Peroxide-based oxygen generating topical wound dressing for enhancing healing of dermal wounds

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Abstract

Oxygen generating biomaterials represent a new trend in regenerative medicine that aims to generate and supply oxygen at the site of requirement, to support tissue healing and regeneration. To enhance the healing of dermal wounds, we have developed a highly portable, in situ oxygen generating wound dressings that uses sodium percarbonate (SPO) and calcium peroxide (CPO) as chemical oxygen sources. The dressing continuously generated oxygen for more than 3 days, after which it was replaced. In the in vivo testing on porcine full-thickness porcine wound model, the SPO/CPO dressing showed enhanced wound healing during the 8 week study period. Quantitative measurements of wound healing related parameters, such as wound closure, reepithelialization, epidermal thickness and collagen content of dermis showed that supplying oxygen topically using the SPO/CPO dressing significantly accelerated the wound healing. An increase in neovascularization, as determined using Von Willebrand factor (vWF) and CD31 staining, was also observed in the presence of SPO/CPO dressing. This novel design for a wound dressing that contains oxygen generating biomaterials (SPO/CPO) for supplying topical oxygen, may find utility in treating various types of acute to chronic wounds.

INTRODUCTION

Physical injuries to skin can compromise the vascular systems of tissues, and in turn may cause hypoxia and ischemia. A typical wound site is characterized by a disrupted vasculature and a high energy and oxygen demand to support the regeneration of the wounded tissue. Following tissue injury, several wound repair mechanisms are sequentially activated and are grouped into three overlapping phases of wound healing: inflammation, proliferation, and remodeling.1 The various factors affecting wound healing have been reviewed in detail before.2–4 Increased oxygen tension in a wound site initiates wound healing by stimulating several processes, including phagocytosis; oxidative microbial killing mediated by neutrophils; degradation of necrotic wound tissue; collagen synthesis; and neovascularization.5 For these tissue repair processes, a situation of high oxygen demand and a low oxygen supply results in extreme hypoxia.6,11 Long-term hypoxia can impair the normal healing sequence and can also result in pathologic wound healing.

Chronic wounds (including diabetic wounds, ischemic ulcers, venous ulcers, neuropathic foot ulcers, infected surgical sites etc.) are a huge clinical concern and a significant burden to patients, health care providers and the health care system all over the world. In the United States alone, chronic wounds affect around 6.5 million people and costs an estimated 25 billion US dollars for treatment annually.12,13 It is now well known that one key factor that can result in wound chronicity is limited oxygenation of the wound site. It has now been well established that administration of externally supplied oxygen can play a crucial role in healing of a wound by providing cells of the ischemic tissue with sufficient oxygen to survive, proliferate and function.13–18 It has been seen that although tissue hypoxia plays a central role in initiating the formation of new blood vessels (neovascularization) in wounded tissue, it cannot sustain it for a long time.19 An additional supply of oxygen will be needed to sustain this process.

