An oxygen-releasing agent promotes healing of skin wounds in rats

Objective: To evaluate the local effect of a slow oxygen-release gel on the healing of standardised skin wounds caused in rats.
Method: Skin wounds were created on the backs of male rats (*Rattus norvegicus*, Wistar) that were randomly allocated into two groups. In the treated (T) and control (C) groups, oxygen gel and distilled water, respectively, were applied to the wounds on alternate days for 28 days. Postoperatively, euthanasia was performed at 5, 10, 14, 21 and 28 days, followed by clinical, histological (Masson's trichrome) and immunohistochemical analysis. Data were subjected to analysis of variance (ANOVA) and Bonferroni's test.
Results: The cohort comprised 50 rats. On clinical and histological analysis, groups C and T showed similar characteristics 5 days post-operation. Subsequently, group T showed better healing at 14, 21 and 28 days and presented more intense inflammatory infiltrate up to 10 days. At days 14, 21 and 28, group T exhibited a reduction in oedema and increased angiogenesis, granulation tissue formation, and deposition of collagen fibres than group C. Immunohistochemical analysis showed the presence of tumour necrosis factor (TNF)-α and vascular endothelial growth factor (VEGF) in both the groups, but the levels were significantly higher in group T ($p<0.05$).
Conclusion: The local application of slow oxygen-release gel accelerated the healing of standardised skin wounds created surgically in rats, with increased angiogenesis and better collagen fibre formation.
Declaration of interest: The authors have no conflicts of interest to declare.

healing • hypoxia • inflammation • oxygen • wound • wound care • wound dressing • wound healing

The cutaneous wound healing process involves complex phases, such as cell proliferation, bacterial defence, angiogenesis and collagen synthesis, which require increased cellular metabolic activity, resulting in high oxygen demand.[1,2]

During the initial stage of healing, platelets and leukocytes release cytokines, such as tumour necrosis factor-alpha (TNFα), which activate the inflammatory response, and growth factors, such as vascular endothelial growth factor (VEGF), which stimulate angiogenesis.[3,4] This entire process significantly reduces oxygen levels in the region of the lesion, causing hypoxia. Although hypoxia is fundamental during the initial stage of healing and stimulation of angiogenesis, oxygen enables the maintenance of the process.

For this reason, it is necessary to re-establish oxygenation of the microenvironment, both in the peripheral and central region of the wound, so that the subsequent steps are not impaired.[5–8]

Studies[9–14] have demonstrated that oxygen supplementation during healing aids in the oxidative killing of bacteria, stimulation of angiogenesis, acceleration of extracellular matrix (ECM) formation, increased proliferation of fibroblasts, and collagen deposition, thereby enabling faster healing.

The literature mentions different treatments, such as oxygen therapy, which accelerate the healing process. Oxygen therapy can be systemic,[2] as in the topical hyperbaric chamber technique,[15] in which oxygen is delivered purely to the wound site and can be carried out with the aid of an air 'bag',[16] using devices[17] or dressings.[12]

Another such treatment is ozone therapy that helps in antimicrobial action, formation of reactive oxygen species, stimulation of growth factors important in healing, increased collagen deposition, and acceleration of the closure of hard-to-heal wounds.[18,19] Similarly, studies on low-power laser have demonstrated that laser therapy accelerates wound healing by stimulating angiogenesis and optimising cell activity and proliferation.[20–22]

Platelet-rich fibrin (PRF) has also been used to optimise healing. It acts by releasing growth factors that stimulate angiogenesis and activate cellular activity.[23,24]

Some studies[25,26] have shown that enamel matrix derivative (EMD) protein assisted in healing by binding to the ECM proteins of the wound, thereby regulating the adhesive properties, enabling the adhesion of fibroblasts on epithelial cells, stimulating the deposition of collagen fibres and accelerating healing.

Oxygen-releasing gel is a commercially available new product that supplies oxygen in a controlled, slow, and direct manner to the site of injury, and its action is expected to optimise the healing process.[27] This form of treatment is known as topical oxygen therapy (TOT).

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There are no previous studies in the literature evaluating the effect of oxygen gel on the healing of skin wounds.

The objective of this study was to evaluate the clinical, histological and immunohistochemical effect of local application of slow-release oxygen gel on the healing of standardised skin wounds in rats.

Method

Ethical approval

This study was approved by the Ethics Committee on Animal Use Research of the Positivo University (CEUA-UP), Protocol 409.

Study design

Fifty male rats (*Rattus norvegicus*, Wistar) (Curitiba/Paraná, Brazil) of a mean age of 5 months and weight ranging between 360–480g were used in this study. The animals received commercial feed (Nuvital; Colombo/PR, Brazil) and mineral water ad libitum. During the entire duration of the study, environmental conditions, such as light, temperature and humidity of the rooms, were controlled by a digital panel to maintain a 12-hour light–dark cycle, temperature range of 18–22°C along with humidity of 65%.

