Ozonated oils as functional dermatological matrices: Effects on the wound healing process using SKH1 mice

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Graphical Abstract

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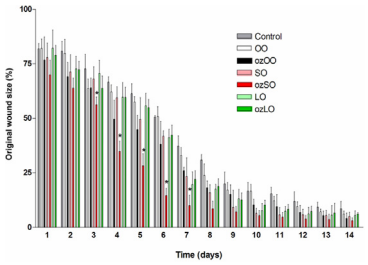

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Ozonated oils as functional dermatological matrices: Effects on the wound healing process using SKH1 mice

Q1 G. Valacchi[a,b,**], I. Zanardi[c], Y. Lim[b], G. Belmonte[d], C. Miracco[e], C. Sticozzi[a], V. Bocci[f], V. Travagli[c,*]

[a] Dipartimento di Scienze della Vita e Biotecnologie, Università degli Studi di Ferrara, Italy
[b] Department of Food and Nutrition, Kyung Hee University, Seoul, South Korea
[c] Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Italy
[d] Dipartimento di Scienze Biomediche, Università degli Studi di Siena, Italy
[e] Dipartimento di Scienze Mediche, Chirurgiche e Neuroscienze, Università degli Studi di Siena, Italy
[f] Dipartimento di Fisiologia, Università degli Studi di Siena, Italy

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ABSTRACT

Wound tissue repair is a complex and dynamic process of restoring cellular structures and tissue layers. Improvement of this process is crucial for several pathologies characterized by chronic delayed wound closure such as diabetes, and the investigation of new approaches aimed to ameliorate the wound healing process is under continuous evolution. Recently, the usage of vegetable matrices in the form of ozonated oils has been proposed and several researchers have shown a positive effect in the wound, based on their bactericidal, antiviral, and antifungal properties. The present study was undertaken to compare the effect that different ozonated oils (olive, sesame and linseed) with the same level of ozonation have on wound healing rate in SKH1 mice. Several histological parameters and the level of key proteins such as VEGF and PCNA have been analyzed. Only treatment with ozonated sesame oil shows a faster wound closure in the first 7 days. This effect paralleled with the increased VEGF and PCNA levels, NFκB nuclear translocation and 4-HNE formation. The present study shows that not only the ozonation grade is of importance for the improvement of wound healing process but also the typical composition of the oil.

1. Introduction

In normal skin, the epidermis (outermost layer) and dermis (inner or deeper layer) exists in a steady-state equilibrium, forming a protective barrier against the external environment. Once the protective barrier is broken, a set of complex biochemical events takes place in a closely orchestrated cascade to repair the damage. Such a process defined wound healing (WH) is immediately set in motion to prevent infectious events. The closure of cutaneous wounds involves complex tissue movements such as haemorrhage, inflammation, re-epithelization (proliferation), granulation tissue formation, and the late remodelling phase of repair (Werner and Grose, 2003).

Previous studies have demonstrated that endogenous factors are able to modulate and coordinate the healing process, such as vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF), cellular cycle proteins and even reactive oxygen species (ROS). All these factors are released and expressed from different cells involved in the WH process such as macrophages, fibroblasts, and keratinocytes at the site of injury and they participate in the regulation of re-epithelization, granulation tissue formation, collagen synthesis and neovascularization (Barrientos et al., 2008; de Melo et al., 2011).

The ability to modulate the levels and the release of the mentioned growth factors can influence the WH process. In addition, infection of the wound will delay the wound closure (Edwards and Harding, 2004).

Ozone (O_3) is widely recognized as one of the best bactericidal, antiviral, and antifungal molecules used in the therapy of chronic wounds. The beneficial effects of O_3 on WH might be assumed to be due to decreased bacterial infection, ameliorated dermal WH rate and increased oxygen tension by O_3 exposure in the wound area (Valacchi et al., 2012). In an aqueous environment, O_3 does not penetrate the cells but it instantaneously reacts with the double bonds of polyunsaturated fatty acids (PUFA) leading to the formation of

* Corresponding author at: Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Viale Aldo Moro, 2, 53100 Siena, Italy.
Tel.: +39 0577 234317; fax: +39 0577234254.
** Corresponding author at: Dipartimento Scienze della Vita e Biotecnologie, Università degli Studi di Ferrara, Via Borsari, 26, 44100 Ferrara, Italy.
Tel.: +39 0532455482; fax: +39 0532455550.
E-mail addresses: giuseppe.valacchi@unife.it (G. Valacchi), valter.travagli@unisi.it (V. Travagli).

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ROS and bioactive products such as aldehydes (Pryor and Church, 1991). These "second messenger" of O_3 reactivity, via the activation of redox transcription factors such as nuclear factor-kappaB (NFκB), can induce the synthesis of growth factors and accelerate the cell cycle.

