Low flow oxygenation of full-excisional skin wounds on diabetic mice improves wound healing by accelerating wound closure and reepithelialization

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ABSTRACT

Oxygen-based therapies have proven effective in treating chronic and difficult-to-heal skin wounds, but the current therapeutic approaches suffer from major limitations and they do not allow for continuous wound treatment. Here we examined whether the continuous treatment of wounds with pure oxygen at low flow rates accelerates wound closure and improves wound healing in a murine model of diabetic skin wounds. Two full-excisional dorsal skin wounds were generated on 15-week-old diabetic db/db mice and treated for 10 weeks continuously with pure oxygen (>99·9%) at low flow rates (3 ml/h). After 6 days, oxygen treatment resulted in a mean reduction of the original wound size by 60·2% as compared with only 45·2% in wounds on control mice that did not receive pure oxygen.($P = 0{\cdot}022$). After 10 days, oxygen-treated wounds were 83·1% closed compared with 71·2% in wounds on control mice. While reepithelialisation was complete after 10 days in over 57% of wounds receiving low flow oxygen treatment, significant epithelial gaps remained in 75% wounds from mice that did not receive oxygen. Continuous low flow oxygenation significantly improves healing of diabetic skin wounds in mice and may therefore be an effective treatment for chronic cutaneous and possibly other slow-healing wounds in diabetic patients.

Key words: Diabetes • Oxygen • Reepithelialisation • Wound closure

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INTRODUCTION

Slow- and non-healing chronic wounds associated with diabetes are a major health problem in the USA. Multiple factors can contribute to impaired wound healing in diabetic patients, including edema, infection and metabolic disorders, but the availability of oxygen appears to be a key rate-limiting step in wound healing (1–3).

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Key Points

- in this study,we explored the hypothesis that providing diabetic skin wounds only locally but continuously with a saturated oxygen environment at ambient pressure and at low O_2 flow rates improves wound healing
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emerged as an effective adjunct therapy for many types of wounds (4). However, costs associated with this treatment and limited access to high-pressure chambers have prevented its routine use, highlighting the need for more user-friendly and cost-effective therapies.

Physiological oxygen delivery to wounds is dependent on multiple factors including blood perfusion of the tissue, capillary density, arterial partial oxygen pressure (pO_2), oxyhaemoglobin dissociation conditions and local oxygen consumption (5). Oxygen diffuses into the tissue and the concentration decrease is inversely proportional to the square of the diffusion distance, resulting in pO_2 as low as 0–5 mmHg in devascularised central regions of the wound. However, a minimal pO_2 is required for normal cell functions. For example, normal cell division requires pO_2 of approximately 30 mmHg, at pO_2 of 25 mmHg the O_2-dependent hydroxylation of proline and lysine required for collagen synthesis is impaired, and if pO_2 drop below 20 mmHg cells switch to anaerobic metabolism, resulting in lactate production and reduced pH, which inhibit wound healing (1,4,6). It is therefore not surprising that increasing oxygen delivery to wounds, for example by HBO_2-T or topical (high flow) O_2 therapy (TOT), has been reported to improve wound healing. Pure oxygen can directly impact wound healing because of its antimicrobial properties, but its major beneficial properties appear to be because of its ability to affect multiple molecular targets and cell types, resulting in an overall improvement (or restoration) of cellular functions, which together result in accelerated tissue repair. Oxygen has been reported to increase fibroblast migration and replication (7), increase the rate of collagen production and tensile strength of collagen fibres (6,8), stimulate angiogenesis (7), promote macrophage chemotaxis (9), enhances the antibacterial activities of leukocytes, including phagocytic function (10), thereby increasing the removal of cell debris and promoting physiological wound debridement. Thus, an extensive body of experimental data suggests that improving the supply of oxygen throughout the repair process should restore and improve overall cell functions in diabetic wounds, resulting in improved tissue regeneration and accelerated wound closure. A reduction in wound healing time would be of clinical significance, potentially reducing cost and improving outcomes. In this study, we explored the hypothesis that providing diabetic skin wounds only locally but continuously with a saturated oxygen environment at ambient pressure and at low O_2 flow rates improves wound healing. Here we report that local low flow O_2 treatment dramatically accelerated reepithelialisation and wound closure of full-excisional skins wounds in diabetic mice.