Clinical use of oxygen to promote wound healing began as early as the 1960s, with the use of hyperbaric oxygen therapy (HBOT) that delivers systemic oxygen to the whole body at elevated pressures (2-3 atmospheres) for treating various types of wounds.20–24 However, the widespread use of HBOT has been limited by several issues, which include oxygen toxicity20–22; psychological stress that can impair its effectiveness23 and the overall cost of the therapy. On the contrary, topical oxygen therapy (TOT), where oxygen is locally administered to an affected region of the body surface, usually under normal atmospheric pressure (1.03) or slightly elevated pressures, has been gaining prominence as an alternate form of oxygen therapy to HBOT. The advantages of TOT include a lack of systemic oxygen toxicity (as seen in HBOT), low cost and self-administration. TOT has also been used clinically for more than four decade (similar time as HBOT) treating different types of skin wounds, including pressure sores and ulcers,24,25 burn wounds,26 recalcitrant open wounds,27 and also as an adjunct to normal wound healing.28 The widespread therapeutic use of TOT was lacking until now
due to insufficient scientific data to support its therapeutic benefits over other methods of oxygen therapy, mainly HBOT. A study by Fries et al.,29 using topically applied pure oxygen on open wound sites in pigs showed that oxygen partial pressure (pO2), when measured at 2 mm below the surface of the wound, increased from a baseline of 5–7 mmHg to levels >40 mmHg as early as 4 minutes into treatment, along with increases in vascular endothelial growth factor (VEGF) expression and formation of new blood vessels. This result was an important proof of principle that TOT can oxygenate superficial wound tissues, which was also supported by the studies of several other groups.18,30–32 An additional boost to TOT for wound healing comes from the decision of the United States FDA’s proposal to reclassify TO devices from the most stringent class III (premarket approval) to a safer class II (http://www.fda.gov/cdrh/ode/guidance/1582.html). A major drawback with most of the current TOT devices is that they use gaseous oxygen as the source, which has several limitations, including having a constant supply of pure oxygen over the entire period of wound treatment (usually several days), physically attaching external oxygen supplying devices to the patients that restrict their daily activities and the overall cost of such treatments. More recent TOT devices try to address these issues. For example, Oxyband Wound dressing (Oxyband Technologies, MO) is a bandage that has air pockets filled with oxygen and other device that uses a topical oxygen emulsion (TOE) containing super-saturated oxygen suspension with perfluorocarbons30 or an undisclosed compound3 to treat second-degree burns and for wound repair. However, for all of the above “alternative” strategies, gaseous oxygen still needs to be incorporated into the product during manufacturing.

We have developed a wound dressing that chemically produces oxygen in situ, using particulate oxygen generators (POG) that consist of a mixture of sodium percarbonate (SPO) and calcium peroxide (CPO) in a heterogeneous polymer matrix. The goal of this present study is: (1) To develop a novel design for a topical wound dressing that can oxygenate superficial wound tissues, which was also supported by the studies of several other groups.18,30–32 An additional boost to TOT for wound healing comes from the decision of the United States FDA’s proposal to reclassify TO devices from the most stringent class III (premarket approval) to a safer class II (http://www.fda.gov/cdrh/ode/guidance/1582.html). A major drawback with most of the current TOT devices is that they use gaseous oxygen as the source, which has several limitations, including having a constant supply of pure oxygen over the entire period of wound treatment (usually several days), physically attaching external oxygen supplying devices to the patients that restrict their daily activities and the overall cost of such treatments. More recent TOT devices try to address these issues. For example, Oxyband Wound dressing (Oxyband Technologies, MO) is a bandage that has air pockets filled with oxygen and other device that uses a topical oxygen emulsion (TOE) containing super-saturated oxygen suspension with perfluorocarbons30 or an undisclosed compound3 to treat second-degree burns and for wound repair. However, for all of the above “alternative” strategies, gaseous oxygen still needs to be incorporated into the product during manufacturing.

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Preparation of the oxygen generating wound dressing

The oxygen generating wound dressing consists of four distinct layers, as shown in Figure 1. Layer 1 (L1) is a Gelatin-based layer that contacts the wound. Layer 2 (L2) is the oxygen generating layer that has sodium percarbonate (SPO) and calcium peroxide (CPO) as chemical oxygen source in a polyvinyl alcohol (PVA) and polycaprolactone (PCL) polymer matrix. Layer 3 (L3) is a silicone-based layer that provides mechanical stability and flexibility to the dressing and Layer 4 (L4) is a thin polyvinylidene chloride (PVDC)-based layer that forms the outermost covering of the dressing and would prevent the POGs generated oxygen from leaking out of the dressing due to its very low gas and vapor permeability; (B) Actual POG-based oxygen generating wound dressing, placed on the pig skin and covers a 10 × 10 cm2 full-thickness surgical wound. The white layer in the picture is the oxygen generating layer containing SPO and CPO (L2). The arrows indicate the actual (transparent) edges of the dressing which is composed mainly of L4.