The animals were randomly divided into two equal groups of 25 animals each. In the treated group (T), oxygen-releasing gel was applied, and in the control group (C), distilled water was applied to the skin wound on the same days. Each group of animals was divided into five equal subgroups (n=5) for euthanasia, which was performed at 5, 10, 14, 21 and 28 days postoperatively.

Surgical procedure

The animals were individually induced to anaesthesia by inhalation and were maintained anaesthetised with 3l/min of isoflurane (Cristália, Brazil). Subsequently, the animals were anaesthetised with xylazine (12mg/kg) (Vetbrands, Brazil) and ketamine (90mg/kg) (Vetbrands, Brazil) by intramuscular injection in the posterior part of their thighs. All animals received morphine (0.1ml) subcutaneously before starting the surgical procedure, which was performed by a single trained and calibrated operator.

The animals were submitted to trichotomy and antisepsis (with 10% active iodine) of the dorsal region, which was the area where the surgical wound was performed. A map was made in a radiographic film packaging with an area of 4cm^2 that served as a guide to remove the soft tissue from the dorsal region. The surgical wound was made in the mid-portion of the median sagittal plane of the animal, using a scalpel with blade number 15c.

After the creation of the standardised skin wounds, a thick layer of the oxygen-containing gel (bluem gel; Bluem Europe, The Netherlands) (composition: water, denatured ethanol (96.2%), glycerin, silica, sodium saccharin, sodium perborate, citric acid, polyethylene glycol (PEG)-32, sodium gluconate, cellulose gum, xanthan gum, lactoferrin) was applied with a molt-type spatula on the wounds of group T. In group C, only distilled water was applied to the wounds, using a syringe and insulin needle. The animals were isolated in boxes until euthanasia, to avoid them causing injury to each other.

Postoperatively, each animal received an intramuscular injection of 10mg/kg of enrofloxacin (Baytril Injectable 5%; Bayer S.A., Brazil) daily for 7 days and subcutaneous injection of 5mg/kg of tramadol (twice a day) for 3 days to prevent the animals from feeling pain.

Gel and distilled water were applied in groups T and C, respectively, on alternate days (Monday, Wednesday, and Friday) until euthanasia was performed.

At the experimental timepoints of 5, 10, 14, 21 and 28 days, euthanasia was performed (n=5) by an overdose of isoflurane. It was performed following the precept of animal ethics according to the Declaration of Helsinki.

Clinical evaluation

Clinical evaluation was performed by analysing photographic images taken with a cell phone (iPhone 8). The images were obtained at 5, 10, 14, 21 and 28 days postoperatively. Contraction of the edges, re-epithelialisation and visual features of the wounds were evaluated.

Histological processing

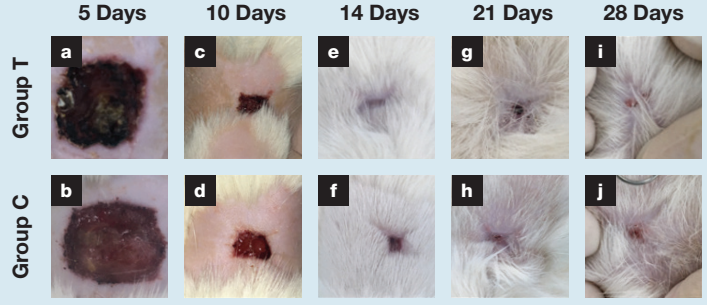
The surgical region of the skin lesion and 1cm^2 of the normal tissue was removed from the dorsal region of each animal, using a scalpel blade number 15 and Dietrich tweezers.

The removed tissues were placed in flasks containing 10% formalin. The formalin-fixed tissues were dehydrated, embedded in paraffin, and cut into 4µm-thick sections.

The tissue sections were deparaffinised in xylene, rehydrated in an alcohol descending series, and stained with Masson's trichrome.

The soft tissues were analysed under light microscopy. The process of inflammation, presence of tissue

Fig 1. Photographic images of the clinical evolution of skin wound healing at 5, 10, 14, 21 and 28 days for both the treated **(T)** and control **(C)** groups after the surgical procedure. After 14 days, healing was already at a more advanced stage in the treated group **(e)** when compared to the control group **(f)**. The best clinical healing is also observed in group T at 21 and 28 days postoperatively **(g, i)**, respectively



necrosis, blood vessel formation, organisation of collagen fibres, wound size reduction and re-epithelialisation were evaluated.