MATERIALS AND METHODS

Animals

Twenty female db/db mice (stock # 000642, BKS.Cg-m+/+ Lepr db) on a C57BL/KS background were obtained from The Jackson Laboratories (Bar Harbor, ME).

All studies were carried out in compliance with and approval of the UTHSCSA Institutional Animal Care and Use Committee. Mice were maintained on regular chow diet and housed in the Central Animal Facility at UTHSCSA. After wounding, mice were housed individually in a special paper-based bedding material (ALPHA-dri™, SSP, Kalamazoo, MI), to prevent contamination of wounds, which occurs frequently with commonly used bedding materials. To reduce the risk of infections, mice were maintained on drinking water containing antibiotics (160 μg/ml sulfamethoxazole and 32 μg/ml trimetoprim) for 1 week prior to wounding and for the remainder of the study.

Wounding

Mice (15 weeks of age) were randomised into two groups of 10 mice and anaesthetised with an intraperitoneal injection comprised of a combination of ketamine (95 mg/kg body weight) and xylazine (5 mg/kg body weight). After shaving, the back of the mice was cleaned with betadine. Excisional full-thickness skin wounds were made on the dorsal skin by picking up a vertical fold of skin between the shoulders and punching through the epidermis and dermis with a sterile disposable biopsy punch with an inner diameter of 4 mm. This generated two wounds simultaneously along the midline, with the wound closer to the head referred to as 'TOP' and the lower wound as 'BOTTOM'.

Immediately after wounding, and also after 6 and 10 days, each wound was digitally photographed and wound areas were quantified

with ImagePro Software (MediaCybernetics, Bethesda, MD). Wound dressings and oxygenation tubing were replaced immediately after images were taken. After the mice were terminated, wound areas surrounded by a margin of healthy tissue were excised with an 8-mm sterile disposable biopsy punch and prepared for histological analysis. A total of six mice died within the first 4 days, three in each group. The likely cause of death was determined to be heart failure.

The mice are severely obese with average weights of $55 \pm 0{\cdot}6$ and hyperglycaemic with average blood glucose levels of 315 ± 20 mg/dl. Pilot studies indicated that once these mice were wounded they became fragile. In these pilot studies, repeated wound analysis and the associated dressing changes resulted in mortality rates of over 75% by day 10, post-wounding. Therefore, only two time points were chosen for this study: day 6 post-wounding, with an expected mean wound closure of 50% in the absence of O_2 treatment, and day 10 post-wounding, the study termination. Our pilot studies indicated that at day 10 we could expect approximately 75% wound closure in the absence of O_2 treatment. Limiting wound analysis to two time points reduced the overall mortality in both groups to less than 30%.

Wound treatment with pure oxygen

All mice received a sterilised, disposable oxygenation cannula (0·76 mm OD; 0·25 mm ID, Disetronic, Portsmouth, NH) with a standard luer fitting. For the treatment group, the cannula was attached to the oxygen delivery device (TransCu-O_2 Tissue Oxygenation System, EO_2 Concepts, San Antonio, TX; patent pending US 61/000695). In the control group, the cannula was attached to a flexible wire above the cage. The distal adapter of the cannula was removed, and the opening positioned so that the tube could supply both wounds directly with oxygen. The tube ending was placed between two layers of a sterile moisture-absorbent gauze sponge ($2'' \times 2''$, Kendall Curity, Mansfield, MA), which was then placed on the wounds; the tube opening pointed towards the head and rested between the two wounds at the lower rim of the upper wound ('TOP'). The entire dressing was covered and sealed with a secondary thin-film occlusive dressing (Opsite Flexifix; 2″, Smith&Nephew, Largo, FL) to maintain high oxygen concentrations over the wound sites throughout the entire treatment period. To ensure complete sealing by the thin-film dressing, the skin around the wounds was pre-treated with Skin Prep (Smith&Nephew, Largo, FL). Pure oxygen (>99·9% O_2 as rated by the manufacturer) was generated with the TransCu-O_2 device by a battery-driven electrochemical reaction and delivered to the wounds at 3 ml/h. Flow rates were verified during the course of the experiment with a rotameter.

