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ORIGINAL PAPER

Development and evaluation of a vegetable oil blend formulation for cutaneous wound healing

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Abstract

This work aimed to evaluate the in vivo capacity of a vegetable oil blend formulation (VOB) developed to accelerate cutaneous wound closure. Total thickness wounds were punctured on the skin on the back side of each animal and topically treated with the VOB formulation, Dersani® ointment or the vehicle control. After 2, 7, 14, 21 days post-wounding, five animals from each group were euthanized, and the rates of wound closure and re-epithelialization were evaluated. The wounds were harvested for histological and biochemical analysis. VOB resulted in faster and greater re-epithelialization in the in vivo excisional wounds, exhibiting significant wound area reduction of 8.9, 8.0, 35.1, 45.2 and 47.0% after 2, 5, 10, 14 and 21 days post-wounding, respectively, when compared with the vehicle control. Histological and biochemical analyses showed that the VOB-treated wounds exhibited a significant increase of granular tissue and controlled inflammatory response by modulation of the release of pro-inflammatory cytokines TNF-α, IL-6 and IL-1. Moreover, VOB-treated wounds showed a significant and concrete increase in the deposition and organisation of collagen fibres in the wound site and improved the quality of the scar tissue. Altogether, these data revealed that VOB accelerates wound healing processes and might be beneficial for treating wound disorders.

Keywords Fatty acids · Vegetable oil · Wound healing · Cytokines · Collagen · Inflammation

Introduction

In response to a tissue injury, the skin is repaired through a sequence of coordinated events involving various cell types, including leukocytes, platelets, fibroblasts and epithelial cells [7, 25]. The wound healing process is divided into three overlapping phases: inflammatory, proliferative, and remodelling [7]. The first cells to appear at the wound site are the polymorphonuclear leukocytes, also called the inflammatory infiltrate, which reach the site of inflammation through diapedesis [15]. Together with macrophages and resident cells, they produce and release different pro-inflammatory cytokines and chemokines, including interleukin-1 and 6 (IL-1, IL-6) and tumour necrosis factor (TNF-α) for the activation of further inflammatory cells, giving rise to the proliferative phase [21, 27]. The proliferative phase involves epithelization and angiogenesis, in which transforming growth factor (TGF) and epidermal growth factor (EGF) are important for the proliferation, migration and differentiation of fibroblasts and keratinocytes [7, 17, 20]. Tissue maturation and remodelling is the last phase of wound healing, where collagen synthesis comes into equilibrium with collagen breakdown [12, 20, 24].

Vegetable oils are considered a rich source of fatty acids that have been used prominently in the medical and cosmetic fields [28]. The fatty acids contained in these oils create an occlusive film on the skin, reducing the transepidermal water loss (TEWL), which contributes to the maintenance of correct hydration of the skin [14, 28]. Linoleic (C18:2n-6) and alpha-linolenic (C18:2n-3) fatty acids are essential for normal cellular functions and act as precursors for the synthesis

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of long-chain polyunsaturated fatty acids such as arachidonic acid (C20:4n-6), eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C20:6-n3), which are involved in numerous cellular functions, such as membrane fluidity and the synthesis of eicosanoids such as prostaglandins, leukotrienes and thromboxanes [9, 16]. Thus, they have the ability to modify inflammatory and immunological reactions, altering leukocyte functions and accelerating the process of tissue granulation. Vegetable oil formulations have been used with great success in the treatment of wounds and pressure ulcers in bedridden patients due to their low cost and widespread availability [12, 13].

Despite several reports in the literature describing the biological properties of vegetable oils, few reports are available about in vivo studies of the use of VOB for cutaneous wound healing. Thus, this study aimed to evaluate the efficacy of a developed VOB formulation in cutaneous wound healing processes using the full-thickness excisional wound model in rats and to compare it to a commercially available reference product (Dersani® ointment) indicated for the treatment of any type of cutaneous lesion.

Materials and methods

Chemicals and biochemicals

Dersani® ointment was purchased from a local pharmacy. IL-1α, IL-6, TGF-β, and TNF-α ELISA kits were purchased from eBioscience (San Diego, CA, USA). Bradford protein assay reagents were purchased from Thermo Scientific (Rockford, IL, USA). Ketamine and xylazine were purchased from Vetbrands (Paulinia, SP, Brazil). All other reagents used in the experiments were obtained from several commercial sources and both are analytical grade.

Vegetable oils

The following vegetable oils were purchased from SM Farmaceutica (Campinas, SP, Brazil) and were accompanied by quality control reports attesting to a degree of purity above 99.2%: sunflower oil (*Helianthus annuus)*, olive oil (*Olea europaea*), rosehip oil (*Rosa aff. rubiginosa)*, linseed oil (*Linum usitatissimum*), black currant oil (*Ribes nigrum)*, and macadamia oil (*Macadamia ternifolia* nut oil).