Immunohistochemical processing

For the immunoperoxidase method, the sections were mounted on silanised slides, deparaffinised in xylene, and rehydrated in gradual alcohol baths. Antigen reactivation was performed using 1% pepsin solution of pH 1.8–2.0, followed by heating in an oven at 37°C for one hour, washing in deionised water, and immersing in phosphate-buffered saline (PBS) for 5 minutes.

Immunolabelling was performed with the following primary antibodies diluted at 1:100 ratio in PBS: mouse-produced monoclonal anti-TNFα (sc-52746; Santa Cruz Biotechnology, US); and mouse-produced monoclonal anti-VEGF (mob308; Diagnostic BioSystems, US).

After overnight refrigerated incubation in a humidity chamber and immersion in PBS for 3 minutes, sections were processed as follows at room temperature, using the REVEAL Biotin-Free Polyvalent DAB kit (SPD-125; Abcam, UK): incubation with complement reagent (REVEAL Complement, DCMT-125; Abcam, UK) and peroxidase-conjugated secondary antibody (REVEAL HRP Conjugate, DHRR-125; Abcam, UK) for 30 minutes each, and immunostaining with 3,3′-diaminobenzidine (DAB) solution containing one drop of chromogen (DAB Chromogen, DABC-004; Abcam, UK) in 1ml of substrate (DAB Substrate, DABS-125; Abcam, UK).

Counterstaining was performed using Harris haematoxylin and slides were photographed using an Olympus BX43 optical microscope with Olympus SC100 camera (Olympus Corp., Japan).

Three representative fields of the central region of the lesion were photographed from each slide at 400× magnification for immunohistochemical analysis. The immunolabelled area was quantified using the freely accessible image processing software, ImageJ (version 1.52k, NIH, US) and using the image colour deconvolution method of Ruifrok and Johnston,[28] that uses the plugin developed by Positano et al.[29] This method performs the separation of haematoxylin and DAB stains by measuring the positive immunolabelled area, which is expressed as a percentage of the total number of pixels for each field.

The immunostaining values represent the entire image captured. The immunohistochemical analysis of TNFα was performed only at days 5 and 10 post-operation when the inflammatory process was more evident. For the VEGF antibody, the analysis was performed at all postoperative timepoints.

Statistical analysis

The mean of the measurements of each blade was statistically analysed, and the treatments, as well as timepoints, were evaluated. The results were analysed using two-factor analysis of variance (ANOVA) without replication. To evaluate the differences between treatment and control, the Bonferroni test was used, which allowed establishment of a minimum significant difference between the two means. Significance was defined as $p<0.05$. The analyses were performed using GraphPad Prisma 6 software (GraphPad, US).

Fig 2. Histological characteristics of the skin wounds stained with Masson's trichrome. At 5 days, in both groups **(a, b)**, we observe reddish borders resulting from the formation of clot/crust (asterisks) and, just below, an area of necrosis. At 10 days, in both groups **(c,d)**, the beginning of re-epithelialisation is already evident. At 14 days, group T showed more organised collagen fibres and more intense angiogenesis (arrows), with formation of the epidermis **(e)**. At 21 days **(g, h)**, the predominance of collagen fibres (blue colour) and regression of blood vessels (arrows) can be seen. At 28 days, complete re-epithelialisation of the wound **(i)**, in group T. (Magnification 10×)

