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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Inhibition of E6 and E7 expressions by inducible shRNA technique alters p53 and pRb protein levels.

Figure S2. Characterization of the effects of viral E6 and E7 gene inhibition on cell proliferation.

Data S1. Materials and methods.

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Letter to the Editor

Effects of a topical aqueous oxygen emulsion on collagen deposition and angiogenesis in a porcine deep partial-thickness wound model

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Abstract: A porcine deep partial-thickness wound model was used to evaluate the effects of a newly developed topical aqueous oxygen emulsion (TOE) on wound repair. The wounds were treated with TOE, which contains super-saturated oxygen or vehicle control. Semiquantitative immunofluorescent staining was performed to examine protein production for type I and type III collagen and vascular endothelial growth factor (VEGF). Immunofluorescent staining revealed higher protein levels of type I and type III collagen and VEGF in the TOE treatment group. Histological analysis also revealed improved angiogenesis and granulation tissue formation with topical TOE treatment and was consistent with the protein expression. In addition, the histology examination demonstrated faster epithelialization in wounds treated with TOE. The study suggests that sustained high levels of oxygen released by TOE may promote the process of wound repair through increasing collagen deposition and angiogenesis as well as stimulating epithelialization.

Key words: collagen – granulation tissue formation – partial-thickness wound – topic aqueous oxygen emulsion – VEGF

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Background

Oxygen plays a crucial role in wound healing (1). Raising oxygen tension (pO_2) has shown to increase the rate of keratinocyte migration, collagen deposition and tensile strength (2,3). Systemic hyperbaric oxygen therapy (HBOT) and topical oxygen therapy (TOT) have been used to deliver oxygen to wounds (4–6). HBOT and TOT both have shown promising effects on wound revascularization and epithelialization. However, HBOT has higher costs associated and may have more side effects including ear discomfort and CNS toxicity (7). TOT is less expensive and can be performed at home (8). However, its ability to penetrate the skin is limited (9,10).

Recently developed topical aqueous oxygen emulsions (TOE) can overcome these limitations (9–11). TOE contains super-saturated oxygen which can be delivered topically. The formulation is based on perfluorocarbon droplets being encapsulated within an aqueous continuous phase allowing slow release of oxygen over time. The oxygen solubility of the perfluorocarbon is relatively high, approximately twenty times greater than water; therefore, it has a high oxygen-carrying capacity (9,11). We have previously reported that TOE increased the healing rate in partial-thickness wounds in a porcine study *in vivo* (9). However, the mechanisms involved and molecular effects of the TOE on wound healing are still unknown.

Questions addressed

This study was to examine the molecular effects of the TOE in wound epithelialization, angiogenesis and granulation tissue formation.

Experimental design

Effects of TOE on wound healing were studied using a porcine partial-thickness excisional wound model. The expressions of types I and III collagen and VEFG were analysed by immunofluorescent staining. Histological analyses were conducted in parallel to examine the wound epithelialization, angiogenesis and granulation tissue formation. Please see supporting information for methods (Data S1).

Results

TOE stimulated wound epithelialization, angiogenesis and granulation tissue formation

There was a significant statistical difference between the TOE treatment and vehicle control groups ($P < 0.01$). Histological examination revealed a significant enhancement of epithelialization with TOE treatment. Epithelialization occurred earlier and faster in TOE-treated wounds compared with vehicle alone in percentage of epithelialization (Fig. 1a,b). It showed a significant increase as early as day 4 in TOE-treated wounds ($P < 0.01$).