Histological analysis

The circular tissue biopsies (8 mm diameters) were cut into two equal halves, fixed in 10% buffered formalin, processed and embedded in paraffin blocks using standard protocols. Tissue sections (4 μm) were cut along the cross-section spanning the entire biopsy diameter and were stained with haematoxylin and eosin (H&E) or Masson-Trichrome.

Blood glucose analysis

Mice were fasted for 8 h prior to glucose measurements using a Contour®meter (Bayer).

Statistics

All data are presented as mean ± SE. Student's t test was used to compare control and treatment groups (SPSS 16·0). Results were considered statistically significant at the $P < 0{\cdot}05$ level.

RESULTS

Low flow oxygen treatment accelerates wound closure in diabetic mice

The sizes of the fresh wounds varied considerably between each individual animal, ranging from 11·3 to 20·2 mm^2. The wounds closer to the head (TOP) were significantly smaller, $13{\cdot}1 \pm 0{\cdot}3$ mm^2, as compared with the lower wounds (BOTTOM), $16{\cdot}0 \pm 0{\cdot}4$, even though both sets of wounds were generated simultaneously. However, we observed no statistically significant differences in the size of initial wounds between the control group (TOP: $13{\cdot}2 \pm 0{\cdot}5$ mm^2, BOTTOM: $15{\cdot}8 \pm 0{\cdot}6$ mm^2, $n = 10$) and the mice randomised to the O_2-treatment group (TOP: $13{\cdot}1 \pm 0{\cdot}3$ mm^2, BOTTOM: $16{\cdot}3 \pm 0{\cdot}5$ mm^2, $n = 10$).

After 6 days, diabetic mice in the O_2-treatment group showed significantly smaller

Key Points

- in this study, we examined the effect of sustained low flow oxygenation on wound healing of full-excisional skin wounds in diabetic mice
- continuous, low flow O_2 treatment accelerated both wound closure as well as reepithelialisation of these slow-healing wounds
- furthermore, histological evidence indicates improved collagen organisation in O_2-treated wounds
- overall, our findings are in line with a significant body of experimental data suggesting that increasing oxygen supply to diabetic wounds improves wound healing, both in animal models and patients
- our data suggest that neither high-pressure nor high flow rates are required for O_2 to improve wound healing
- in our study, O_2 supply was improved only locally by maintaining a constant delivery of pure O_2 to the wounds and thus increasing the pO_2 directly over the site of injury

wounds as compared with the control group (Figure 1A). Morphometric analysis of the wounds showed that wound closure for both TOP and BOTTOM wounds was significantly faster in the O_2-treated mice (Figure 1B). TOP wounds were 18% smaller and BOTTOM wounds were 34% smaller in O_2 treated than in control animals. However, within each group, the difference in wound closure rates between TOP and BOTTOM wounds did not reach statistical significance. After 6 days, the mean reduction in wound size for both TOP and BOTTOM wounds relative to the initial wound size was 60·2% in O_2-treated mice but only 45·2% in control mice (Figure 1B, BOTH). This difference corresponds to a 33% acceleration of wound closure induced by the O_2 treatment.

By day 10 post-wounding, both TOP and BOTTOM wounds in the O_2 treatment group had closed by over 82% of their initial size, whereas wound closure had only reached 73% for TOP and 70% for BOTTOM wounds in the control group. Again, within each group, we observed no statistically significant difference in wound closure rates between TOP and BOTTOM wounds.

Low flow oxygen treatment accelerates reepithelialisation of cutaneous wounds in diabetic mice

To investigate the possible mechanisms underlying the accelerated wound closure rates induced by the low flow O_2 treatment, we examined the reepithelialisation of both TOP and BOTTOM wounds in H&E cross-sections from the biopsied wound tissue. Because the wound samples were dissected through the middle of the wound, the cross-sections represented the full width of each wound (Figure 2A). In the control group, the TOP wounds of only three mice were completely reepithelialised and the BOTTOM wounds in all the mice showed epithelial gaps. In contrast, O_2 treatment resulted in the complete reepithelialisation of both TOP and BOTTOM wounds in three mice, two mice showed complete reepithelialisation of TOP wounds, with small epithelial gaps remaining in their BOTTOM wounds, and only two mice showed epithelial gaps in both their TOP and BOTTOM wounds. Thus, after 10 days O_2 treatment resulted in complete reepithelialisation of over 57% of wounds, whereas without O_2 treatment only 25% of wounds showed continuous epithelial layers over the site of injury. The mean epithelial gap was 59% smaller in TOP wounds and 69% smaller in the BOTTOM wounds of O_2-treated mice as compared with the respective wounds on control mice (Figure 2B). These results suggest that low flow O_2 treatment may increase epithelial cell proliferation.