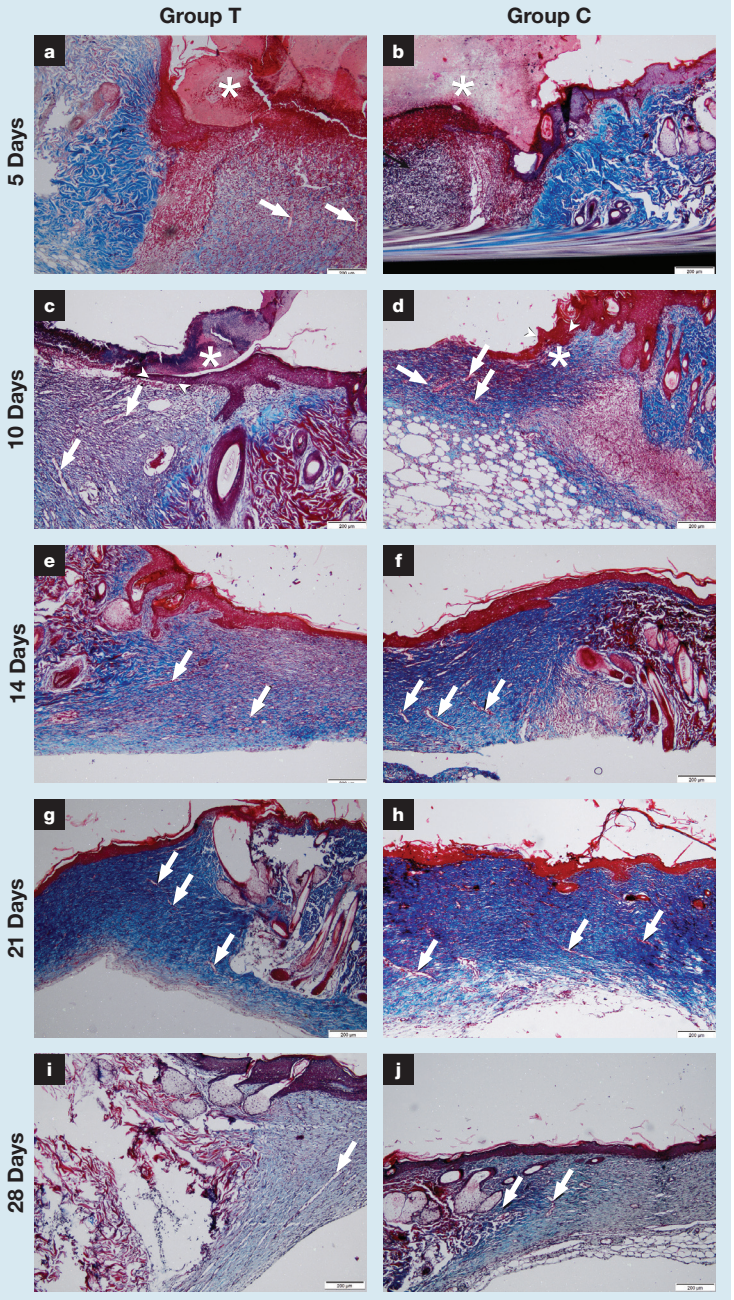
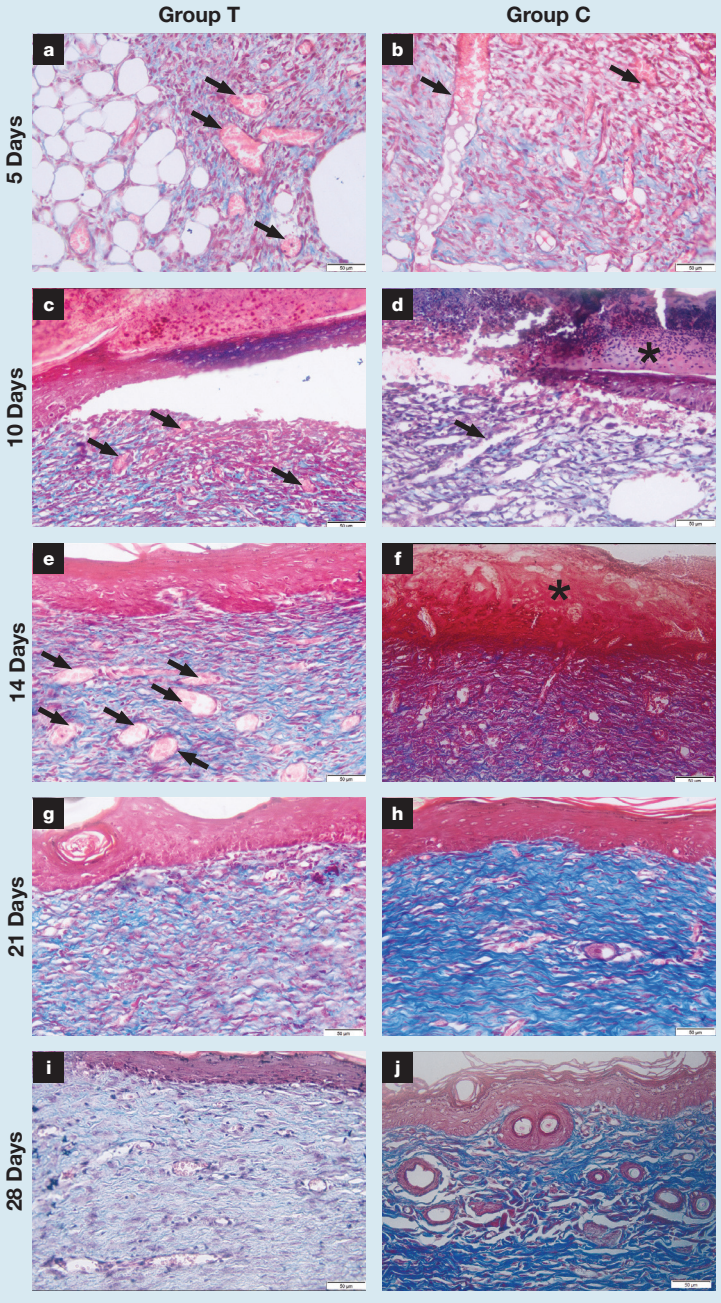