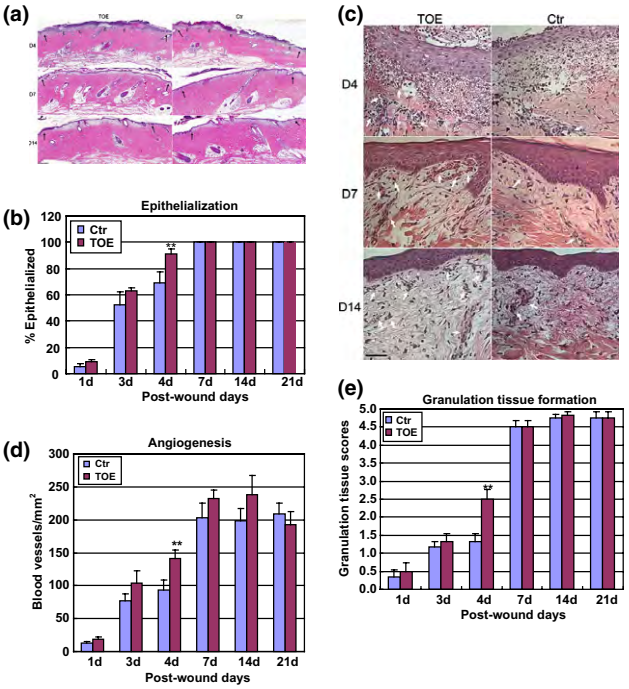

Figure 1. Wound histology analysis. (a) Representative whole wound scan pictures of H&E staining sections. TOE treatment (TOE), vehicle alone (Ctr), at day 4 (D4), day 7 (D7) and day 14 (D14), at 50× magnification. Figure shows wounds have 80% or 70% re-epithelialized with TOE treatment or vehicle alone at day 4, respectively. At day 7 and day 14, all wounds were 100% re-epithelialized. E: denotes the newly formed epidermis from edges of the wound or from the remaining hair follicles in the wound. Black errors indicate the wound margins. Scale bar = 100 μm. (b) Graphical analysis of percentage of epithelialized. (c) Representative H&E histological pictures under light microscope showing wound angiogenesis and granulation tissue formation. TOE treatment (TOE), vehicle alone (Ctr), at day 4 (D4), day 7 (D7) and day 14 (D14), at 200× magnification. White errors indicate the new capillary blood vessels. The images are representative for their corresponding treatment groups. Scale bar = 50 μm. (d) Graphical analysis of angiogenesis, number of capillary blood vessels/mm^2. (e) Graphical analysis of granulation tissue formation, scores 0–5. (b, d, e) Data are expressed as mean ± SE of values from six pigs, one sample from each pig ($n = 6$). Ctr: vehicle alone control; TOE: topical aqueous oxygen emulsion which contains super-saturated oxygen. 1d to 21d denote day 1 to day 21 postwounding. Each bar expressed as mean value ± standard error (SE). *, ** and *** denote statistically significant changes with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

TOE-treated wounds exhibited earlier and better signs of angiogenesis compared with wounds of vehicle alone control group. At day 4, it demonstrated significant increase in angiogenesis with TOE treatment compared with vehicle alone ($P = 0.01$; Fig. 1c,d). Moreover, wounds treated with TOE demonstrated earlier and better granulation tissue formation with marked increase at day 4 compared with vehicle group ($P = 0.0024$; Fig. 1c,e). There was a good correlation between angiogenesis and granulation tissue formation (Fig. 1c–e).

TOE stimulated the production of types I and III collagen and VEGF

To further study the role of TOE in wound angiogenesis and granulation tissue formation, we analysed the expressions of types I and III collagen and VEGF using immunofluorescence analysis. Our results demonstrated that wounds treated with TOE had significant stronger staining for all three types of protein examined compared with the vehicle control group.

The expression of type I collagen peaked during day 7 through day 21. TOE-treated wounds had markedly higher expression of type I collagen than those controls with vehicle alone at day