Increase deposition of collagen at wound base after continuous low flow oxygen treatment

To determine if low flow O_2 treatment affects matrix formation and deposition in wounds, we analysed the sections adjacent to the H&E-stained sections for collagen depositions. We found dense collagen deposits at the base of most O_2-treated wounds but to a much lesser extent in control wounds (Figure 2C). Furthermore, the collagen deposits in O_2-treated wounds had a more organised, fibrous appearance (Figure 2D), suggesting that wound remodelling was significantly more advanced in the O_2 treatment group.

DISCUSSION

In this study, we examined the effect of sustained low flow oxygenation on wound healing of full-excisional skin wounds in diabetic mice. Continuous, low flow O_2 treatment accelerated both wound closure as well as reepithelialisation of these slow-healing wounds. Furthermore, histological evidence indicates improved collagen organisation in O_2-treated wounds. Overall, our findings are in line with a significant body of experimental data suggesting that increasing oxygen supply to diabetic wounds improves wound healing, both in animal models and patients. Previous approaches to increase wound pO_2 levels used either very high systemic O_2 pressure or high O_2 flow rates applied to entire extremities (4). Our data suggest that neither high-pressure nor high flow rates are required for O_2 to improve wound healing. In our study, O_2 supply was improved only locally by maintaining a constant delivery of pure O_2 to the wounds and thus increasing the pO_2 directly over the site of injury. We don't know how large the actual increase in pO_2 was that resulted in the very significant acceleration of wound healing we observed. However, measurements by other investigators of pO_2 in both intact and wound tissues suggest that even marginal increases in tissue pO_2 in wounds