Fig 3. Histological characteristics of the two groups stained with Masson's trichrome. At, 5 days, group T presented a more intense inflammatory infiltration than group C **(a, b)**. At 10 days **(c)**, there is intense inflammatory infiltrate and a crust in the process of loosening. In group C, there is a region of clot/crust **(d)** (asterisk) that still remains at 14 days **(f)**. At 14 days **(e)** there is intense angiogenesis (arrows), as well as bluish colouration, suggesting the deposition of collagen fibres. In group C, there is less angiogenesis **(f)**. At 21 days, in both groups **(g, h)** there is the presence of collagen fibres, which are more disorganised in group C **(h)**. At 28 days, collagen fibre deposition is more organised in the treated group, with the presence of more blood vessels (arrows) **(i)**. (Magnification ×40)



Results

Clinical evaluation

On clinical evaluation, 5 days post-operation, different aspects of healing were observed to be similar in both C and T groups (Fig 1a, b). However, group T was already showing contraction of the wound edges and crust formation (Fig 1a). At 14 days post-operation, the wound in group T showed more advanced features (approximation of the edges and reduction in size) of healing than that in group C (Fig 1e).

Subsequently, on days 21 and 28, better healing was observed in group T with coaptation of the edges and re-epithelialisation of the lesion (Fig 1g, i).

Histological analysis

On histological analysis at 5 days post-operation, both the groups showed the presence of oedema, hyperaemia, congested blood vessels and leukocyte infiltration, in addition to the formation of a clot and an area of necrosis just below it (Fig 2a, b). Group T presented a more intense inflammatory infiltration than group C (Fig 2a). On day 10 postoperatively, group C still had congested blood vessels, leukocyte and mononuclear cell infiltration, clot drying and crust formation, and granulation tissue formation (Figs 2d, 3d). Group T showed increased angiogenesis and granulation tissue, the beginning of the re-epithelialisation process, reduction of oedema, and dryness of the clot and crust in the process of loosening (Figs 2c, 3c).

On day 14, group C showed the presence of crust, a region of tissue necrosis, reduction of oedema and inflammatory cells, as well as angiogenesis (Figs 2f, 3f). However, at the same timepoint, group T showed the presence of collagen fibres and more intense angiogenesis; the tissues also presented a more organised structure, and it was possible to observe the formation of the epidermis (Figs 2e, 3e).

On day 21, re-epithelialisation and reduction of blood vessels were observed in group C. However, the collagen fibres present were still disorganised (Figs 2h, 3h). In group T, the epidermis was already fully regenerated without the presence of a clot or crust. In addition, a reduction in the size of the lesion and the beginning of the organisation of collagen fibres was observed (Figs 2g, 3g). On day 28, re-epithelialisation was evident in group C, but the collagen fibres were still disorganised (Figs 2j, 3j). However, in group T, the collagen fibres were fully organised and abundant, suggesting complete healing of the lesion (Figs 2i, 3i).

Immunohistochemical analysis

The quantitative immunohistochemical data (represented as percentage) for the presence of TNFα and VEGF using anti-TNFα and anti-VEGF antibodies are presented in Fig 4 and Fig 5, respectively ($p<0.05$). The quantitative and qualitative data are described below.

TNFα: At 5 and 10 days postoperatively, on analysis of both groups, positivity for the antibodies was observed as seen in Fig 6. The positive reaction was

more intense (Fig 6a, c), and the percentage of TNFα positivity was significantly higher ($p<0.05$) in group T than in group C (Fig 6b, d) at both timepoints (Fig 4).

VEGF: During analysis of both the groups at all postoperative timepoints, they were observed to be positive for this antibody, as seen in Fig 7. At 14, 21 and 28 days, the immunopositivity was more intense (Fig 7e, g, i) and the percentage of VEGF positivity was significantly higher ($p<0.05$) in group T than in group C after 14, 21 and 28 days (Fig 5).

Discussion

Active oxygen gel is used in the treatment of lesions in the human oral cavity, as it controls the infection and accelerates healing.[27]

Our study evaluated the effect of local application of oxygen gel on standardised skin wounds caused in rats and observed that the gel therapy stimulated the inflammatory response, angiogenesis and granulation tissue formation, as well as accelerating the onset of the re-epithelialisation process. These results are unprecedented since no studies have been conducted previously that have evaluated the action of the active oxygen gel on the healing of skin wounds.

The slow release source of oxygen comes from sodium perborate monohydrate, which is one component of the gel. In contact with water or tissue fluid, sodium perborate is converted into sodium borate and hydrogen peroxide, and small quantities of hydrogen peroxide (0.015%) are released very gradually; this slow oxygen release stimulates cell migration, angiogenesis and collagen fibre formation.

