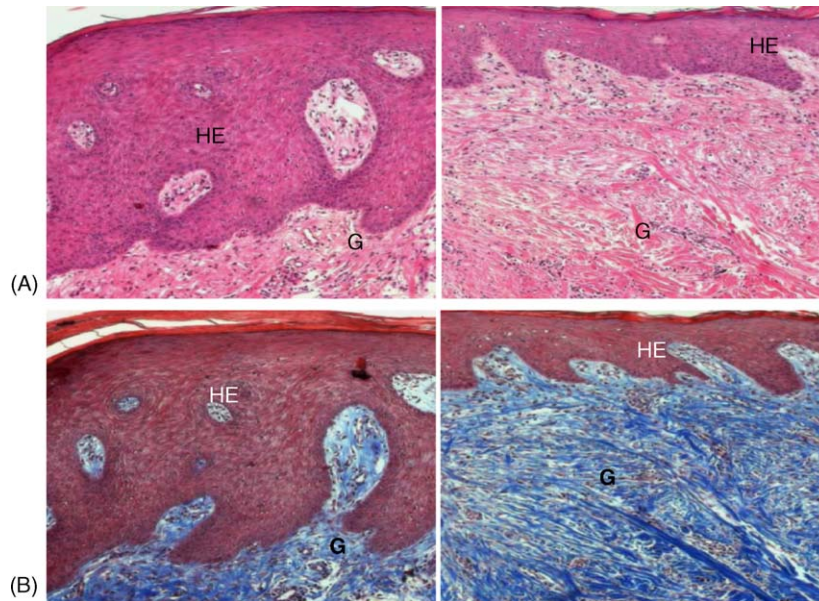


Fig. 4. Pig dermal wound histology in response to oxygen treatment. The dermal wound model is described above in Fig. 2. Three millimetres punch biopsies of the regenerated tissue were taken on day 22 from control and treated wounds. Formalin fixed paraffin sections were stained using (A) H&E or (B) Mason Trichrome. Note the architectural differences in the epidermis between the control and treated wounds, supporting advanced remodeling and healing in the treated as compared to the control group. HE, hyperproliferative epidermis; G, granulation tissue.

responsible for the tensile strength achieved in healed wounds. Of the  $O_2$  dependent enzymatic processes, the rate of collagen synthesis is reflected by the rate at which prolyl hydroxylation occurs [36]. The amount of  $O_2$  at which collagen synthesis is half-maximal ( $K_m$  using Michaelis-Menton equation) has been determined to occur at a  $pO_2$  of 20–25 mmHg [37,38], with  $V_{max}$  occurring at levels approaching 250 mmHg. This represents levels of  $O_2$  availability that exceeds the  $pO_2$  normally present in wounds and suggests that adequate wound tissue oxygenation is crucial to support collagen synthesis. Indeed, increasing wound oxygenation results in increased collagen deposition and tensile strength [39–41].

Wound tissue oxygenation is an extremely sensitive indicator for the risk of infection in surgical patients [21,42]. The ability of supplemental  $O_2$  to reduce infection is mediated by reactive oxygen species (ROS) such as  $H_2O_2$  generated by NADPH oxidases in wound neutrophils and macrophages. The concentration of  $O_2$  necessary to achieve half maximal ROS production (the  $K_m$ ) is in the range of 45–80 mmHg, with maximal ROS production seen at  $pO_2$  at >300 mmHg

[30]. Thus, just as with the enzymes regulating collagen synthesis, the maximal effects of this biologic process can only be achieved through the administration of supplemental  $O_2$  to attain wound  $pO_2$  levels

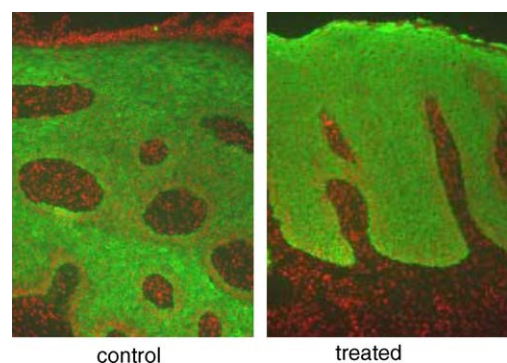


Fig. 5. Effect of oxygen treatment on epidermal remodeling during the healing process. The dermal wound model is described above in Fig. 2. Three millimetres punch biopsies were taken on day 15 from control and treated wounds. Formalin fixed paraffin sections were stained using antibody against keratin-14 (green) to stain for epidermis. Nuclei were stained with DAPI (red). Note more defined epidermis in treated side compared to the control.