Recently, it has been suggested that ozonated oils could be functional matrices to slowly deliver O_3 messengers and improve WH due to the ability of the oil to stabilize O_3 in a suitable chemical form (Travagli et al., 2010). Moreover, in an experimental, well-characterized skin lesion in mice it had been demonstrated that the repeated application of ozonated sesame oil (ozSO) was able to significantly accelerate the first phase of the wound closure which is more susceptible to infections (Valacchi et al., 2011). This effect was mainly observed at a certain level of ozonation, emphasizing that the peroxidation grade is an essential parameter to keep under consideration in the modulation of wound closure process. In fact, the peroxide value is an indirect outcome of the 1,2,4-trioxolane moiety that represents the active compound of the ozonated vegetable matrices (Travagli et al., 2009). Apparently, when this annular ozonide comes into contact with the altered skin in the presence of exudates at the body temperature, it decomposes to reactive ozone derivatives, which readily dissolve in the aqueous biological milieux generating both hydrogen peroxide and a variety of oxidized compounds that improve the local metabolism and enhances healing, even in the presence of skin infectious diseases. In the present paper, the relevance of three different oils such as olive oil, sesame oil and linseed oil at a specific degree of ozonation in a wound healing model using SKH1 mice had been studied in terms of: (i) wound closure rate; (ii) skin structure; (iii) activation of the transcription factor NFκB, together with the expression of key proteins involved in the wound healing process such as VEGF (vascularization) and proliferating cell nuclear antigen (PCNA) as well as the levels of oxidative stress markers such as 4-hydroxynonenal (4-HNE).

2. Materials and methods

2.1. Animals

Hairless female SKH-1 mice (6 weeks old) were purchased from Orient Bio Inc. (Gyeonggi-do, Korea) and lodged in individual plastic cages at temperature- and humidity-controlled conditions (22 ± 1 °C, RH 50–60%, 12 h light/dark cycle) with allowed access to distilled water and food. Mice were acclimated for 10 days before initiation of the treatment. Mice (n = 36) were divided in 4 groups, one group (n = 9) for each time-point (d0, d3, d7 and d14, as reported in Section 2.3). The animals were randomly selected at the start of the experiment and they were used in accordance with animal protocols approved by the Kyung Hee University Institutional Animal Care and Use Committee.

2.2. Wound biopsy and measurement of wound closure

The skin was pinched and folded on the dorsum (anaesthetization with isoflurane, sterile biopsy punch 3.5 mm diameter, Miltex Instrument Company, York, PA), below the shoulder so as to obtain height circular wounds identical in size, as previously described (Lim et al., 2006).

This area was selected for wounding as it cannot be reached by the mice thereby preventing self-licking and wound irritation. Wounds in individual mouse were photographed digitally every day, beginning on the day of wounding (d0) with a standard dot equivalent to the initial wound area placed beside the wound. The quantification of wound closure was performed as previously described (Lim et al., 2006). Wound closure was quantified by Canvas 11SE software (Deneba, Miami, FL). The rate of wound closure was expressed as the % ratio of wound area (each day after wounding) compared with the initial wound area. Decreased wound ratio indicates the amelioration of wound closure.

As to concern the immunohistochemical analysis skin samples were harvested at the following time points: days 0, 3, 7, and 14 (d0, d3, d7, and d14, respectively).

2.3. Ozonated oil and topical application

Olive oil (OO), sesame oil (SO), and linseed oil (LO) of pharmaceutical grade were purchased from Galeno Srl (Comeana, Italy). Table 1 summarizes the main chemical–physical properties of the different samples used. Specifically, the oils have been selected for their compositions in terms of n-3, n-6 as well as n-9 fatty acids. The real percentages as stated in the manufacturer's data sheet compared to the intervals reported in the literature (in brackets) have been reported (Crews et al., 2006; European Pharmacopoeia, 2010, 2011). Ozonated samples of olive oil (ozOO), sesame oil (ozSO), and linseed oil (ozLO) were obtained according to Zanardi et al. (2008) and Sega et al. (2010). The ozonation has been conducted until a peroxide value of about 1500 was reached. Physical characterization has been performed by viscosity measurements (Viscomate VM-10AL, CBC Europe) at both the temperatures of 22 and 35 ± 0.2 °C. Eight microlitres of untreated or ozonated oil was applied twice a day on the corresponding wound. As control groups, nothing was applied on the seventh and eight wound.

2.4. Immunohistochemical analysis

Surgical excision of the wounded area was performed at days 0, 3, 7, and 14 (d0, d3, d7, and d14, respectively) in each group. Skin tissue was immersion fixed in 10% NBF (neutral-buffered formalin) for 24 h at RT and embedding in paraffin block. Sections (4 μm) were deparaffinized in xylene and rehydrated in alcohol gradients (Sticozzi et al., 2012). For immunohistochemistry, after dewaxing, sections were incubated overnight at 4 °C with the following antibodies: VEGF (polyclonal antibody; Millipore, Milan, Italy; dilution factor 1:300; 0.05% trypsin–$CaCl_2$ buffer pH 7.8 pretreatment for 10 min at 37 °C, for antigen retrieval); NFκB (polyclonal antibody; Santa Cruz, DBA, Milan, Italy; dilution factor 1:100; Na-citrate buffer pH 6 pretreatment for 10 min at 95 °C); PCNA (polyclonal antibody; Biovision, Milan, Italy; dilution factor 1:100), and 4-HNE (polyclonal antibody; Millipore, Milan, Italy; dilution factor 1:200). Then the slides were washed three times with PBS and endogenous peroxidase was blocked with 3% hydrogen peroxide in distilled water for 10 min at RT. Finally, after aspecific binding block, they were incubated with the EnVision + System-HRP (DAKO, Glostrup, Denmark) for 1 h at RT. The reaction products were stained with diaminobenzidine (DAB), counterstained with Mayer's Haematoxylin and after drying were mounted with Eukitt mounting medium. The semi-quantitative analysis was performed by measuring the saturation of reaction by using Thresold colour HSB of ImageJ software.