The acute inflammatory response has healing as its natural outcome.[30] In contrast, chronic inflammation is responsible for hypertrophic scar formation or, in some cases, non-healing wounds.[1,30]

The healing of skin wounds is a complex process that involves various cell types that act in harmony. The occurrence of haemostasis and clot formation, soon after tissue injury, is responsible for the formation of a provisional matrix that acts as a support structure for the migration of leukocytes, keratinocytes, fibroblasts and endothelial cells.[5,15] The chemotaxis of neutrophils, the fundamental cells at the beginning of an inflammatory response, is performed by mediators, such as TNFα and interleukins (IL-1 and IL-6) that amplify the inflammatory response and stimulate VEGF and IL- 8 for adequate healing to occur.[3]

In our study, throughout the 10 days, both the groups showed positive reactions to the TNFα antibody. However, immunopositivity was higher in the treated group, indicating that the inflammatory process was greater in the group that received the gel (Fig 4). The wound closure was faster in the presence of the gel (Fig 1e). It is possible that a more intense initial inflammatory response, by the action of the gel itself, activated the inflammatory pathway and ultimately favoured adequate healing. During and after the inflammatory response, angiogenesis must occur to restore the vascular system in the wound bed to allow nutrient perfusion to occur at the site of injury. This process is triggered by growth factors, such as VEGF.[3]

In our study, after 14 days, a higher expression of VEGF and intense angiogenesis was observed in the treated group that was offered oxygen (Figs 5, 7). On clinical evaluation, adequate formation of granulation tissue and an advanced stage of wound healing were observed (Fig 1e, i). We suggest that supplementation with oxygen stimulated angiogenesis and favoured the healing process.

Fig 4. The graph represents the result of immunohistochemistry for TNFα antibody. The percentage of TNFα positivity was significantly higher in group T than in group C ($p<0.05$) after 5 and 10 days. C—control group; T—treated group; *$p<0.05$

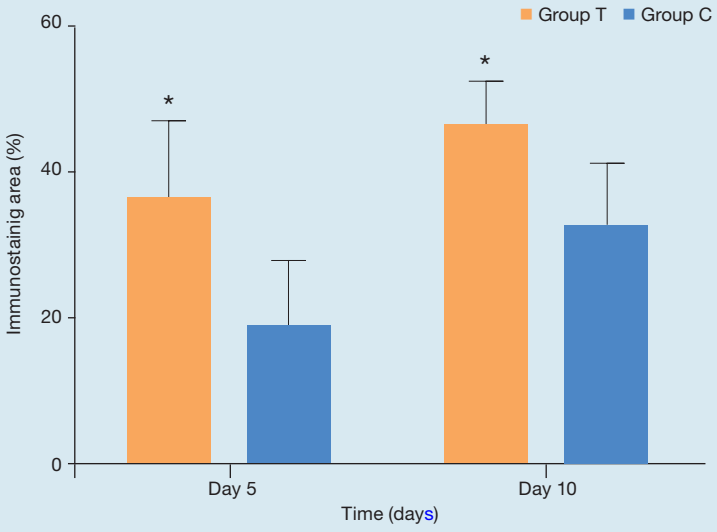


Fig 5. The graph represents the result of immunohistochemistry for vascular endothelial growth factor (VEGF) antibody. The percentage of VEGF positivity was significantly higher in group T (treated) than in group C (control) after 14, 21 and 28 days ($p<0.05$). *$p<0.05$

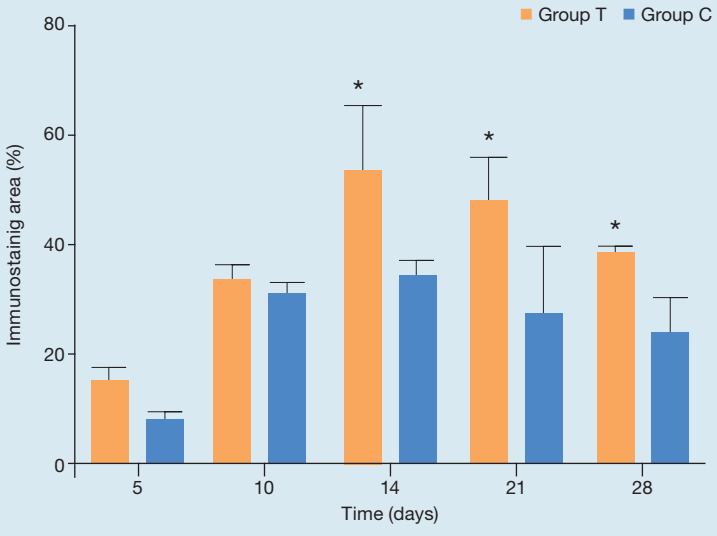
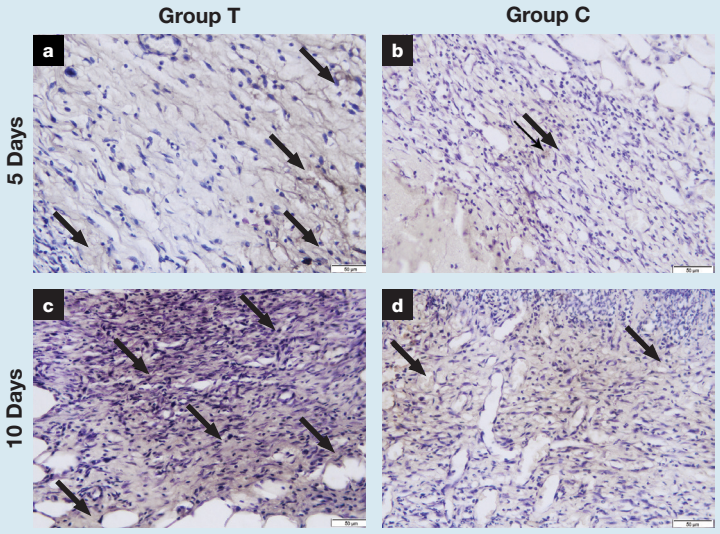