Figure 1. Design of the POG-based oxygen generating wound dressing. (A) The dressing is composed of four distinct layers (L): L1 is a Gelatin-based layer containing manganese chloride (MnCl2) that is placed directly on top of the wound. It is hydrophilic, oxygen permeable and can provide cushioning to the wound. L2 is the oxygen generating layer that has sodium percarbonate (SPO) and calcium peroxide (CPO) as chemical oxygen source in a polyvinyl alcohol (PVA) and polycaprolactone (PCL) polymer matrix. L3 is the silicone layer that provides flexibility to the dressing and can protect the wound site from minor external injuries. L4 is a thin polyvinylidene chloride-based layer that forms the outermost covering of the dressing and would prevent the POGs generated oxygen from leaking out of the dressing due to its very low gas and vapor permeability; (B) Actual POG-based oxygen generating wound dressing, placed on the pig skin and covers a 10 × 10 cm2 full-thickness surgical wound. The white layer in the picture is the oxygen generating layer containing SPO and CPO (L2). The arrows indicate the actual (transparent) edges of the dressing which is composed mainly of L4.
11 cm² film. The gelatin layer was sterilized separately as assembling the wound dressing, L1 was cut to 11 cm² at room temperature (22–24°C) for 15 minutes, followed by placement on a hot plate (70°C) for 15 minutes at room temperature (22–24°C) before removing for use. For the oxygen generating SPO/CPO (also referred as POG) dressing or control dressing, followed by secondary wound dressing study. For pain management, a transdermal fentanyl patch (5–75 mcg/individual) was placed in the 10 mL syringe was measured every 24 hours for over a 3–7 day period. The amount of water displaced is directly proportional (mL) to the amount of oxygen being generated.

Experimental animal model, surgery, and treatment protocol

All animal-related experiments were carried out using protocols approved by the Institutional Animal Care and Use Committee at Wake Forest University Health Sciences (IACUC protocol # A12-136) and strict adherence to the guidelines and recommendations. All surgeries, biopsies and wound dressing changes were performed under the supervision of trained animal care staff, with appropriate anesthesia and postoperative pain medication given to the animals.

Preparation of animals for surgery and study

For this wound healing study, 2–3 week old non-SPF (specific pathogen free) pigs were obtained and housed in common cages. Animal care and handling was according to the institutional guidelines (and the approved protocol as stated above) and the pigs were saddle-trained for about 2 weeks before surgery.

Animal surgery

Around 5-6 week old pigs (average weight 30 kg) were used for creating the full-thickness surgical wound and subsequent wound healing study. For pain management, a transdermal fentanyl patch (5–75 mcg/individual) was placed on the cervical dorsum or rear flank of the pig 24 hours prior to surgery. On the day of the surgery, the pigs were anesthetized using ketamine (10–15 mg/kg), xylazine (2 mg/kg IM), and acepromazine (0.2–0.5 mg/kg IM), and maintained under anesthesia using inhaled isoflurane (3–5% induction; 1–3% maintenance) via endotracheal tube. After immobilizing the animals in a dorsal position, shaving their backs, sterilizing the skin with β-iodine and 70% alcohol, four 10 cm² squares were marked using a skin marker pen in the central back along the thoracic and lumbar area, followed by tattooing incisions were made along the markings with a surgical blade and the overlying skin was excised to the panniculus carnosus layer, thereby creating four 10 cm² full-thickness surgical wounds. Excessive bleeding was controlled using a small amount of topical epinephrine (0.01% topical). These wounds were subsequently covered with the oxygen generating SPO/CPO (also referred as POG) dressing or control dressing, followed by secondary bandages that included in sequence a hypoallergenic skin adhesive surgical tape (3M MEDIPORE-H surgical tape; 3M Healthcare, St. Paul, MN), 2-3 layers of absorbent material (WEBRIL cotton undercast padding; Covidien,
Minneapolis, MN), 2 layers of elastic self-adherent wraps (3M Corban), an in-house made suture (to protect the wounds from external injuries in cage) and a jacket (Lomir Biomedical, Quebec, Canada). Pain management after the surgery or bandage change included buprenorphine injection (0.005–0.01 mg/kg IM) given 6–8 hours and either an injection of Carprofen SC (2–4 mg/kg) or oral Carprofen (2–4 mg/kg) and oral famotidine (0.5 mg/kg) for 3–5 days. For each pig, there were two wound sites for each type of dressing (POG or control) and a total of five pigs were used in the study where the wound healing was monitored 2 times per week, for a total of 8 weeks.