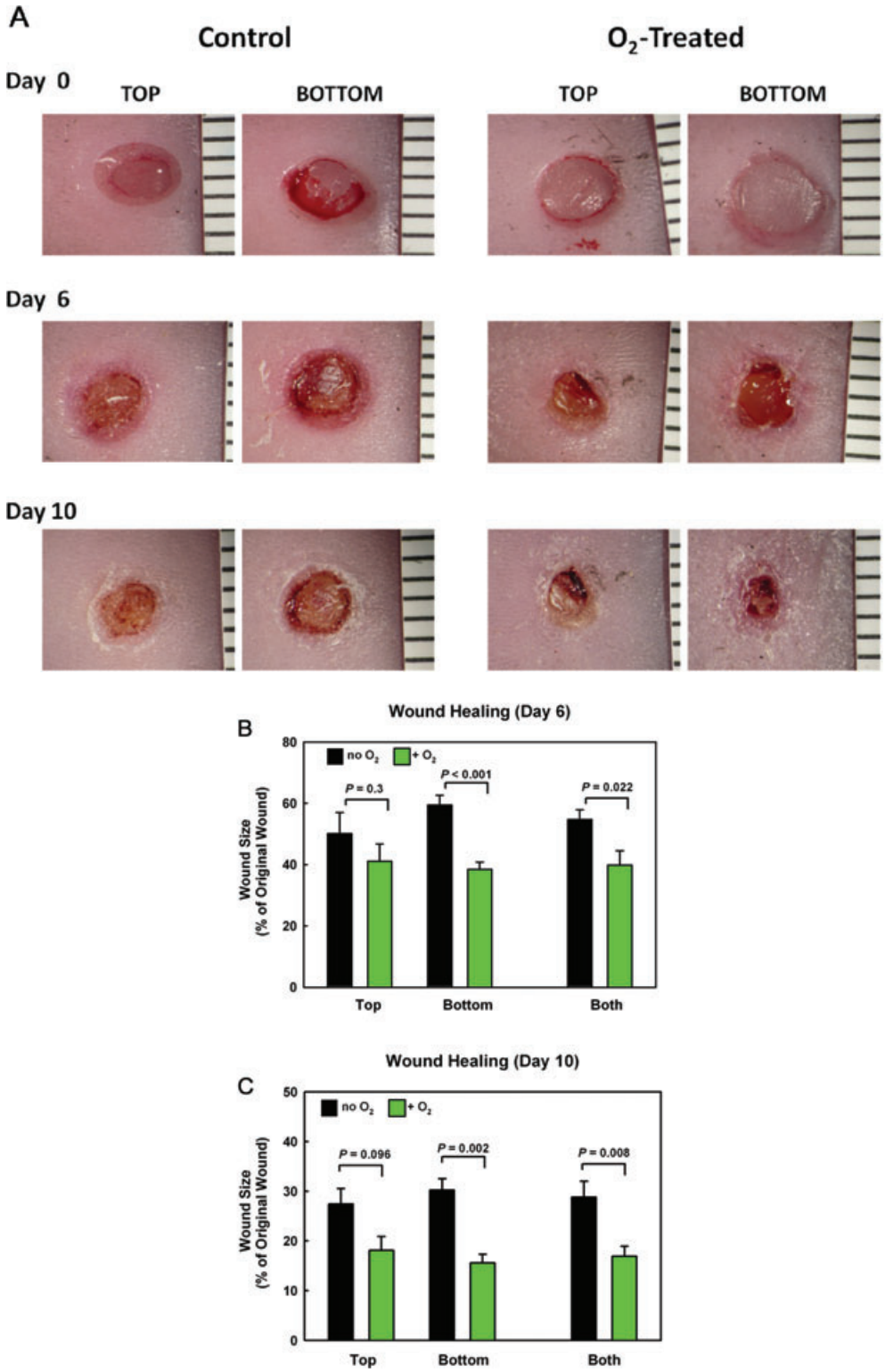

Figure 1. Accelerated closure of diabetic skin wounds treated with pure oxygen. (A) Images of wounds taken immediately after wounding (day 0) and after 6 and 10 days post-wounding from a representative untreated mouse (Control) and a mouse that received pure oxygen treatment (O_2-Treated). Both the upper (TOP) and lower (BOTTOM) wounds of the same mouse are shown. The smallest unit on the scales shown is 1 mm. (B) Wound areas were determined by morphometry using digital images on day 6 and (C) day 10 post-wounding. Results for the upper (TOP), lower (BOTTOM) wounds as well as the average for both wounds (both) of the same mouse are shown ($n = 7$).