2.5. Preparation of total and nuclear extracts for Western blotting

For Western blotting analysis, the tissue was homogenized and lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease and phosphatase inhibitors for total protein extracts, as previously described (Valacchi et al., 2009).

For nuclear extracts, tissue was homogenized and then lysed in hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.3% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, protease inhibitor cocktail, 1 mM orthovanadate, 5 mM β-glycerophosphate. The lysates

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Table 1
The main chemical–physical properties of the different samples used.

Sample	Chemical composition			Peroxide value (mEq/kg)	Viscosity (mPa s)	
	n-3 content	n-6 content	n-9 content		22 ± 0.2 °C	35 ± 0.2 °C
Olive Oil (OO)	0.6% (max 1.2%)[a]	8.8% (3.5–20%)[a]	72.9% (56.0–85.0%)[a]	143 ± 3	78.7 ± 1.2	37.5 ± 0.5
Sesame Oil (SO)	0.5% (0.2–1.0%)[b]	48.1% (36.9–46.8%)[b]	43.1% (34.1–44.7%)[b]	198 ± 9	59.9 ± 1	34.2 ± 0.3
Linseed Oil (LO)	53.7% (35.0–65.0%)[c]	14.3% (11.0–24.0%)[c]	21.5% (11.0–35.0%)[c]	163 ± 7	55.9 ± 0.5	30.8 ± 0.1
Ozonated Olive Oil (ozOO)	n.a.[d]	n.a.[d]	n.a.[d]	1612 ± 146	980 ± 23	470 ± 15
Ozonated Sesame Oil (ozSO)	n.a.[d]	n.a.[d]	n.a.[d]	1543 ± 44	108 ± 1.3	61.3 ± 0.3
Ozonated Linseed Oil (ozLO)	n.a.[d]	n.a.[d]	n.a.[d]	1535 ± 78	81.3 ± 0.5	38.2 ± 0.2

[a] European Pharmacopoeia (2011).
[b] Crews et al. (2006).
[c] European Pharmacopoeia (2010).
[d] Not available.

were incubated for 15 min on ice and then centrifuged at 1500 × *g* for 5 min at 4 °C for collection of the supernatants containing cytosolic proteins. Pellets containing crude nuclei were resuspended in extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.6 M KCl, 0.2 mM EDTA, 20% glycerol, and 0.5 mM phenylmethylsulphonyl fluoride, protease inhibitor cocktail, 1 mM orthovanadate, 5 mM β-glycerophosphate and then were incubated for 30 min on ice. The samples were centrifuged at 10,000 × *g* for 15 min to obtain supernatants containing nuclear fractions.

2.6. Western blot analysis

Briefly, 60 μg boiled protein was loaded onto 10% sodium dodecyl sulphate-polyacrylamide electrophoresis gels and separated by molecular size. Gels were electro-blotted onto nitrocellulose membranes and then blocked for 1 h in Tris-buffered saline, pH 7.5, containing 0.5% Tween20 and 5% milk. Membranes were incubated overnight at 4 °C with either VEGF (Millipore Corporation, Billerica, MA, USA) or β-actin (Cell Signalling; Celbio, Milan, Italy), and with either p65 subunit (Santa Cruz, CA, USA) or laminin B (Cell Signalling; Celbio, Milan, Italy). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h, and the bound antibodies were detected by chemiluminescence (BioRad, Milan, Italy). β-actin was used as loading control. 4′,6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI, 1:10,000, Sigma–Aldrich D9642) was used to counterstain the nuclei. Images of the bands were digitized and the densitometry analysis was performed using Image-J software.

2.7. Histological evaluation of wound healing

Histological qualitative assessment of wound healing on skin biopsies was performed using 4 μm thick H&E-stained sections. They were examined for the presence of the following features: ulceration, necrosis, re-epithelization, inflammatory cells, myofibroblasts, vascularization, mastocytes, and collagen fibre bundle organization. VEGF and PCNA-positive cells were counted in immunostained sections in five to ten high power fields (HPFs).