Preparation of the VOB formulation and controls

The vegetable oil blend formulation (VOB) was prepared by simple mixing of the vegetable oils in the following proportions: sunflower oil (*Helianthus annuus*) 30%, olive oil (*Olea europaea*) 20%, rosehip oil (*Rosa aff. Rubiginosa*) 10%, linseed oil (*Linum usitatissimum*) 15%, black currant oil (*Ribes nigrum*) 10%, macadamia oil (*Macadamia ternifolia* nut oil) 15%. To prepare the ointment, the VOBs were heated to 45 °C, and 10% Compritol ATO 888 (glyceryl dibehenate, tribehenin) was added. Upon returning to room temperature, the VOB acquired an ointment texture. The commercially available Dersani® ointment was used as a positive control (caprylic acid, capric acid, soy lecithin, vitamin A, vitamin E, caproic acid—and sunflower oil—linoleic acid). Mineral oil was formulated with the same gelling agent used in the VOB formulation to obtain the same ointment consistency and was then used as the vehicle control.

Formulation stability testing

The stability of the formulation and the expiration date were determined using the accelerated stability method according to the Brazilian Health Regulatory Agency [2]. The VOB was conditioned in a transparent, neutral glass bottle with a cover that guaranteed a good seal, therefore, avoiding the loss of gases and evaporation to the medium. The VOB was submitted to heating in an oven at 45 °C ± 2 °C, alternating with cooling in the refrigerator at 5 °C ± 2 °C, with cycles of 24 h each over 4 weeks. Organoleptic characteristics such as colour, odour, and appearance and physical–chemical parameters such as pH and viscosity were evaluated [2]. Moreover, the VOB fatty acid profile was analysed by gas chromatography before and after the accelerated stability test.

VOB fatty acid profile

The fatty acid content of the VOB was analyzed by a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) coupled with a flame ionisation detector (FID). VOB fatty acid methyl esters (FAME) were prepared by methylation with boron trifluoride (12% BF_3) in methanol. FAME were identified by comparing the retention times to a known FAME standard (GLC-85 reference standard, NU-CHEK PREP INC., Elysian, USA). The internal standard used was methyl tricosanoate (C23:0 reference standard, NU-CHEK PREP INC., Elysian, USA). FAME were separated on a capillary column DB-5 Agilent (30 m × 0.25 mm d.i. × 0.25 µm). Nitrogen was used as a carrier gas at 0.6 mL min^{-1}. The chromatographic conditions were as follows: injector 250 °C, split 1:50, injection volume 1 µL; oven: 100 °C for 0.5 min, followed by an increment of 3 °C min^{-1} to 260 °C; FID was maintained at 280 °C.

Animals

All experiments involving the use of animals were conducted in agreement with the Brazilian Animal Care Committee and were approved by the Committee of Ethics, Bioethics

and Animal Welfare of the Universidade Vila Velha (UVV) (CEUA-UVV protocol 381/2016). A total of 60 adult male Wistar rats (*Rattus norvegicus*) weighing about 270–330 g and aged 7–8 weeks were obtained through Central Biotério of the Universidade Vila Velha, Vila Velha, Brazil. The animals were kept under standard temperature-controlled conditions (22 ± 2 °C) with 12-h light/dark cycles and with free access to food and water. Two weeks prior to the experiment to produce the lesions, the animals were housed in separate cages to avoid injury by contact with other individuals, which could interfere with the progress of the experiment.

In vivo wound healing experiment

Prior to the production of the wounds, the rats were weighed and anaesthetized with 4% hydrated chloride. After shaving and cleaning the skin with 70% ethanol, four full-thickness excision wounds were created on the dorsum region of each rat with a sterile 15-mm punch biopsy. Then, the rats were randomly divided into three distinct groups ($n = 20$) and evaluated for 21 days according to the standard protocols [3, 8]. The rats were treated daily with VOB, Dersani® ointment (positive control), or the vehicle control. The wounds were covered with gauze and tape to keep them protected. The choice of dry gauze dressing was based on the pilot study and because it is considered the most economically option and largely used by the population. Five rats from each group were euthanized on days 2, 7, 14 and 21 after the surgical procedure, and the wounds and their surrounding areas were collected and stored for future histological and biochemical investigations, frozen to − 80 °C [3, 8].

Wound area studies

The wound areas were calculated using ImageJ software (NIH, USA). The morphometric analysis of the wounds was performed using images of the wounds at 0, 2, 5, 7, 10, 14 and 21 days post-wounding. The rate of wound closure that represents the percentage of wound reduction from the original wound size was calculated using the following formula: ((wound area day 0 − wound area at days 2, 5, 7, 10, 14 and 21)/wound area day 0) × 100. Values were expressed as the percentage of healed wounds.

Histological processing

Two wound biopsies from each animal in each group and treatment time were conditioned for 24 h in 3.7% phosphate buffered formaldehyde, followed by histological processing and paraffin inclusion. Serial histological sections with thicknesses of 3–5 µm were mounted on glass slides and stained with haematoxylin and eosin (H&E) (for evaluation and quantification of the inflammatory infiltrate) and with a solution of Sirius Red F3BA saturated in aqueous picric acid (for quantification of collagenase) [4, 10].

Evaluation of inflammatory infiltrate

Paraffin-wound sections stained with haematoxylin–eosin (H&E) were photographed using image capture software (Honestec VHS to DVD 3.0 SE) in a blinded fashion at 100 × using a digital camera attached to a light microscope (Model Leica Mikroscope Type 501095). About 35 different fields (from the superficial dermis to the deep dermis, and an uninjured area) were examined, and a region of interest was selected for each field. The images from each group on their respective day were loaded and analysed using the open source software CellProfiler (CP) (version 2.1.1), designed for the quantitative analysis of biological images, to identify the size and shape of the cells in the inflammatory infiltrate [6]. Six different microscopic slides were used for each treatment at each time point ($n = 20$ wounds/group), and the data were reported as the average of the total number of inflammatory cells per group [8].