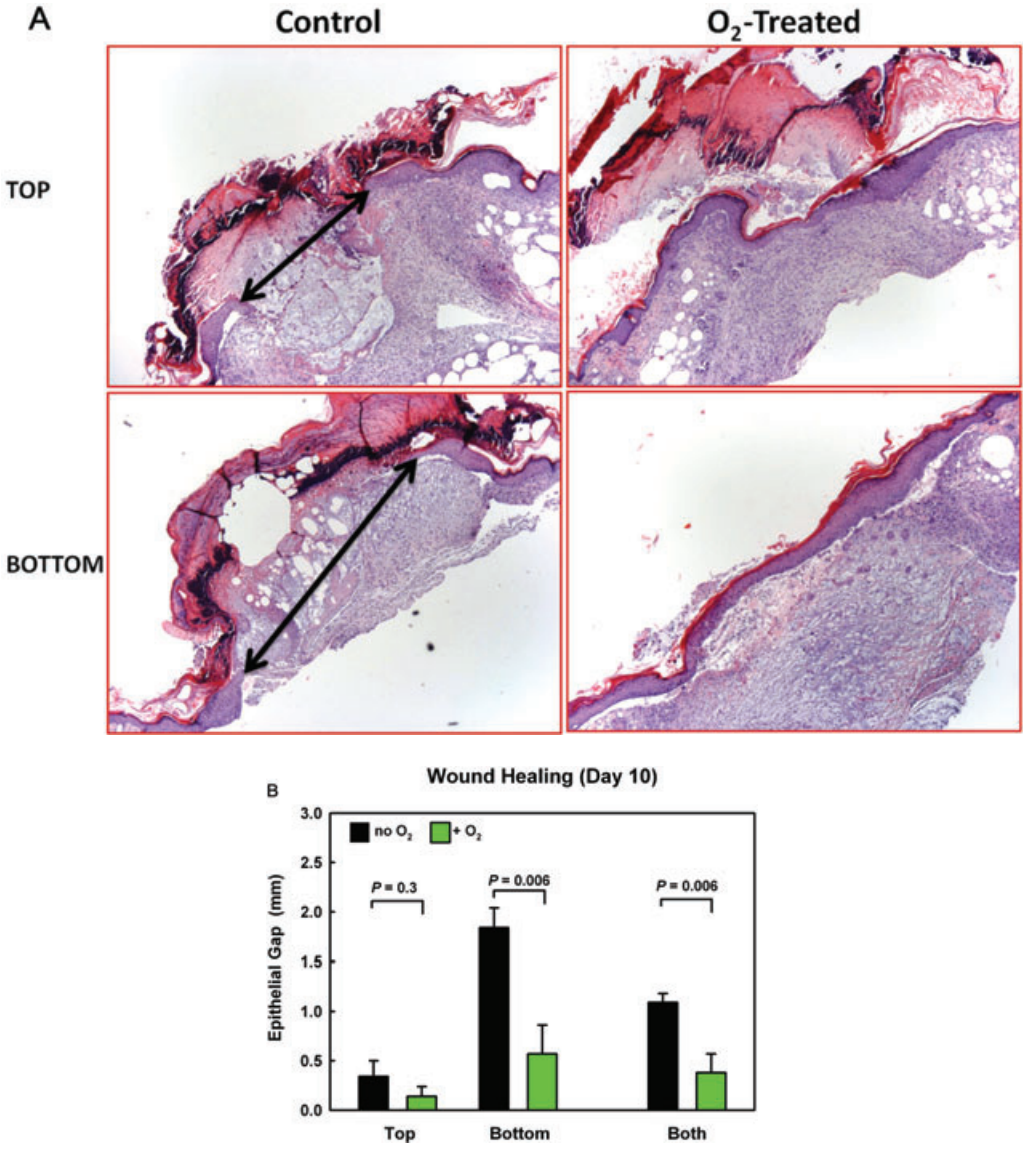

Figure 2. Accelerated reepithelialisation and healing of diabetic skin wounds treated with pure oxygen. (A) Representative images of sections of wounds stained with haematoxylin and eosin (H&E; magnification: 40×) from untreated (Control) and O_2-treated diabetic mice (O_2-Treated) 10 days post-wounding. Both the upper (TOP) and lower (BOTTOM) wounds of the same mouse are shown. The double-sided arrow indicates the epithelial gap. (B) Morphometric analysis of the epithelial gap was performed on H&E sections (magnification: 40×) from untreated (■) and O_2-treated diabetic mice (■). Results for the upper (TOP), lower (BOTTOM) wounds as well as the average for both wounds (both) of the same mouse are shown ($n = 7$). (C) Collagen formation was assessed in adjacent sections stained with Mason-Trichrome (magnification: 40×). Arrows indicate sites of dense collagen depositions at the base of the treated wounds. (D) Areas containing collagen depositions were imaged under higher magnification (magnification: 400×).

Key Points

- normalising wound pO_2 to 45–65 mmHg should be sufficient to significantly improve or even normalise wound healing, if insufficient O_2 supply, for example because of impaired blood flow, is the only underlying cause of poor wound healing

are likely to show significant benefits on the healing response. Mean subcutaneous pO_2 levels in normal skin at 3–4 mm depth are in the range of 45–53 mmHg (11). pO_2 levels less than 25–45 mmHg are associated with poor wound healing (12). If pO_2 levels drop to 5–25 mmHg, typical for most chronic wounds, cells switch to anaerobic metabolism, lactate production is increased, and the resulting decrease in tissue pH further inhibits wound healing (4). One would, therefore, expect that normalising wound pO_2 to 45–65 mmHg should be sufficient to significantly improve or even normalise wound healing, if insufficient O_2 supply, for example because of impaired blood flow, is the only underlying cause of poor wound healing.