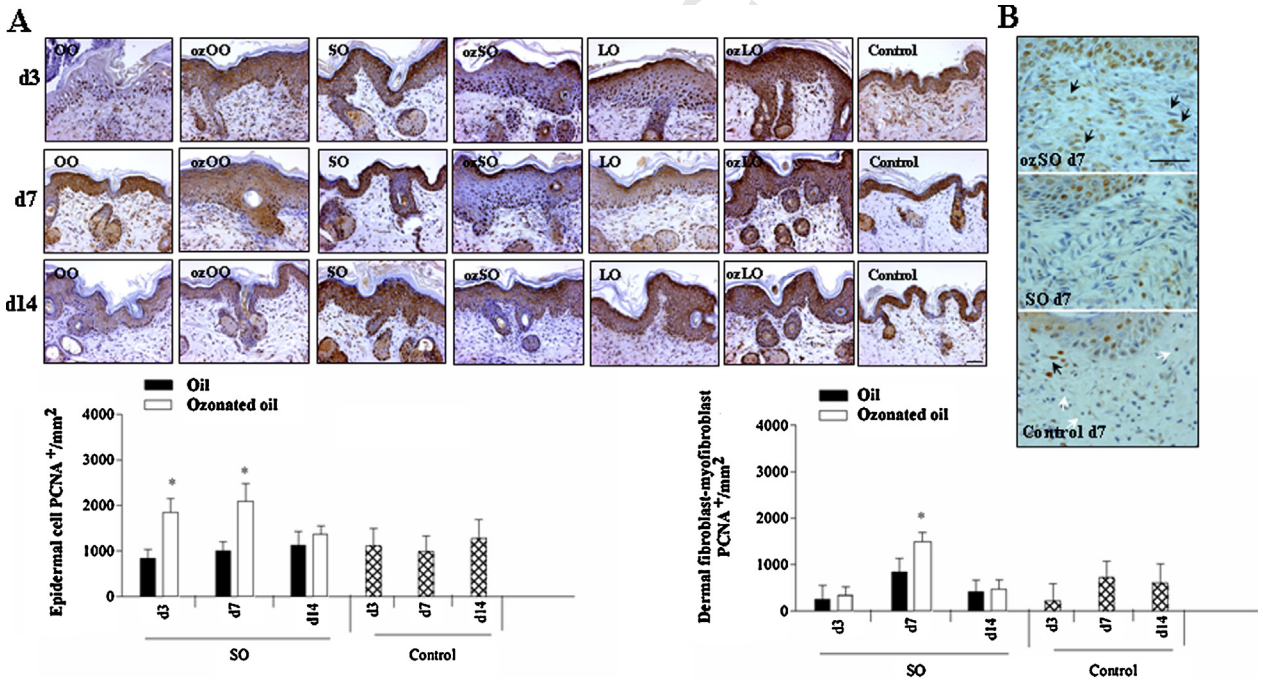

Fig. 1. Effects of ozonated oils on PCNA levels. PCNA staining at d3, d7 and d14 of murine skin after the different treatments. (A) A higher number of PCNA-positive nuclei of keratinocytes are evident in basal/suprabasal epidermal layers in ozSO, when compared to the other groups (original magnification, ×200; scale bar: 100 μm). Left diagram reports the number of cell PCNA positive in epidermis. (B) A higher number of PCNA-positive nuclei of fibroblasts/myofibroblasts are evident in dermal layers in ozSO (black arrows) with respect to control and SO treatment, where it is observed a persistence of inflammatory cells (white arrows; original magnification, ×400; scale bar: 50 μm). Right diagram reports the number of cell PCNA positive in dermis. Values represent mean ± SEM for three separate experiments. *$P < 0.05$.

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2.8. Picrosirius red staining of collagen fibres

Picrosirius red staining was performed on paraffin embedded sections. The sections were deparaffinized and rehydrated through five changes of decreasing gradient ethanol and the nucleus stained with Weigert's haematoxylin. Then, the sections were incubated in a solution containing picrosirius red 0.1% (Sigma–Aldrich Inc.) for 1 h at RT. Sections were then washed in two changes of acidified water, dehydrated in absolute ethanol, cleared in xylene, and mounted with Permount (Fisher Scientific Company). The sections were observed under light and polarization microscopy (Zeiss).

2.9. Statistical analysis

The results of quantitative parameters were statistically analyzed. The Kolmogorov–Smirnov test was applied to verify the normal distribution of sample data. In each group, the mean value per field was considered for epidermal thickness and microvessel density, and the median value per field for all the other parameters, for which a normal distribution was not observed. The Kruskal–Wallis and the paired Student's *t*-test were applied. A significance level of $p < 0.05$ was chosen.

3. Results

3.1. Effect of different ozonated oils on wound closure rate

Wound area was calculated from day 0 (d0) until complete closure of wound (d14), in terms of original wound size. As shown in Table 2 at d14 a wound closure greater than 90% was reached in all cases. It is evident that ozSO showed a significant increase in closure rate compared to SO already at d1 and it was statistical significant from d3 to d7 ($p < 0.05$). In detail, the increased WH rate was about 18% at d3, 42% at d4 and d5, 65% at d6 and 57% at d7. As far as ozOO, there was a similar trend observed for ozSO, but the wound closure rate was not statistically significant with respect to both air and OO controls. On the other hand, ozLO did not show any effect on wound closure rate.

3.2. Effect of ozonated oil on PCNA expression

Being WH process characterized by cellular proliferation, we have performed immunohistochemistry (IHC) analysis for PCNA, a marker of cellular proliferation and wound repair (Moldovan et al., 2007). As shown in Fig. 1, ozSO was able to clearly increase skin PCNA levels as also reported by the semi-quantification of positive keratinocytes (panel A) and fibroblasts/myofibroblasts (panel B). This increase was statistically significant at d3 (2.5-fold increase, $p < 0.05$) and at d7 (2-fold increase, $p < 0.05$) for keratinocytes and at d7 for fibroblasts/myofibroblasts ($p < 0.05$) going back to the control levels in the latest time points. Both ozOO and ozLO did not show any significant effect on PCNA levels, although ozLO seems to induce PCNA levels in the last part of wound healing process (data not shown).