Fig 6. Immunolabelling analysis of tumour necrosis factor (TNF)-α at 5 and 10 days. Note more pronounced immunolabelling in group T at 10 days. Arrows exemplify immunolabelling. The more brownish spots, the more positive immunolabelling. C—control group; T—treated group



Patel et al.[4] found higher levels of VEGF protein in a cell culture that was offered oxygen, demonstrating that the protein is sensitive to oxygen. VEGF is a long-term angiogenic stimulus in the presence of oxygen.

Similarly, some authors[12,13] who performed oxygen supplementation through the use of oxygen-containing dressings in porcine models, found that the dressing stimulated angiogenesis in addition to promoting collagen deposition. This suggested that increased oxygen supply may be beneficial for local wound healing, as shown in the results of this study.

On the other hand, Sano et al.[31] found that oxygen-deprived wounds also increased angiogenesis, but there was less granulation tissue and delayed wound closure compared to that in the normoxia group. According to Sano et al.,[31] the intensified neovascularisation in the hypoxia group may have been a compensatory response to the permanent insufficient supply of oxygen and may not be a factor favouring healing. Thus, they suggested that adequate oxygen supply is preferable for the maintenance of angiogenesis and facilitating healing.

In our study, the presence and orientation of collagen fibres was consistently assessed using Masson's trichrome staining.[32] Histological analysis suggested that collagen deposition occurred earlier in group T (Figs 2, 3).

Studies showed that after sufficient collagen was produced during the synthesis and degradation of the ECM, collagen prevailed in the wound extension and fibroblast proliferation was reduced.[8] At this stage, there was a reduction in the blood vessels and the collagen fibres had an organised and resistant architecture, which was similar to the tissues adjacent to the lesion region.[33]

Rao et al.[14] showed that the collagen fibres were better organised and deposited in larger quantities in a shorter period of time, in the group treated with oxygen. They also observed a reduction in blood vessels at this stage of healing. Thus, the same was concluded for this study on obtaining similar results using the gel (Figs 1e, 2e).

Hard-to-heal wounds with failed fibroblast production and improper collagen deposition usually lack oxygen.[34] However, on supplying oxygen, satisfactory results have been observed in the healing of hard-to-heal wounds that are usually hypoxic and difficult-to-heal.[16,17,35]

There are different forms of oxygen delivery that accelerate skin wound healing.[2,11,16,17,35,36] The source of the slow-release active oxygen in bluem gel is hydrogen peroxide.[27] Peroxides are generally unstable, and they decompose and produce molecular oxygen on contact with water or heat. It is possible to control the rate of decomposition of peroxides by adjusting the amount of water available. In this way, they can sustainably deliver oxygen to cells by slow release.[37]

When delivered in low concentrations, hydrogen peroxide assisted in the removal of pathogens and cellular debris, as well as favouring the secretion of cytokines that assisted in tissue regeneration.[38]

Topical oxygen therapy has also been shown to be important in the improvement and healing approach for the efficient recovery of hard-to-heal diabetic wounds. Diabetic wounds are challenging to treat due to a wide range of pathophysiological changes. Hypoxia is one of the predominant contributing factors of poor vascularisation and chronicity in diabetic wounds.[39]

Thus, this therapy, involving local application of oxygen-containing gel on skin wounds, was demonstrated to be promising, accessible and easy to handle, as well as to apply; it also exhibited faster formation of well-organised and structured collagen matrix, as well as obtaining a healed tissue similar to the original, when compared to the control group.