Evaluation of collagenesis by imaging

The morphometric analysis corresponding to the area occupied by the collagen fibres was determined by analysing the colour density by digitally converting the images first to grey scale, and then to black and white using the CellProfiler (CP) software (version 2.1.1) [6]. Four different fields of each wound tissue stained with Sirius red were photographed using image capture software (Honestec VHS to DVD 3.0 SE) at 100x using a digital camera attached to a light microscope (Model Leica Microscope Type 501095). The distribution of collagen (red colour) was quantified using the Image Math and Measure Image Intensity functions of the CellProfiler (CP) software (version 2.1.1). Five different rats were used for each treatment at each time ($n = 20$ wounds/group), and the results were reported as the average distribution of collagen per treatment [4, 8].

Cytokine measurements

Two wound specimen biopsies collected from each animal at days 0, 2, 7, 14 and 21 post-wounding were immediately frozen at − 80 °C. Next, fragments of these biopsies were homogenised on ice using Lysing Matrix A tubes and a Fast Prep-24 homogenizer (MP Biomedicals, Santa Ana, CA) and then centrifuged (1500 g). The homogenate fluid obtained was used to measure the IL-1, IL-6, TNF-α, and TGF-β using the enzyme-linked immunosorbent assay (ELISA) following the manufacturer's specifications for each assay (eBioscience, San Diego, California, USA). Optical densities were measured at 450 nm in a microplate reader device

(Molecular Devices Spectra MAX 190, USA). The cytokine levels were expressed in pg; sensitivities were > 10 pg mL^{-1}.

Total protein quantification

The total protein contents of the homogenate fluid obtained from the tissue sections of the wounds treated with the VOB, the positive control, and the vehicle control were estimated using the Coomassie protein assay reagent (Rockford, USA) according to the manufacturer's instructions. Experiments were performed in 96-well plates, and protein concentrations were calculated by regression analysis using as standard curve a solution of bovine serum albumin (BSA) by colorimetric measurements at a length of about 595 nm in an ELISA plate reader (Molecular Devices Spectra MAX 190).

Biochemical measurement of myeloperoxidase (MPO)

The density of the neutrophilic infiltrate in the homogenate fluid obtained from the tissue sections was determined through the myeloperoxidase (MPO) assay, as previously described by Dos Santos Gramma et al. [8]. The results were described as the total number of neutrophils $\times 10^3$ mg^{-1} tissue by comparing the absorbance of the tissue homogenate to a standard curve generated using rat peritoneal neutrophils.

Statistical analysis

Statistical analyses were performed using GraphPad software (San Diego, CA, 176 USA). Data are presented as the mean ± standard error of mean (SEM) or standard deviation (SD), and statistical comparisons were carried out using one-way analysis of variance (ANOVA) followed by Tukey's post-test or two-way ANOVA when appropriate. The level of significance was $p < 0.05$.

Results

Accelerated stability test

Different physicochemical properties of the VOB formulation were evaluated after the accelerated stability test. The results indicated that no changes in colour, appearance, odour or viscosity were observed. The pH of the VOB was also determined to ensure that the formulation would not produce any irritation of the skin. The freshly prepared VOB exhibited a pH of 3.85 ± 0.2, and after the accelerated stability test, the pH was found to be 3.87 ± 0.3. These results indicate that the pH and organoleptic characteristics of the VOB were markedly stable during the test period.

Fatty acid composition of the VOB formulation

Characterizations of the fatty acid composition in the freshly prepared VOB and after the stability testing were expressed as the percentage of total methyl esters and were analysed by GC-FID (Table 1). Table 1 shows that although the VOB that underwent the stability test was stressed at high temperatures and cooling, the fatty acid profile of the sample did not change, and the characteristics of the polyunsaturated fatty acids were preserved.

In vivo wound closure assessment

Topical application of the VOB allowed faster and greater re-epithelialization in in vivo full-thickness excisional wounds compared to the vehicle control and the positive control groups, as shown in Fig. 1. In addition to accelerating the wound closure, the VOB led to a rapid recovery of the mature epidermal structure, with the lesions becoming progressively less inflamed and producing fewer scars than those wounds treated with either the positive control or the vehicle control. Significant wound area reductions of 8.9, 8.0, 35.1, 45.2 and 47.0% were observed in the VOB-treated group compared to the vehicle control group after 2, 5, 10, 14 and 21 days post-wounding, respectively. On the other hand, wounds treated with the positive control exhibited

Table 1 Relative percentages of FAME in the freshly prepared vegetable oil blend (VOB) and after the stability testing