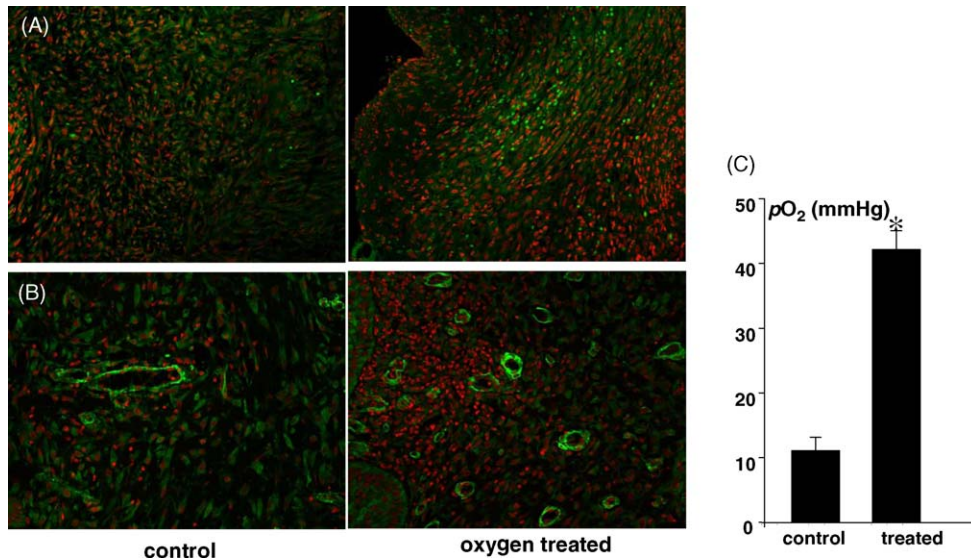


Fig. 6. Angiogenic response at the wound site following topical oxygen treatment of full-thickness dermal wounds. The dermal wound model is described above in Fig. 2. Three mm punch biopsy from wound margins were harvested. Formalin-fixed paraffin sections were stained using antibody against (A) vascular endothelial growth factor (VEGF, green, day 7 post wounding) or (B)  $\alpha$ -smooth muscle actin (SMA, green, day 16 post wounding). Counterstaining of nuclei was performed using DAPI (red). Note that compared to the control side more VEGF and SMA stain in the treated side; (C) wound site  $pO_2$  levels were measured under resting conditions on day 22. Mean  $\pm$  S.D. \* $p < 0.05$ . Baseline skin  $pO_2 = 40$ –50 mm Hg.

beyond those encountered when breathing room air. In fact, approximately 98% of the  $O_2$  consumed by wound neutrophils and macrophages is utilized for respiratory burst [30]. At the wound-site, ROS are generated from oxygen by almost all wound-related cells. Recently, first evidence indicating that ROS may contribute to several facets of wound healing including angiogenesis has been reported [18,24,43]. Of importance, numerous wound healing related growth factors including PDGF $\beta$  (Regranex gel, Johnson & Johnson, Indianapolis, IN) rely on ROS for the execution of its biological function [44]. Oxidation plays a central role in promoting TGF $\beta$  function [26]. Indeed, strategies to raise wound  $pO_2$  show a synergistic effect to benefit wound healing in conjunction with both TGF $\beta$  as well as PDGF therapy of wounds [45]. Fig. 7 presents a schematic illustration of the oxygen and ROS-sensitive pathways that are relevant to the current study.

From a diagnostic standpoint, many surgeons already use measurements of wound oxygenation to guide their treatment planning when they obtain TCOM with non-invasive vascular studies. TCOM measure-

ments provide reliable prognostic information regarding the ability of wounds to heal and this has been used to determine amputation levels [17,19,46]. It is important to note though that TCOM does not reflect wound-site  $pO_2$  like we have measured by placing a probe directly at the center of the wound. Standard TCOM measurements are conducted under conditions where the skin is warmed to 42 °C. This warmth factor contributes to overestimation of  $pO_2$  especially because typically  $O_2$  therapy to the wound is not accompanied with warming of the wound site [2]. There is a fundamental difference between the intact skin in the perimeter of the wound compared to the wound core. While the former is well vascularized, wound cores are typically characterized by disrupted vasculature and therefore suffer from poor blood perfusion.  $pO_2$  measurement performed in this study and TCOM has another significant contrasting feature. TCOM is based on the Clark electrode technology [47]. This technology is particularly not best suited under hypoxic conditions because it consumes oxygen while measuring it. This may lead to artifacts especially under conditions where oxygen availability is limited [17]. In contrast,