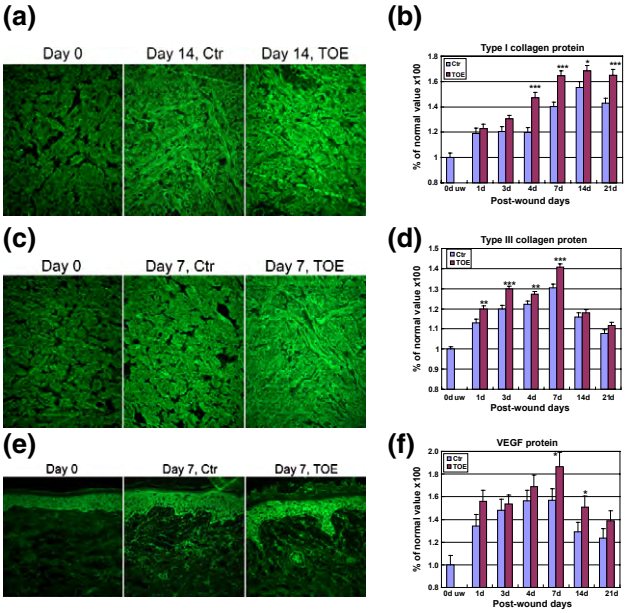

Figure 2. TOE stimulated expressions of type I collagen, type III collagen and VEGF in porcine partial-thickness wounds. (a, c, e) Immunofluorescent staining for type I collagen, type III collagen and VEGF, respectively. Ctr: vehicle only control; TOE: topical aqueous oxygen emulsion which contains super-saturated oxygen. Magnification 200×. (b, d, f) Graphic analyses of relative protein expression compared with the normal unwound control (the percentage of control value) for type I collagen, type III collagen and VEGF, respectively. The intensities of staining were measured by optical density as green value per pixel. The mean value for unwound normal skin was set at 100% (or 1). Each bar expressed as mean value ± SE. *, ** and *** denote statistically significant changes with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. (a, b) Immunofluorescent staining for type I collagen. Figure shows the staining of type I collagen fibres in the dermis on day 0 normal unwound skin and on day 14 two treatment groups, with the strongest staining in TOE-treated wound. (c, d) Immunofluorescent staining for type III collagen. Figure shows the staining of collagen III in the dermis on day 0 normal unwound skin and on day 7 two treatment groups, with the strongest staining in TOE-treated wound. (e, f) Immunofluorescent staining for VEGF. Figure shows the staining of VEGF in both the epidermis and the dermis on day 0 normal unwound skin and on day 7 two treatment groups, with the strongest staining in TOE-treated wound.

4 ($P < 0.001$), day 7 ($P < 0.001$), day 14 ($P < 0.05$) and day 21 ($P < 0.001$; Fig. 2a,b). Expression of type III collagen reached its peak at day 7 then gradually decreased to a level close to the normal at day 21. Wounds treated with TOE had significantly higher expression of type III collagen than those of vehicle controls at days 1 ($P < 0.01$), 3 ($P < 0.001$), 4 ($P < 0.01$), and 7 ($P < 0.001$; Fig. 2c,d).

Increased expression of VEGF was observed in both the dermis and epidermis throughout the entire 21 days of experiment with peak at day 7. Wounds treated with TOE had significantly higher expression than those of the vehicle control group at days 7 and 14 (both $P < 0.05$; Fig. 2e,f).

Conclusions

Each step of the wound healing requires proper levels of oxygen to achieve optimal results (12). Our data have demonstrated that TOE has a beneficial impact on the proliferative phase of the wound healing. The histological evaluation revealed that wounds treated with TOE improved angiogenesis (Fig. 1c,d) and granulation tissue formation (Fig. 1c,e), which are characteristics of dermal repair. Consistently, our immunofluorescent staining showed increased deposition of types I and III collagen (Fig. 2a–d) and higher level of VEGF production (Fig. 2e,f) in TOE group compared with controls. The data suggests that TOE improves wound angiogenesis and granulation tissue formation via stimulating VEGF expression and types I and III collagen production. The sequence of the protein expressions is in consistence with that of normal wound repair occurring in a timely fashion (13,14), indicating that TOE accelerates but does not alter the orderly healing process.

Oxygen has been shown to stimulate angiogenesis and enhance the ability of fibroblasts to produce collagen (15–17). The expression pattern of types I and III collagen detected in this study is similar to that seen in normal wound healing. The total amount of collagen increased early, reaching a maximum between 2 and 3 weeks after injury. Our results are also consistent with previous reports that increased oxygen resulted in higher level of VEGF production in wounds (17–22).

In addition, the histological examination revealed faster epithelialization. The data are consistent with our previous report of porcine *in vivo* study with salt-split technique (9). The stimulatory effects could be the direct effect of TOE and its subsequently generated reactive oxygen species (ROS) on keratinocyte migration and proliferation (2,23–26). It is also possible secondary to the improved angiogenesis or granulation tissue formation. In conclusion, our study suggests unique therapeutic value of TOE in wounds especially for poorly perfused chronic wounds that often lack an adequate oxygen supply (27).

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Authors' contributions

Experiment performance carried out by LZ, KR, study design carried out by JL, SCD, PMM, data analysis performed by LT, JOS, BES, WLM, manuscript by JOS, LT and editing was performed by JL, SCD, PMM, KR, BES.

Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplemental methods.