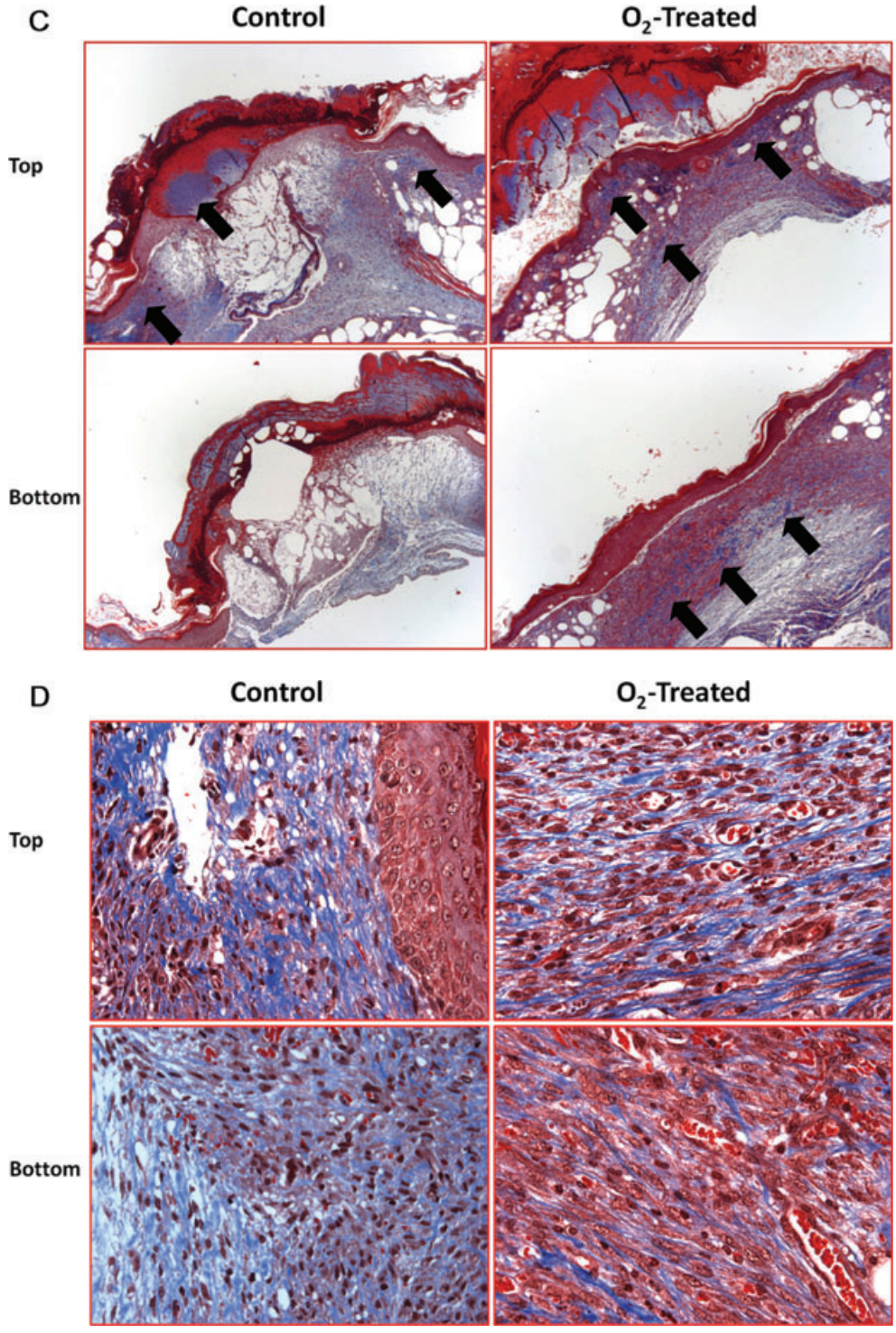

Figure 2. (*Continued*)

This raises the question whether increasing wound pO_2 levels beyond 45–65 mmHg would have any significant additional benefit for cell function and tissue repair. Fibroblast proliferation and protein production have been reported to be optimal at 160 mmHg, that is at pO_2 levels two- to threefold higher than those found in healthy tissues (13). The optimum pO_2 for angiogenesis, effective leukocyte killing and fibroblast and endothelial cell replacement is