**Bandage changes and biopsy**

For each pig, the wound dressings (SPO/CPO and control) were changed every 3–4 days, under general anesthesia without, tracheal intubation. Briefly, pigs were anesthetized with ketamine (10 mg/kg) and dexmedetomidine (0.05 mg/kg) and maintained under Isoflurane with nose cone. After removing the old bandages, the wounds were cleaned measured, and photographed. For both wound models, 2–3 mm diameter punch biopsies were taken from each wound at 1, 2, 3, 4, 6, and 8 weeks, and two or less 12 mm diameter punch biopsies will be taken from the edge of the wound. Placement of the test wound dressings and secondary bandage was done as described under animal surgery. After returning the animal to the cage, an injection of atipamezole (0.5 mg/kg IM) was given to reverse the effects of dexmedetomidine. The pigs were given oral carprofen (2–4 mg/kg) and oral famotidine (0.5 mg/kg) for 3–5 days.

**Morphological assessment; measurement of wound closure and reepithelialization**

After the surgery and during every subsequent bandage changes, the wounds were cleaned and digitally photographed for a total of 8 weeks. Using a ruler that was part of the wound images, the total area and the change in area was calculated. Placement of the test wound dressings and secondary bandage was done as described under animal surgery. After returning the animal to the cage, an injection of atipamezole (0.5 mg/kg IM) was given to reverse the effects of dexmedetomidine. The pigs were given oral carprofen (2–4 mg/kg) and oral famotidine (0.5 mg/kg) for 3–5 days.

**Corrected area of the wound on Day 0 = M₀ × 1 = M₀**

% of wound area on Day Z = M₀/Mz × 100

The results of the wound healing are expressed as percentage (% of the original wound size (original surgical wound size is 10 × 10 cm²). For measuring the reepithelialization of the wounds, the area excluded by a new epithelial layer (area of open wound-represented here as O) was traced and measured using Polygon selection tool in ImageJ along with the wound area. The area covered by the new epithelial layer (reepithelialization) was calculated as follows:

Area of open wound on day Z = O
Corrected area of open wound on Day Z (represented here as Oz) = O × C (see above)

Area covered by new epithelial layer on Day Z (represented here as Ez) = Mz – Oz

% of epithelial layer (reepithelialization) = Ez/Mz×100

The data for wound closure and reepithelialization during the course of the study (8 weeks) include areas measured from five different pigs, with 6–7 different wound sites on each type of dressing (Oxygen generating SPO/CPO and control).

**Histological analysis of healing skin tissue**

Biopsies of the healing skin tissue (treated with oxygen generating SPO/CPO dressing or control dressing) were taken using 5 mm disposable biopsy punch (Integra Millex, York, PA), placed in 10% neutral buffered formalin (Fisher Scientific) and fixed overnight (12–14 hours). Samples were then placed in a Leica ASP300S tissue processor and embedded in wax using the Leica EG1160 (Leica Microsystems, IL). After cutting 5 μm thick sections The tissue sections were deparaffinized and rehydrated on a Leica ST5010 Autostainer XL (Leica Microsystems, Buffalo Grove, IL), fixed in Bouin’s Fixative (Sigma-Aldrich) for 1 hr and stained with Hematoxylin & Eosin or Masson’s Trichrome reagent, using standard procedures. Stained tissues were imaged using either a Zeiss AxioImager 2 microscope (Carl Zeiss Microscopy GmbH, Gottinghen, Germany) or a Leica microscope (Leica Microsystems).

**Collagen quantification**

Pig skin biopsies were collected, processed and stained using the Masson’s Trichrome reagent, as mentioned in section “Animal surgery.” Images of collagen staining were taken using brightfield optics in a Zeiss AxioImager 2 microscope (100× total magnification), starting from the epidermal layer region to the lower dermis, in increments of 1 mm (each image representing about 1 mm depth of the skin tissue) which were then meshed together to get an overview of the collagen distribution in different layers of the pig skin. For quantifying collagen content, the skin dermis was divided into upper dermis (UD), middle dermis (MD), and lower dermis (LD), where each region was approximately 2 mm thick. For image analysis, ImageJ software was used where “Blue” stained areas (collagen staining) were selected. Using the “Analyze” function in
Image J, the “Threshold” was adjusted to remove the other color (reddish-brown representing cell staining), color channels were split and the green channel image was selected for further analysis. The green channel image was converted to a 16-bit image (grayscale), the image was Inverted (black & white color reversed) and then the mean gray values were calculated using an area fraction of 0.4 mm$^2$ per image. The collagen staining intensity is represented as mean grey value per 0.4 mm$^2$ of the skin tissue.