FAME	VOB ointment freshly prepared (%)	VOB ointment stability testing (%)
Saturated		
10:00	0.04	0.03
12:00	0.31	0.27
14:00	0.28	0.26
15:00	0.05	0.06
16:00	9.64	9.84
17:00	0.08	0.08
18:00	3.79	3.91
20:00	1.05	1.21
22:00	6.67	8.95
Total	21.91	24.61
Monounsaturated		
16:1n-7	1.94	1.96
18:1n-9	32.88	31.24
20:1n-9	0.45	0.44
Total	35.27	33.64
Polyunsaturated		
18:2n-6	34.53	34.21
18:3n-3	7.57	7.09
Total	42.1	41.3

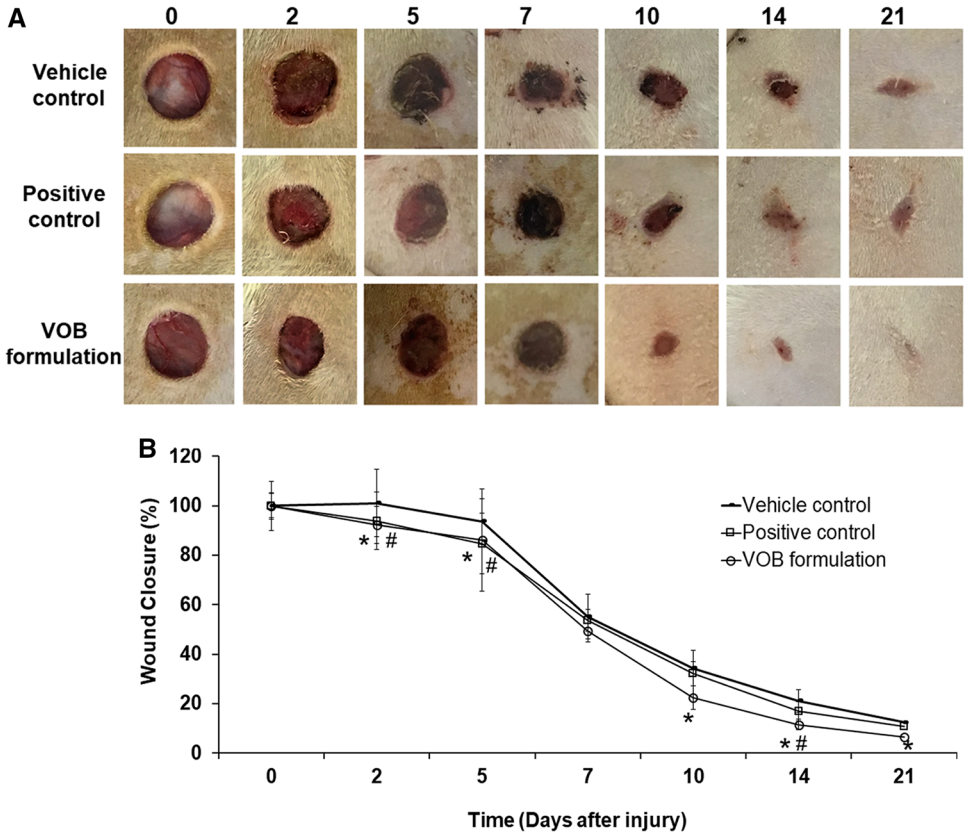

Fig. 1 Topical application of the VOB formulation accelerates excisional wound closure. **a** Photographic representation of the wounds on the indicated days post-wounding. **b** Percentage of wound closure after daily topical application of the VOB, positive control and vehicle control at days 0, 2, 5, 7, 10, 14, and 21. Data are expressed as percent area reduction from the original wound size (day 0). Mean values ± SEM ($n = 20$ wounds/group), $^{*}p < 0.05$, VOB formulation compared to vehicle control; $^{\#}p < 0.05$, positive control compared to vehicle control

significant reductions of 7.2, 9.6 and 20.1% in the wound area after 2, 5 and 14 days, respectively. Thus, it is evident that the wound healing rate of the VOB-treated lesions was similar to or even greater than that of the positive control, especially during the re-epithelization stage after 10 days and at the maturation stage on the 21st day.

Vegetable oil blend (VOB) positively influenced the inflammatory phase

Histological analysis of the wound biopsies stained with H&E were used for quantitative analysis of the cellular density at the wound site (Fig. 2a, b). Analysis of the inflammatory cellular infiltrate in the wound biopsies treated with the VOB showed a proportional reduction in the inflammatory infiltrate at the wound site after 2, 7 and 14 days, when compared to the vehicle control and the reference drug groups ($p < 0.05$). Reductions of 24.2, 14.4 and 26.6% were observed in the cellular densities on days 2, 7 and 14, respectively, compared with the vehicle control group. No significant differences were observed in the wounds treated with the positive control compared to those treated with the vehicle control. The cellular density after 21 days post-wounding was similar between all groups and decreased to nearly the physiological cell number (Fig. 2b).

Subsequently, the presence and involvement of neutrophils in the inflammatory process were estimated by investigating the activity of the enzyme myeloperoxidase (MPO). Very low MPO levels were identified in the animals with preserved skin (day 0) (Fig. 3). However, on days 2 and 7 post-wounding, the MPO levels presented considerably increased levels of MPO in all groups, although no significant difference was observed among the tested groups. At 21 days post-wounding, the concentrations of MPO decreased back to the physiological levels observed on day 0.