At the end of the wound healing process (d14) while in ozSO the skin presents a normal architecture with only few inflammatory cells, in control the reparative phenomena is still evident with persistence of inflammatory cells as well as of myofibroblasts and fibroblasts (panel B).

3.3. Effect of ozonated oils on VEGF levels

VEGF has been shown to be the main player in the regulation of all the phases of the wound closure process. As shown in Fig. 2, both ozSO and ozOO increased VEGF production in the first 7 days

Table 2
Quantification of wound closure in terms of % of original wound size.

	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11	d12	d13	d14
Air	81.8 ± 2.5	80.7 ± 4.6	72.6 ± 6.7	66.6 ± 2.5	61.3 ± 4.6	50.6 ± 0.7	37.2 ± 4.7	30.8 ± 2.5	19.9 ± 5.4	16.5 ± 4.0	15.3 ± 3.0	11.9 ± 4.4	9.25 ± 2.2	8.47 ± 3.4
OO	82.1 ± 4.3	79.7 ± 6.6	63.6 ± 4.5	62.0 ± 3.2	57.5 ± 2.5	50.7 ± 4.6	33.0 ± 3.7	23.8 ± 5.3	17.1 ± 3.5	16.5 ± 3.2	12.1 ± 2.1	9.49 ± 2.2	7.17 ± 1.2	6.02 ± 1.5
OzOO	76.8 ± 10.6	69.1 ± 6.7	63.9 ± 4.4	49.7 ± 8.7	44.8 ± 6.6	38.1 ± 10.6	26.0 ± 1.5	18.1 ± 3.0	15.0 ± 4.4	10.3 ± 2.5	9.49 ± 5.4	6.82 ± 3.2	5.39 ± 2.1	4.06 ± 2.4
SO	77.8 ± 6.7	71.3 ± 7.8	68.1 ± 5.5	59.5 ± 5.0	49.6 ± 9.9	41.8 ± 2.5	23.3 ± 8.6	15.9 ± 3.7	9.21 ± 7.8	6.44 ± 2.3	5.70 ± 3.3	5.73 ± 2.5	5.39 ± 2.5	4.97 ± 1.2
OzSO	70.0 ± 6.7	63.9 ± 4.5	56.2 ± 3.6*	34.8 ± 4.6*	28.2 ± 5.6*	14.5 ± 3.3*	9.96 ± 4.7*	8.51 ± 3.4	7.1 ± 2.4	5.41 ± 2.3	4.62 ± 2.2	3.80 ± 1.56	3.62 ± 2.5	3.04 ± 1.3
LO	82.2 ± 8.3	72.6 ± 5.8	70.6 ± 6.0	59.7 ± 6.8	55.8 ± 5.7	41.2 ± 3.7	19.6 ± 5.8	17.4 ± 2.3	13.0 ± 4.4	7.68 ± 3.2	7.45 ± 2.1	6.18 ± 3.0	5.57 ± 4.4	5.40 ± 2.2
OzLO	78.8 ± 4.6	72.3 ± 3.7	63.7 ± 5.7	59.6 ± 4.7	54.9 ± 3.7	42.3 ± 4.6	21.9 ± 4.0	18.7 ± 3.5	12.5 ± 3.2	10.2 ± 2.1	8.30 ± 2.17	7.30 ± 2.45	6.46 ± 4.6	6.08 ± 0.7

Values represent mean ± SEM for three separate experiments.
* $P < 0.05$.