Immunostaining and Immunofluorescence

Von Willebrand factor (vWF) staining

Pig skin biopsies were collected, sectioned and prepared for antigen binding. Then, the primary antibody, mouse monocolonal to recombinant porcine CD31 (Abcam Plc., Cambridge, United Kingdom) was added at 1:30 dilution and incubated for 1 hour at room temperature, in a humidified chamber. A biotinylated secondary antibody (1:300 dilution) was then added and incubated similarly for 30 minutes, followed by washing and staining with VECTASTAIN Elite Avidin/Biotin peroxidase system (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Finally, the chromogen (3, 3'-diaminobenzidine/DAB) was added and the color was allowed to develop for 10–15 minutes. After that, the slides were washed in water, dehydrated and mounted in MM24 mounting media (Leica Microsystems).

Statistics

Data shown in the bar graphs are mean ± standard deviations, unless otherwise noted. Student’s paired $t$-test was used to determine significance of difference between means. A $p < 0.05$ was interpreted as significant difference between data means.

RESULTS

Oxygen generating wound dressing with a novel design was created and used for treating full-thickness surgical skin wounds in pigs. The dressing is composed of four distinct layers (L) represented as L1, L2, L3, and L4, each with distinct properties and purpose (Figure 1A). When placed on top of a dermal wound, the wound exudate is absorbed by the gelatin-based layer (L1) and reaching L2, where the aqueous environment initiates the decomposition of SPO and CPO to release oxygen according to the following reaction:

\[
\text{SPO} + \text{CPO} \rightarrow 2\text{O}_2 + \text{H}_2\text{O} + \text{CO}_2
\]

CD31 Staining

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CaO$_2$ + $2$H$_2$O $\rightarrow$ Ca(OH)$_2$ + H$_2$O$_2$

2 H$_2$O$_2$ $\rightarrow$ 2 H$_2$O + O$_2$ ↑

2(Na$_2$CO$_3$ + 1.5H$_2$O$_2$) $\rightarrow$ 2Na$_2$CO$_3$ + 3H$_2$O$_2$

2 H$_2$O$_2$ $\rightarrow$ 2 H$_2$O + O$_2$ ↑

The intermediate hydrogen peroxide (H$_2$O$_2$) produced from SPO and CPO quickly decomposes in the presence of an alkaline environment to give oxygen, which diffuses unidirectional through the gelatin layer to the wound tissue. The chances of any hydrogen peroxide leaking from L2 into the wound are minimized by incorporating manganese chloride (MnCl$_2$) in L1, which catalyzes the decomposition of hydrogen peroxide in the gelatin layer. The polyvinylidene chloride-based layer (L4), which has very low gas and vapor permeability, further ensures that oxygen generated in the dressing is preferentially available to the wound site. The oxygen generating SPO/CPO layer (the main active component of the dressing) had an even distribution of sodium percarbonate and calcium peroxide particles, as seen from the SEM analysis of the L2 films (Figure 2) for generating oxygen continuously for 3-4 days. Oxygen release studies done using the L2 layer of wound dressing over a 3 day period in a high water environment showed that around 70% of the oxygen is released within the first 24 hour. (Day 1), after which the level of oxygen increases and is sustained over the remaining 48 hour. (Figure 3). In a low water environment (a situation represented by dry wound surface), the level of oxygen remained constant after increasing for the first 24 hour. (Day 1).