To analyse whether the decrease in inflammatory infiltrate might have some correlation to the edema and the inflammatory phase of wound healing, the total protein content in the tissue biopsy homogenates was analysed. Large amount of total protein content in the wound bed may be correlated with edema formation [8, 10]. The wounds of the VOB-treated group exhibited an apparent reduction in the total protein content at 7 days post-injury when compared to the vehicle control group (Fig. 4). No significant differences were observed in the positive control group compared to the vehicle control group during the entire experimental time.

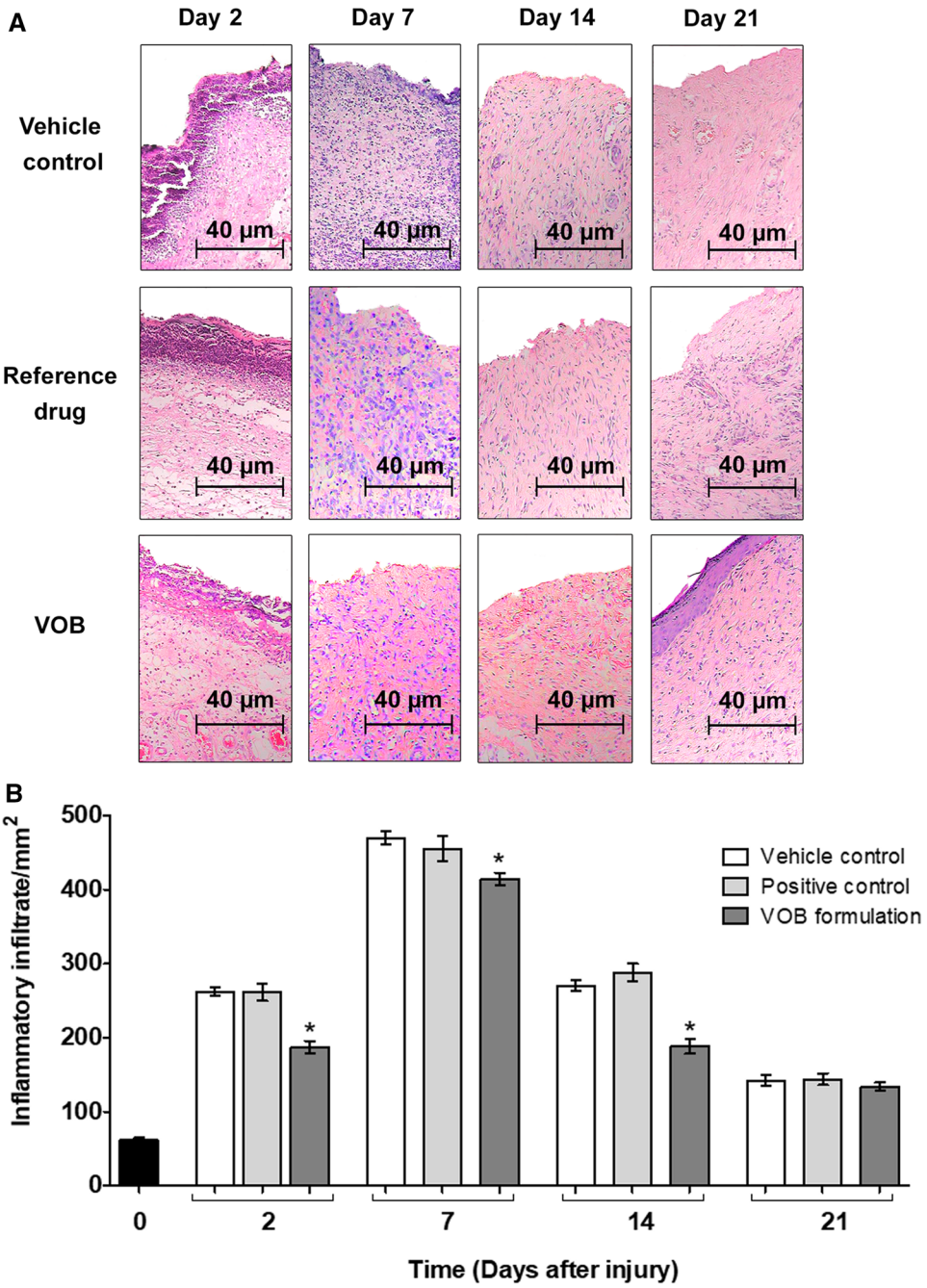

Fig. 2 Vegetable oil blend formulation (VOB) affects polymorphonuclear recruitment at the wound site. **a** Representative photomicrography of the wound sections stained with H&E (×400). **b** Quantitative analysis of inflammatory infiltrate. The values represent the mean ± SEM ($n = 20$ wounds/group), $^*p < 0.05$ compared to the vehicle control group

Cytokine quantitation in the skin wound biopsies

Cytokines are mainly produced by macrophages and lymphocytes, although they can also be produced by polymorphonuclear leukocytes (PMN), endothelial cells and epithelial cells. Therefore, following the investigation of the influence of the VOB on the production and release of different cytokines and growth factors, such as TNF-α, IL-1α, IL-6 and TGF-β, the wound biopsy homogenates were examined. In fact, the VOB modified the release of pro-inflammatory cytokines at the wound site. As observed in Fig. 5a, the concentrations of the TNF-α detected in the homogenate tissues prepared from the wound biopsies following exposure to the VOB after 2, 7, and 14 days were significantly reduced compared to the vehicle control group ($p < 0.05$). The positive control group exhibited a significant decrease in TNF-α concentrations only after 14 days. IL-1α cytokine concentrations after 2 and 7 days also showed a significant decrease after VOB treatment compared to the vehicle control treatment ($p < 0.05$) (Fig. 5b). No significant effects were observed in the positive control group. Concentrations of IL-6 cytokine were significantly suppressed after