Key Points

- because beneficial effects of oxygen on cell function have now been reported in a large number of different cell types, we predict that the improvement and acceleration of wound healing we observed in our study cannot be explained by a single mechanism in a single cell type, but rather is the combined effect of improved O_2 supply to many if not all cell types involved, affecting multiple cellular processes at different stages of the wound healing process
- our data would also suggest that maintaining elevated pO_2 levels close to the pO_2 required for optimal cell function over the entire healing period may prove more effective than intermittently inducing supraphysiological pO_2 levels in wounds
- in summary, our study shows that oxygenation of diabetic skin wounds directly at and limited to the wound site with pure oxygen at low flow rates significantly accelerates wound closure and re-epithelialisation, resulting in overall improved wound healing
- continuous low flow oxygenation may offer a new treatment option for patients with chronic and difficult to-heal wounds

also estimated at 50–100 mmHg, that is above the pO_2 of normal tissue exposed to room air (14). Assuming that throughout our experiment the thin-film occlusive dressing allowed the atmosphere above the wound to saturated with pure oxygen, i.e. $pO_2 = 760$ mmHg or fivefold above normoxic levels, and further assuming a similar O_2 gradient into the tissue was maintained as under normoxic conditions, then one would expect tissue pO_2 also to be elevated to approximately a fivefold higher steady state pO_2 (i.e. 225–325 mmHg). Thus, in principal, low flow oxygenation should be able to achieve pO_2 levels in skin wounds that at least for fibroblasts are in the range for optimal cell function with respect to wound repair. Wound surface and subcutaneous pO_2 measurements will be part of future studies of this device.

The ability to maintain elevated pO_2 levels throughout all phases of the healing process may also have contributed to the significant acceleration in wound healing we observed in our model. It is likely that during low flow oxygenation physiologically relevant pO_2 gradients within the injured tissues are maintained albeit at an overall higher pO_2 level. These pO_2 gradients are important to initiate and promote a number of physiologically processes critical for wound healing, including the recruitment of macrophages which are required for early inflammatory responses, wound debridement, and to promote angiogenesis. Because beneficial effects of oxygen on cell function have now been reported in a large number of different cell types, we predict that the improvement and acceleration of wound healing we observed in our study cannot be explained by a single mechanism in a single cell type, but rather is the combined effect of improved O_2 supply to many if not all cell types involved, affecting multiple cellular processes at different stages of the wound healing process. Our data would also suggest that maintaining elevated pO_2 levels close to the pO_2 required for optimal cell function over the entire healing period may prove more effective than intermittently inducing supraphysiological pO_2 levels in wounds.

Although it remains to be shown that continuous low flow O_2 treatment is effective in patients, the local treatment of difficult-to-heal wounds using this therapeutic approach would have several major advantages over the two O_2-based therapies currently used in clinical practice, HBO_2-T and TOT (4). HBO_2-T requires patient to be placed in specialised chambers in pure O_2 atmospheres at high pressures. While at least two clinical trials support the effectiveness of HBO_2-T, the required presence of trained physicians and specialists make this an expensive therapy that requires repeated visits to the clinic. Furthermore, HBO_2-T is associated with frequent adverse effects, including reversible myopia, rupture of the middle ear or cranial sinuses, tracheobronchial symptoms and claustrophobia (4).

TOT, on the other hand, is less expensive, allows for treatment at home, and does not suffer from any risk of systemic O_2 toxicity. To date, only few complications have been reported and the rates of complications associated with TOT were low (15). However, the boot, bag or chamber that usually covers the affected extremity dramatically limits the patient's mobility. Furthermore, high O_2 flow rates (10 l/min) are required to maintain O_2 saturation above the wound site, necessitating oxygen tanks to supply the oxygen for the treatment, further limiting the patient's mobility and making TOT unsuitable for continuous O_2 treatment. The TransCu-O_2 device used in our study offers all the advantages of TOT, with the added benefits of continuous wound treatment and maintained patient mobility, but without the need for oxygen tanks or large wound covers.

In summary, our study shows that oxygenation of diabetic skin wounds directly at and limited to the wound site with pure oxygen at low flow rates significantly accelerates wound closure and reepithialisation, resulting in overall improved wound healing. Continuous low flow oxygenation may offer a new treatment option for patients with chronic and difficult-to-heal wounds.

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