Pig skin is very similar in structure to human skin and a full-thickness surgical wound model represents an extreme form of wound that can be seen in human patients. In our study, we observed that the wound created by surgical removal of skin tissue was completely filled with a provisional extracellular matrix (mostly consisting of collagen and fibronectin) in the first week itself (Figure 4). By week 2 postsurgery, the healing process had begun and the wounds had started to close. Morphological assessment showed that the healing skin tissues were better vascularized and the edges were closing more uniformly in the POG dressings, as compared to control dressings (Figure 4, week 2, 4, 6, and 8). To get a more quantitative insight into the effect of topical oxygen treatment on wound healing we calculated relative wound closure and reepithelialization from the digital images of the wounds (as shown in Figure 4). Right from week 1, the POG dressing treated groups showed faster wound closure, compared to controls. In the POG treated group, the average size of the wound

Figure 4. Progression seen during healing of the full-thickness surgical wounds (10 $\times$ 10 cm$^2$) created in pigs and treated with either a control dressing (does not contain SPO and CPO and does not generate oxygen) and the oxygen generating (SPO & CPO) dressing. Wound healing was followed for a total of 8 weeks and the test dressings (control and SPO/CPO), along with the secondary dressings and coverings, were replaced every 3-4 days. Digital images of the wounds were taken during each dressing change. The results clearly show the benefits of using the oxygen generating (SPO & CPO) dressing in better and more uniform wound closure. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
Oxygen-generating wound dressing

Figure 5. Effect of topical oxygen treatment on quality of the wound healing, as determined by measuring wound closure (decrease in area of the original wound) and reepithelialization (covering of the open wound by the re-formation of the skin epithelial layer). (A) Treatment of the wound with oxygen generating (POG) dressing shows faster wound closing compared to control dressing and the difference in the rate of closing was significant during the most part of the 8 week study period (except week 4). *p ≤ 0.05 and **p ≤ 0.01 for the two groups during the same time point of analysis (week). Also, among the control groups and POG treated groups analyzed separately, p ≤ 0.01 during weeks 1-3 (***) and p ≤ 0.05 between week 5 and 8 (***). (B) Treatment of the wound with oxygen generating (POG) dressing shows faster reepithelialization. The difference between the control and oxygen generating (POG) dressing treated groups was highly significant during most part of the 8 week study period, except week 5. The original wound was 10 × 10 cm² full-thickness surgical wound. For both analyses, a total of 6 independent wound sites (each for control and POG treated groups) were analyzed from 4 different pigs. *p ≤ 0.05 and **p ≤ 0.01 for the two groups during the same time point of analysis (week).

was 63% at week 4 (original wound size is 100%), compared to 74% when no oxygen was supplied and by week 8, the oxygen treated wounds were on an average 54% of their original size, as compared to 63% for control groups. At week 4 posttreatment, we did not find any significant difference between the sizes of healing wounds between oxygen treated and nontreated groups (Figure 5A). When reepithelialization of the wounds was quantitatively measured, it was seen that treatment with POG dressing resulted in faster reepithelialization of the wounds (more area of healing tissue covered with new epithelial layer) as compared to controls (Figure 5B). During the first 4 weeks there was an average of a 12% increase in reepithelialization (area wise) in POG group, as compared to control groups (no oxygen). The same trend was seen during most of the 8-week treatment period, except week 7. A faster wound closure and increased reepithelialization translates into faster tissue healing and a reduced chance of infection, mainly during the early phases of healing. Hence, this novel oxygen generating wound dressing high relevance towards clinical treatment of severe and chronic wounds.

To test the quality of the regenerated tissue, we performed Hematoxylin-Eosin (H&E) and Masson-Trichrome staining of the tissues derived from the center and sides of the healing wounds during each week of the 8 week treatment (study) period. A broad region of hyper-proliferative epithelial layer is a hallmark of the regenerating wound edge.34 As the wound matures, this epidermal region narrows until it is reduced to a very thin layer, typically seen in intact skin. In POG treated groups, we could clearly see a well-defined hyper-proliferative epidermis at the wound edges at week 4 (Figure 6B- Week 4). By week 6, this thick layer starts to compress, showing the formation of epidermal papillae (Figure 6B- Week 6; H&E staining) and becomes thinner by week 8 and assumes the structure that is typical seen in normal pig skin (Figure 6B- Week 8; H&E staining). In contrast, for wounds treated with control dressing (no topical oxygen supplied), the epidermal layer was not hyper-proliferative at week 4 and by week 8, no clearly defined epidermal papillae could be seen, indicating a still unmatured epidermal layer (Figure 6A). Hence, we could observe that wounds treated with topical oxygen were in a more advanced stage of healing.