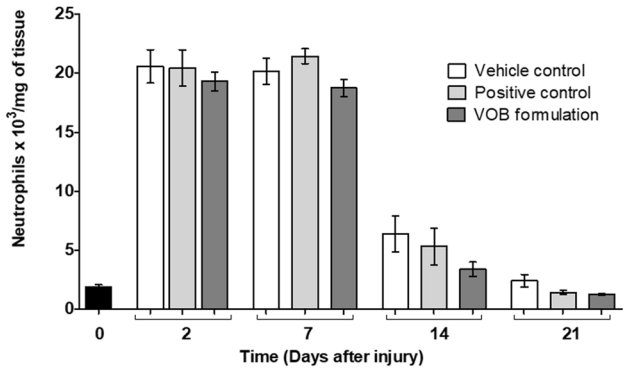

Fig. 3 Tissue neutrophil accumulation determined by myeloperoxidase (MPO) concentrations in the wound biopsies treated with the VOB formulation, the positive control and the vehicle control at 0, 2, 7, 14, 21 days. Values represent mean ± SEM ($n = 10$ wounds/group)

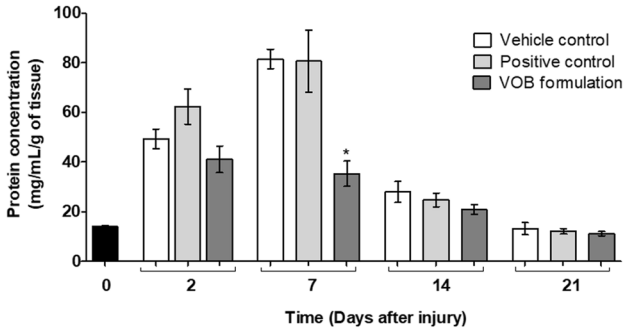

Fig. 4 Total protein content in the wound tissue treated with the VOB formulation, positive control and vehicle control on days 0, 2, 7, 14 and 21 post-wounding. Total protein was measured according to the Coomassie assay in homogenates prepared from the wound biopsies. Values represent mean ± SEM ($n = 10$ wounds/group). *$p < 0.05$ compared to vehicle control group

2 and 21 days post-injury in the group treated daily with VOB compared to the vehicle control group, whereas the positive control group only presented significant effects at day 21 (Fig. 5c). No significant effect on the production of TGF-β was observed after topical treatment of the wounds with VOB and the positive control during the experimental procedure. Although no significant differences were observed in the TGF-β production, VOB treatment positively influenced the production of TGF-β after 2, 7 and 14 days.

Collagenesis

The synthesis, degradation and deposition of collagen at the lesion site are considered an important stage in the proper healing process. Throughout the experimental period, the intense production of collagen and the formation of new tissue at the wound site were observed, especially in the group treated with the VOB (Fig. 6). At days 2, 7 and 14 post-injury, the collagen production in the VOB-treated group was significantly higher compared to the vehicle control group, whereas after 21 days, the collagen concentration was significantly lower ($p < 0.05$). No significant effects on the collagen amount were observed after wound treatment with the positive control. Moreover, the VOB-treated wounds elicited more organised and dense collagen fibres compared to the wounds treated with the positive control or the vehicle control (Fig. 6a).

Discussion

Wound healing is a highly dynamic process and involves different cell types and interactions of extracellular matrix molecules, growth factors, soluble mediators and cytokines to build up the repair of the injured tissue. Millions of people each year suffer from impaired wound healing that is considered a challenge to healthcare systems worldwide [20]. The continuous source and use of alternative therapies for skin wound care has greatly increased over the last decade [22, 23]. Vegetable oils, which are natural sources of fatty acids, have always been an effective, low-cost alternative for the treatment of skin wounds. The idea of having a complex vegetable oil composition was based on the possible synergistic effects and taking into account that each selected oil has a specific or pronounced pharmacological activity due to its specific fatty acid profile. Therefore, the VOB formulation developed here, which has a unique fatty acid profile with very high levels of monounsaturated (35.27%) and polyunsaturated fatty acids (42.1%), demonstrated the provision of a broad spectrum of action in all phases of the healing process, contributing to the adequate healing of the skin.