Limitations

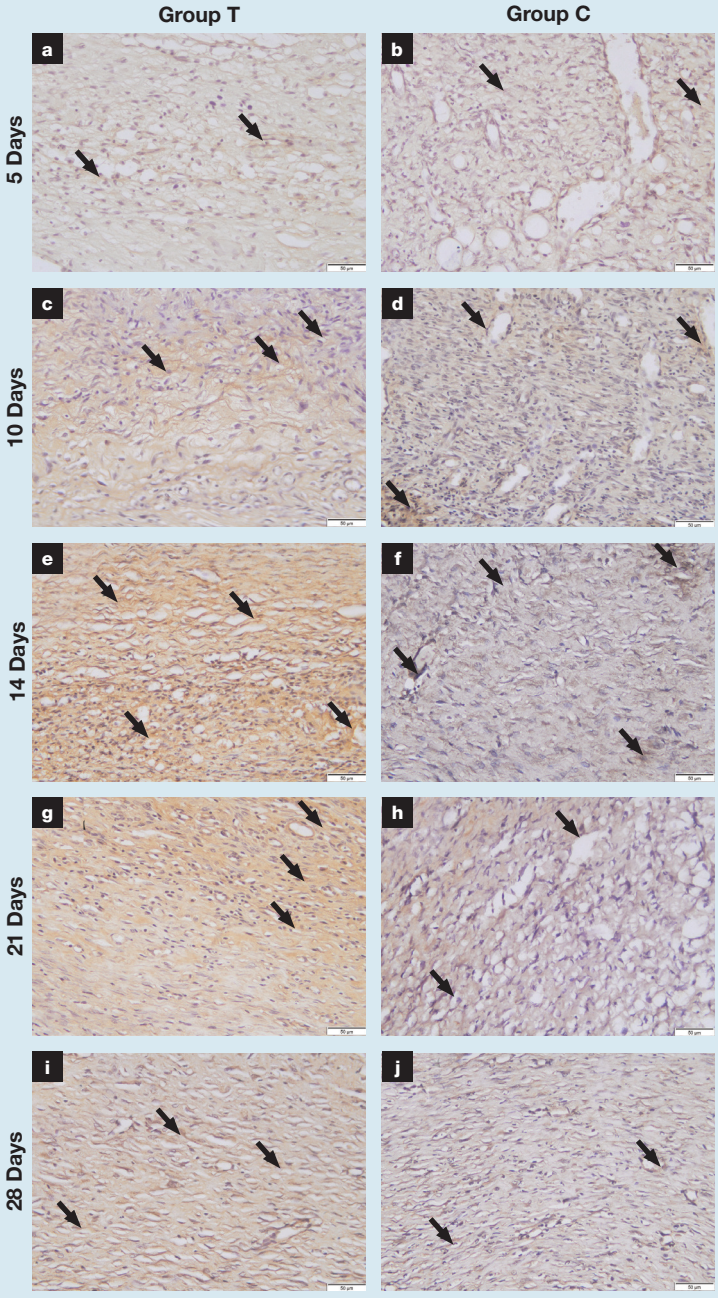
This is the first study that carries out histological and immunohistochemical evaluation of the healing process in rat skin using oral gel with oxygen release. Further animal studies and randomised clinical trials are required to confirm, consolidate and validate the protocol.

It is difficult to compare, and more studies on animals are needed to confirm the results found in the present study, in order to use the gel for treating skin wounds.

Conclusion

Despite a few limitations of the present study, it can be concluded that local application of slow oxygen-release gel accelerated the healing of standardised skin wounds created surgically in rats, with increased angiogenesis and better collagen fibre formation. JWC

Fig 7. Analysis of vascular endothelial growth factor (VEGF) immunolabelling in both groups and times. Note more pronounced immunolabelling in the Group T (14 days **(e)** >21 days **(g)** >28 days **(i)**). Arrows exemplify Immunolabelling. The more brownish spots, the more positive immunolabelling



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Reflective questions

- The hard-to-heal wound is a result of the disordered inflammatory process of wound healing. Prolonged hypoxia and bacterial colonisation may be related to the failure of healing. Since oxygen supplementation enables angiogenesis and fights infection, could the application of oxygen gel correct hypoxia as well as control bacterial infection of hard-to-heal wounds and favour healing of these wounds?
- Considering that hyperglycaemia can interfere with angiogenesis and collagen deposition and that oxygen favours this process, could topical oxygen supplementation with the use of the gel be a treatment of choice for the healing of cutaneous wounds in patients with diabetes?
- In the study presented, occlusive dressing was not chosen after application of the gel. Could the use of the gel followed by occlusive dressing show even better results in the healing of cutaneous wounds?

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