Collagen deposition is a fundamental step in wound healing that provides the matrix for tissue remodeling and also angiogenesis. We quantitatively measured the total amount of collagen in the different layers of the healing pig skin using an indigenously developed analysis method. The purpose for dividing the skin dermis into three hypothetical regions, as UD, MD, and LD was to assess the amounts of collagen in healing tissues as a factor of depth. It has been previously established that topically applied pure oxygen to open wound sites in pigs could increase the oxygen partial pressure (pO2) from a baseline of 5–7 mmHg to levels > 40 mmHg up to about 2 mm below the surface of the wound,30 which could be a limit of oxygen penetration into a soft, undifferentiated tissue. In our experiments, when the 10 × 10 cm² full-thickness wounds were treated with the oxygen generating (POG) dressings, there was a significant increase in the amount of collagen detected in the UD and LD layers of the healing skin tissue at week 2, as compared to control dressing treated wounds (Figure 7). The UD is about 2 mm thick from the epidermal layer and this observation seems consistent with the studies of Davis et al.30 At week 4, higher collagen amounts were seen prominent only in the LD layer, while at week 8, both the UD and MD layers showed significantly higher amounts of collagen in topical oxygen (POG) treated groups. At week 6, the amounts of collagen were similar in all layers, in both the treatment groups. Two interesting trends were seen in this quantitative collagen analysis, where at week 6, the collagen content of all the regions was the lowest during the entire 8 weeks study period; while for the LD layer, the collagen amounts were highest at weeks 2 and 8, while they seem to be reduced during other times. These observations, as interesting as they seem, needs further validation using other methods. However, our analysis supports outcomes from other
studies that show that during tissue remodeling; the amount of collagen changes substantially and is cross-linked to give the skin its characteristic strength and flexibility, along with other components.

To examine the extent of angiogenesis in the healing skin tissue, the formation of new blood vessels was examined using staining for the vWF and with the CD31 antibody. Treatment with oxygen generating POG dressing clearly show a higher density of new blood vessels (Figure 8B), as compared to control dressing (Figure 8A). This was seen in all the three regions of the dermis, UD, MD and LD. Staining for the vWF factor was more intense in the oxygen treated wounds and was clearly seen in the UD regions of a 2 week old healing tissue (Figure 8B, UD). These observations were also supported by the results from CD31 staining, which showed a higher density of new blood vessels in the topical oxygen treated groups (Figure 9). In tissues of week 2 posttreatment, there were higher numbers of small diameter vessels in the POG group, mainly in the UD and LD regions (Figure 9-B; Week-2: UD & LD). However, by week 8, the number of blood vessels in UD and LD decreased, but LD showed a good amount of large diameter vessels (Figure 9-B; Week-8: LD). In wounds treated with control dressing, the

Figure 6. Morphology of the epidermis in the healing wound tissue. (A) Wounds treated with control dressing (no oxygen). (B) Wounds treated with the oxygen generating (SPO/CPO) dressing. Skin tissues from weeks 2 and 4 were stained using Massons-Trichrome, whereas the skin tissues from weeks 6 and 8 were stained with Hematoxylin-Eosin (H&E). The arrows in (B), week 6 & 8 tissue sections indicate the epidermal papillae, which are an indicator of a well-differentiated and maturing epidermis. The vertical bars are qualitative depiction of the epidermis thickness in each of the image. Scale bar 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
number of new blood vessels was relatively less throughout and during the 8 week study period and the LD had very few large diameter vessels in a tissue that seemed less consistent and differentiated.