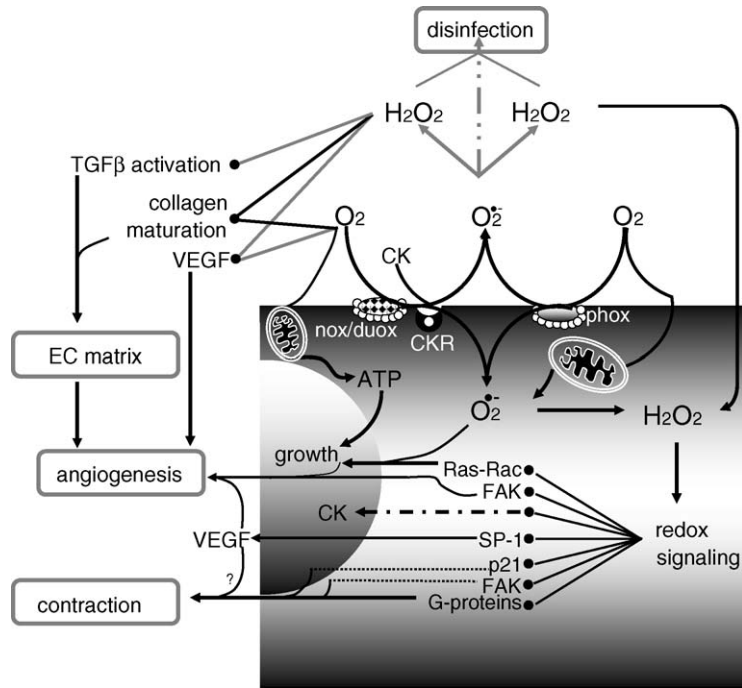


Fig. 7. Schematic illustration of select possible pathways by which oxygen and its reactive derivatives may influence wound healing related processes. The specific processes have been recently reviewed [43]. Excess generation of ROS, such as in cases where the inflammatory phase is not resolved in a timely manner, may cause oxidative damage and impair healing. CK, cytokine; CKR, cytokine receptor; EC, extracellular; FAK, focal adhesion kinase; phox, phagocytic NADPH oxidases; nox/duox, non-phagocytic oxidases.

the oxymetry system we employed is based on fiber-optics  $pO_2$  probes which provide a continuous measure of  $O_2$  partial pressure coupled with fast (<5 s) response times for real-time monitoring of temporal  $O_2$  changes [48]. Fluorescence lifetime is longest at low  $pO_2$ , making these probes most sensitive in the physiological range 0–60 mmHg. Also, because the measurement is based on fluorescence lifetime rather than fluorescent intensity it is much less prone to artifacts (e.g. because of variation in the intensity of the light source, ambient lighting, or photo-bleaching). Compensation for the effects of temperature is required since fluorescent lifetimes are affected by changes in temperature. Temperature is measured by a fully integrated thermocouple, allowing simultaneous monitoring of tissue  $pO_2$  and temperature as well as automatic temperature correction.

Results of this pre-clinical study present first evidence indicating that topical applied pure oxygen is capable of oxygenating the superficial wound tissue

but not deep tissue. Because regeneration of new tissue is expected at the wound surface, it is reasonable to conclude that topical application of oxygen to open wounds had some favorable impact on the overall healing process. These findings suggest that treatment of open wounds with topical oxygen may provide beneficial results provided supply of oxygen to the superficial wound tissue is the key limiting factor. This hypothesis is consistent with previously reported clinical observation that topical oxygen treatment seems to be effective in many but not all cases [19]. If proven to be effective, topical  $O_2$  therapy has the added advantage of caring for much larger potential patient population especially under conditions of public disaster and in a field-setting where HBOT may not be applicable. In addition, topical oxygen based therapeutics has the potential to bypass HBOT related risk of systemic toxicity [20,49]. Further studies testing the potential of topical oxygen in pre-clinical and clinical settings are warranted.



## Acknowledgment

Supported by P50 GM27345 and RO1GM 69589 to C.K.S.

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