Polyunsaturated fatty acids (PUFAs), that are, similar to arachidonic acid, 20 carbon units in length, in addition to their structural function, can modulate cell–cell interactions and intracellular signalling. Thus, altering the fatty acid composition of membrane phospholipids can modulate their fluidity by modifying the binding of cytokines to their receptors [13, 17]. In addition, PUFAs are primary precursors of important lipid mediators of the inflammatory process, such as prostaglandins, thromboxanes and leukotrienes. Previous reports have shown that increasing the availability of n-3 polyunsaturated fatty acids results in a decreased proportion of arachidonic acid (20:4n-6) and an increased proportion of n-3 fatty acids in immune cell phospholipids, including neutrophils, monocytes, T lymphocytes and B lymphocytes [5]. Eicosanoids are also involved in modulating the intensity and duration of inflammatory and immune responses. The effects of PGE_2 and LTB_4 have been widely studied, demonstrating that PGE_2 has many pro-inflammatory effects that increase the vascular permeability and vasodilation; suppress lymphocyte proliferation and natural killer cell

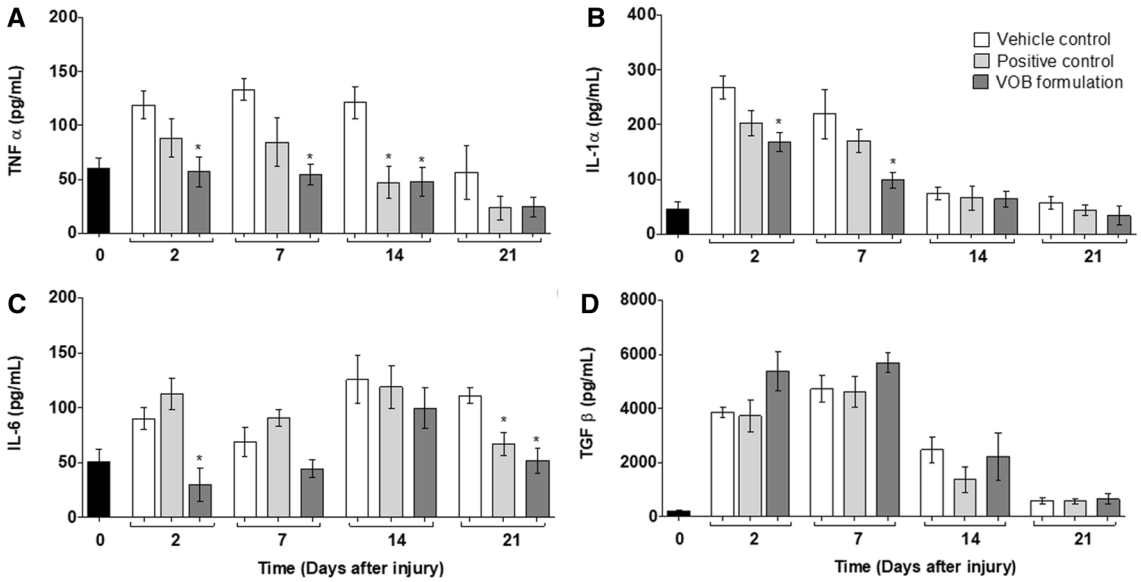

Fig. 5 VOB modulates cytokine production in the skin wound biopsies. Tissue homogenates were prepared from the wound biopsies obtained from animals treated with the VOB formulation, positive control or vehicle control at days 0, 2, 7, 14 and 21 post-wounding. **a** TNF-α, **b** IL-1 α, **c** IL-6, **d** TGF-β were assayed by ELISA. Data are mean ± SEM ($n = 10$ wounds/group). *$p < 0.05$ compared to vehicle control group

activity; and inhibit the production of tumour necrosis factor (TNF-α), interleukin-1 (IL-1), and IL-6 [5, 23].

Thus, during cutaneous wound healing process, an excessive production of inflammatory mediators (cytokines, chemokines, prostanoids) can lead to impaired wound healing resulting in aesthetic or even hypertrophic scars [23]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are n-3 fatty acids that can trigger the proper activation of these cells and, therefore, contribute to the healing process [1]. The influence polyunsaturated fatty acids on the functional behaviours of different cell types participating in inflammation and on the production of chemical mediators has been widely studied demonstrating that they may act in an anti-inflammatory manner, and they may be involved in the resolution of inflammation [5]. Therefore, the present results suggest that the VOB formulation may modulate the inflammatory response by inhibiting the chemotaxis of inflammatory cells and controlling the production and release of pro-inflammatory cytokines, especially IL-1, IL-6 and TNF-α, in the wound site and could, therefore, control the degree and duration of the inflammatory response, contributing to successful wound closure.

Many studies have shown that growth factors, such as TGF-β, keratinocyte growth factor, and platelet-derived growth factor, play roles in both physiological and pathological healing. TGF-β is considered a multifunctional cytokine that plays a central role in wound healing and in tissue repair. TGF-β expression both increases and reduces wound inflammation depending on the time and location of its expression [11, 18]. However, the production of TGF-β is vital for the control of the production of extracellular matrix components, and the massive and constant presence of TGF-β at the wound site can interfere with matrix deposition, in which case it does not provide benefits for healing and may even produce hypertrophic scarring [26]. Therefore, the VOB was shown to be effective in controlling the concentration of TGF-β, in modulating the presence of this growth factor in the initial and final stages, and in promoting an organised re-epithelization of the tissue formation and avoiding the formation of atrophic scars.

The proliferative and remodelling phases are important for full tissue recovery. During these phases, there is a large, gradual deposition of collagen newly synthesised by fibroblasts and keratinocytes [19]. Collagen is the most important protein in the connective tissue that forms the skin, and proper healing depends directly on the process of the production, regulation and deposition of this protein [19]. Therefore, the production of well-organised collagen fibres observed in the VOB group compared to the positive control and vehicle control groups is due to the VOB's ability to promote the proliferation and migration of responsive cells by the production of this protein.