**DISCUSSION**

The advancement in the use of TOT as a mainstream treatment option for multiple types of wound will ideally depend on development of devices that are: (1) simple to use; (2) can provide a sustained levels of oxygen to the healing tissue; (3) are cost-effective and available to the widest patient population as a nonprescription treatment; and (4) that does not use gaseous oxygen as a starting source. Our studies describe the development, in vitro testing and in vivo use of novel oxygen generating wound dressing that uses a chemical-based oxygen source; is simple to use; can provide sustained levels of oxygen to the healing tissue for 3–4 days and has a potential to be made in a cost-effective manner. Using the POG dressing, oxygen delivery can be tightly controlled using different amounts of SPO, CPO, polymer components in layer L2 and/or by modifying layer L1 in the dressing. This dressing was tested for efficacy in a porcine full-thickness (10 \times 10 \text{cm}^2) surgical wound model. Wound healing was followed for a total of 8 weeks using quantitative measurement of several wound healing parameters. We have attempted to define a methodology for a structured approach to assessment of wound healing at the investigational and preclinical stage. Results from in vivo studies using the oxygen generating POG dressing in a pigs full-thickness wound healing model have shown better wound closure and faster reepithelialization (average 10–12% better than controls), which becomes significant when the large size of the original wound is considered (10 \times 10 \text{cm}^2). Histologically, topical oxygen treatment also showed the presence of a hyper-proliferative epidermis during week 4 post start of treatment and a compact and highly defined epidermis by week 8, which is similar in structure to that seen in normal pig skin.

Collagen deposition is a fundamental step in wound healing that provides the matrix for angiogenesis and
tissue remodeling. It has long been known that increasing wound oxygenation through externally supplemented oxygen results in increased collagen deposition and tensile strength of the skin tissue.\textsuperscript{8,13,35} In our study, we have shown quantitatively that in case of topical oxygen supplementation, there was a pattern in tissue collagen deposition (synthesis) with respect to depth of wound. The most consistent effect of topical oxygen treatment using the POG dressing was seen in the UD of the healing skin tissue, which is about 2 mm from the top of the wound. Fries et al., have also shown similar results via transcutaneous measurement of oxygen partial pressure (pO\textsubscript{2}) at different depths of the wound tissue.\textsuperscript{29} This not only supports our results, but contrary to their assumption that topically applied pure oxygen can only oxygenate superficial wound tissues (up to 2 mm depth) but not deep tissues,\textsuperscript{30} we show that topically applied oxygen can also influence collagen deposition in much lower regions of the dermis, which in a healing pig skin tissue can be up to 6 mm deep. These secondary effects of oxygen could be through the oxygen tension gradient that is seen in normal wound healing.\textsuperscript{36} Angiogenesis is a critical component (and also a rate-limiting factor) of successful wound healing, where the VEGF is thought to be the most effective, long-term stimulator.\textsuperscript{37–39} In our study, we have shown using vWF and CD31 staining of healing wound tissue that treatment with topical oxygen can induce the formation of new blood vessels (neovascularization).

One major application of our TOT wound dressing will be to treat wounds in the field (including battlefield) and in area where an established medical center or medical professional is not available or accessible. As, the technology in our oxygen generating wound dressing is mainly chemical/material-based these SPO/CPO dressings...
can be manufactured, sterilized and packaged to remain stable for extended periods to time (even months). Unlike TOT device or dressing based on gaseous oxygen, our dressings would be stable and functional (generate and supply topical oxygen to wounds) even in extreme environments.

The goal of this study was to develop an ideal TOT dressing and to pursue a quantitative strategy for assessing wound healing in a large animal model (pig), using an extreme wound type (full-thickness $10 \times 10$ cm$^2$), which was based on morphological measurements, histological criteria, wound depth-specific collagen quantification and immunological markers. The real benefit of our chemical-based topical oxygen wound dressing can be realized by further studies and testing, particularly in clinical settings. This can substantially increase the likelihood of TOT becoming a mainstream wound healing treatment method.

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**Figure 9.** Distribution of blood vessels in healing pig skin tissue as seen from CD31 antibody staining. Representative images from week 2 and week 8 after treatment with control dressing (A) and oxygen generating SPO/CPO dressing (B). Only blood vessels distribution in upper dermis (UD) and lower dermis (LD) shown. In general, there was a higher density of vascularization in SPO/CPO dressing treated groups throughout the healing skin, compared to control dressing. Arrows in A-LD and B-LD indicate large diameter blood vessels. Also to be noted is the better differentiated epidermis in B-UD week 8 and a well-defined consistent tissue in B-LD week 8, as compared to A-UD week 8 and A-LD week 8, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
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Conflict of Interest: The authors do not have any conflict of interest to disclose.

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