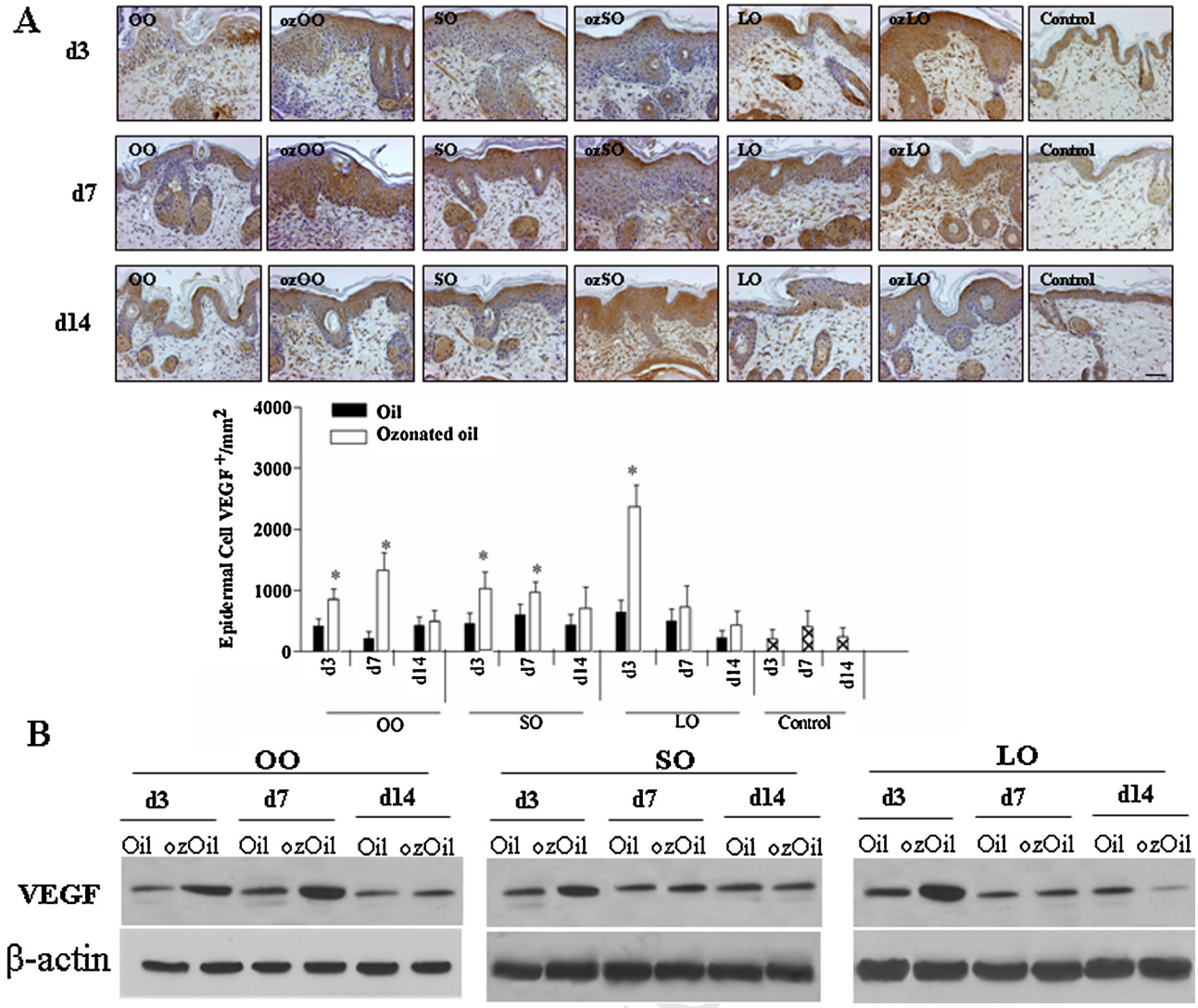

Fig. 2. Effects of ozonated oil on VEGF levels. (A) Vascular endothelial growth factors (VEGF) staining at d3, d7 and d14 of murine skin after the different treatments. VEGF positivity was observed in all the ozonated oils groups (ozOO, ozSO and ozLO) at d3, and only for ozOO and ozSO for d7 (original magnification, ×200; scale bar: 100 μm). The diagram reports the number of cell VEGF positive. Values represent mean ± SEM for three separate experiments; *$P < 0.05$. (B) Western blot for VEGF protein at d3, d7 and d14 of murine skin after the different treatments (ozOO, OO, ozSO, SO, ozLO and LO). Shown is a representative blot from three independent experiments; β-actin was used as loading control.

of the wound closure process. The induction of VEGF by ozOO was more evident than the ozSO at d7 (5-fold and 1.7-fold, respectively) while the effect was similar for d3 (circa 2-fold) (bottom panel). On the other hand, ozLO significantly induced VEGF levels at d3 (3-fold). These data were confirmed by Western blot analysis for VEGF protein, as shown in panel B.

3.4. Effect of ozonated oils on cutaneous 4-HNE protein adducts

Wound healing is a redox controlled process, where oxidative stress, ROS and lipid peroxidation products play a key role in its modulation (Moseley et al., 2004). Therefore, as a marker of lipid peroxidation and an index of oxidative stress (Uchida et al., 2004), we evaluated the levels of 4-HNE by IHC analysis during the wound closure. As shown in Fig. 3, ozSO showed the highest level of 4-HNE during the 14 days of wound closure. Cytoplasmic positivity to 4-HNE was mainly observed in suprabasal epidermal layers and this was much evident than in the samples treated with ozOO and ozLO. Untreated OO, SO and LO did not shown any difference from the controls.

3.5. NFκB activation

NFκB is a redox transcription factor, modulated by oxidative stress and peroxidation products and the role of 4-HNE in the activation of NFκB has been studied in several cells type (Ruef et al., 2001; Ali and Sultana, 2012; Meng et al., 2012). Since the levels of 4-HNE were increased only with the ozSO treatment, the activation of NFκB (p65 subunit) has been performed by both IHC and Western blot analysis only in the samples treated with ozSO. As shown in Fig. 4, ozSO induced a clear activation of NFκB in cutaneous tissues. NFκB nuclear positivity (short arrow) is detected, mainly in the upper epidermal layers at d3; it decreases at d7, and almost disappears on d14; in addition, several inflammatory/reparative cells in the dermis also show nuclear positivity to NFκB (long arrow). On the other hand, the control samples did not show a clear NFκB