Conclusion

In conclusion, under in vivo experimental conditions, developed VOB formulation accelerates the healing of wounds and promotes a rapid and controlled remodelling

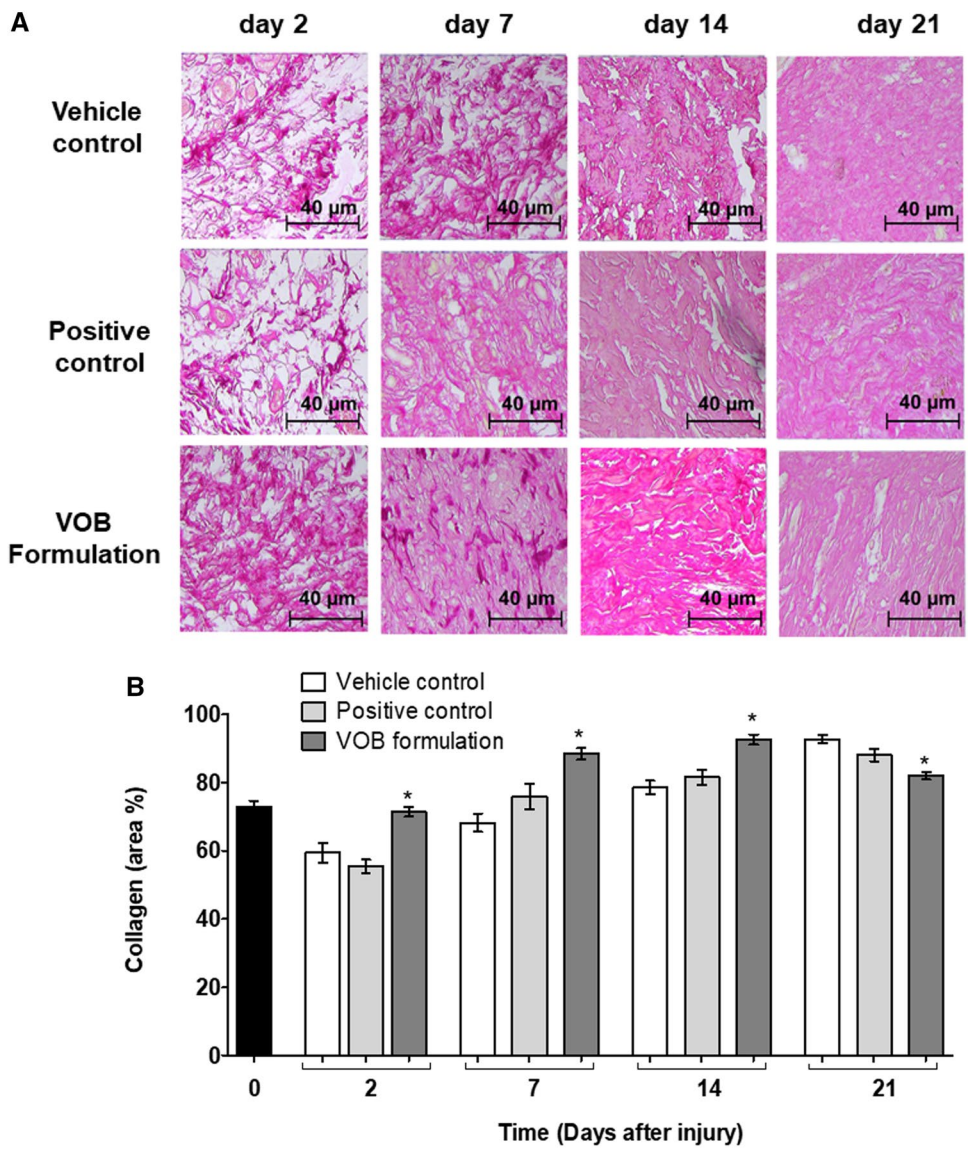

Fig. 6 Effects of VOB formulation on the collagen content in the wound tissue biopsies at days 0, 2, 7, 14, and 21 after injury. **a** Representative photomicrograph of wound tissue sections stained with Picro-Sirius red (×100). **b** Collagen content determined by digital densitometry using CellProfiler (CP) software. Data represent the mean ± SEM ($n = 20$ wounds/group), $^*p < 0.05$ compared to the vehicle control group

of the skin, contributing to the formation of an aesthetically acceptable scar. VOB prevents the overexpression of the inflammatory phase by decreasing the release of pro-inflammatory cytokines, thereby reducing the migration of polymorphonuclear cells to the wound site and promoting proper deposition of the extracellular matrix. In this context, the developed VOB formulation may be a promising and economically viable option for a topical application for wound healing and invasive aesthetic procedures.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards This work follows ethical standards and all experiments involving the use of animals were conducted in agreement with the Brazilian Animal Care Committee and were approved by the Committee of Ethics, Bioethics and Animal Welfare of the Universidade Vila Velha (UVV) (CEUA-UVV protocol 381/2016).

Informed consent Informed consent was obtained from all individual participants included in the study.

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