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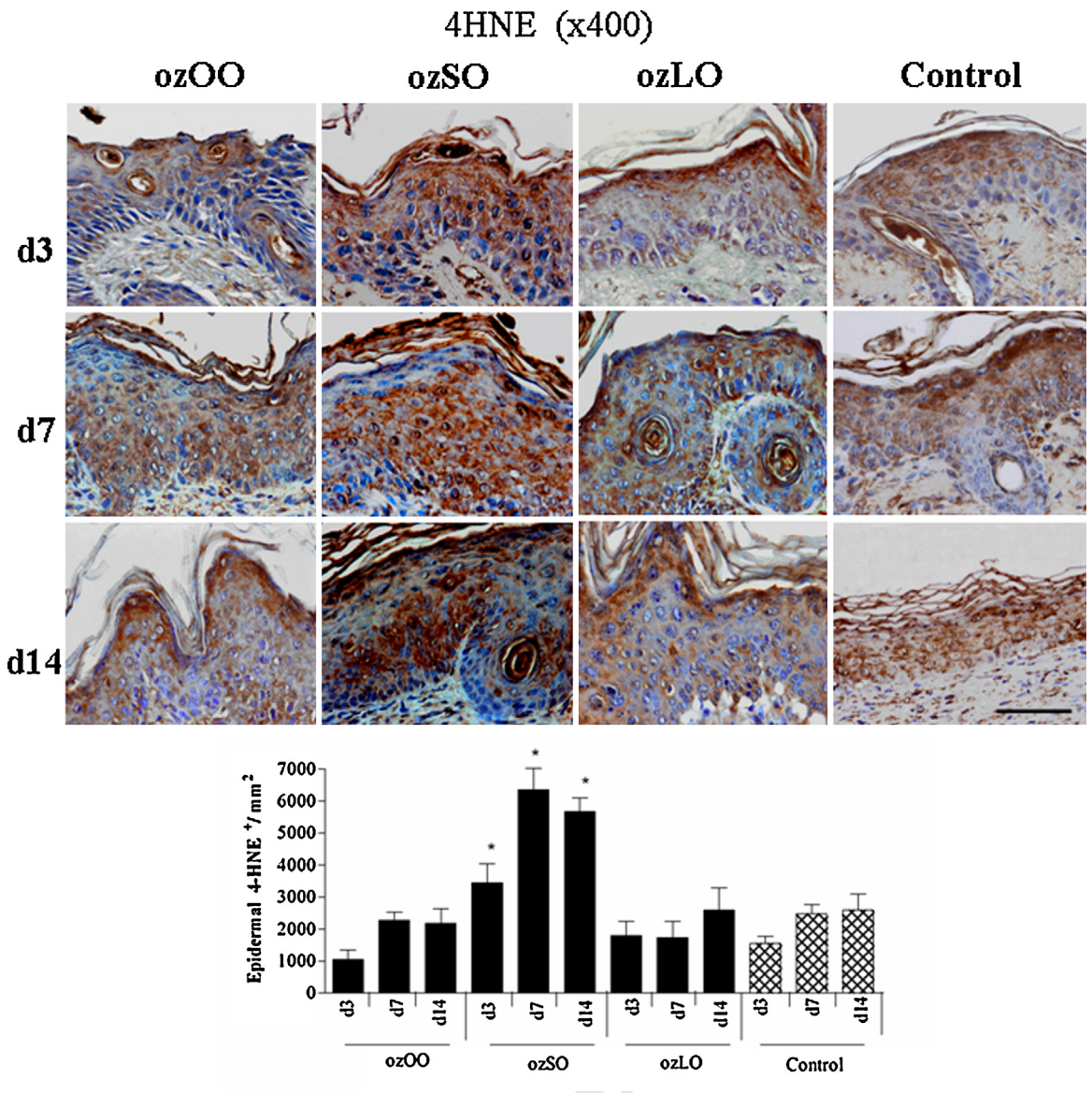

Fig. 3. Effect of ozonated oils on 4-HNE levels. At d3, as well as at d7 and d14, a cytoplasmic positivity to 4-HNE is mainly observable in suprabasal epidermal layers, with ozSO stronger than ozOO and ozLO (original magnification, ×400; scale bar: 50 μm).

nuclear positivity in the epidermis, whereas in the dermis positive nuclei were present in few inflammatory/reparative cells; furthermore, several inflammatory/reparative cell nuclei in the dermis are also positive to NFκB (long arrow). These data were also confirmed by Western blot analysis for p65 nuclear translocation using tissue nuclear extracts.

3.6. Effect of different ozonated sesame oil on skin collagen structure

As shown in Fig. 5 collagen fibre organization and morphology was examined by picrosirius red stained sections of wound closure skin in both ozSO and control wounds. The wounds treated with ozSO showed a less extension of the injury at d3 and d7 compared to the control (arrow). It was possible to note that in ozSO the wound closure rate is faster than in the control as indicated by the collagen fibres (star symbol). Furthermore, under polarization microscopy, the wounds treated with ozSO showed a most amount of type I collagen fibres (yellow/orange appearance) with respect to control. On the other hand, in the wounds treated with SO it is observed a persistence of type III collagen fibres (green appearance) characterizing the granulation tissue.

4. Discussion

This work is the continuance of our previous study where we have shown that only a specific level of peroxidation (expressed as peroxide value about 1500) of ozonated sesame oil is able to significantly accelerate the first phase of wound healing in SKH1 mice (Valacchi et al., 2011). Therefore, this study was performed to evaluate the wound healing properties of different ozonated oils, such as olive oil, sesame oil and linseed oil with the same peroxide values. The question is: is the peroxidation level the only factor responsible for the wound healing properties of ozonated oils?

In general, our data agreed with our previous report, showing the beneficial effect of ozSO on the first phase of the wound healing process (Valacchi et al., 2011). However, the same effect was not appreciable in the other oils used in the present study. The ozOO treatment has to some extent, a similar trend of ozSO, but it was not statistical significant. This is partially in agreement

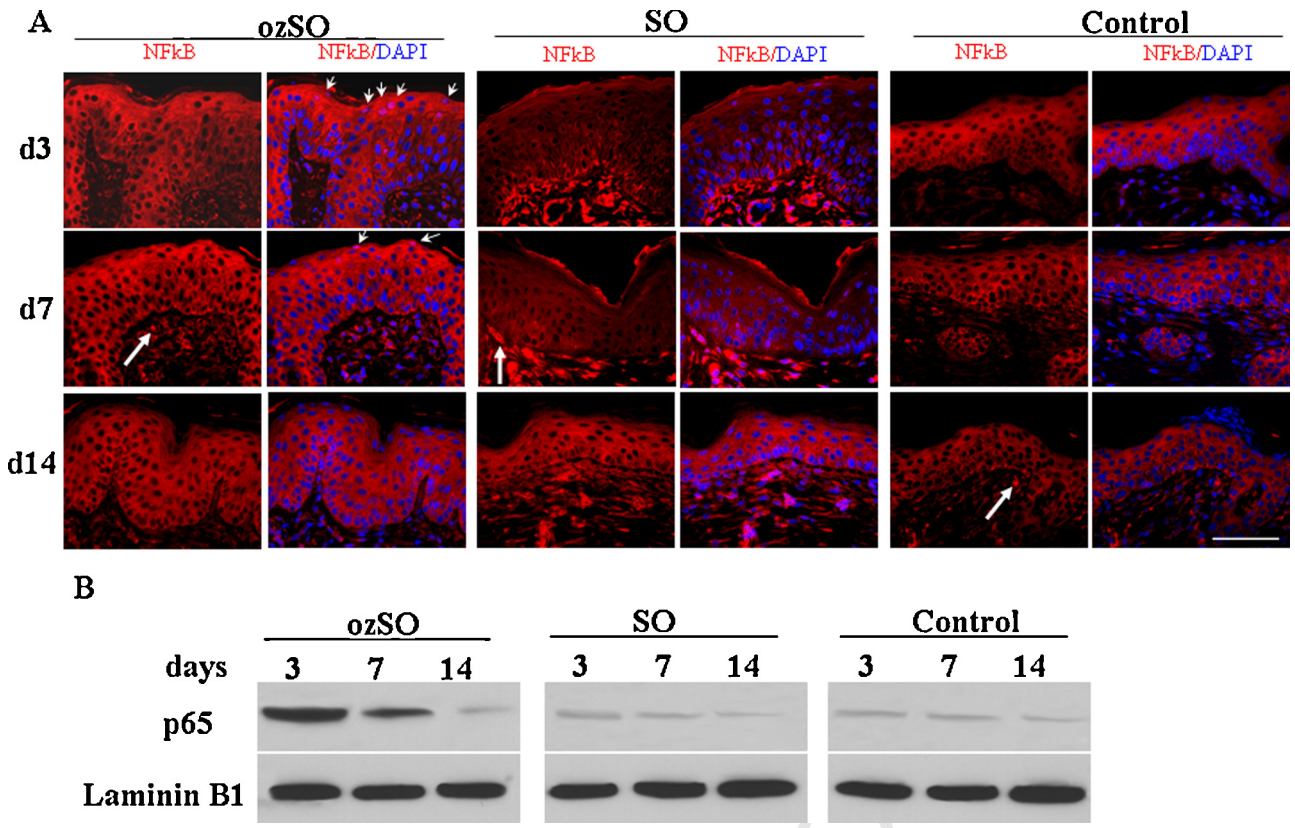

Fig. 4. Effect of ozSO on NFκB activation. (A) In ozSO, an NFκB nuclear positivity (short arrow) is detected, mainly in the upper epidermal layers at d3; it decreases at d7, and almost it disappears on d14; several inflammatory/reparative cells in the dermis also show nuclear positivity to NFκB (long arrow). In the control, no NFκB nuclear positivity is observable in the epidermis, whereas in the dermis positive nuclei are detectable in few inflammatory/reparative cells (long arrow). DAPI was used to blue stain the cell nuclei (original magnification, ×400; scale bar: 50 μm). (B) Activation of NF-κB was determined by the translocation in the nucleus of p65 subunit after the ozSO or SO treatments of murine skin at d3, d7 and d14. Shown is a representative blot from three independent experiments; β-actin was used as loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with the work of Kim et al. (2009) where the therapeutic effects of commercial ozonated olive oil was evaluated in cutaneous wound healing using guinea pigs as an animal model. It is anyway very difficult to compare the two studies since in the study from Kim et al. (2009) the ozonation levels of the olive oil is not indicated. As previously reported (Valacchi et al., 2011), the peroxidation levels of the ozonated oil is one of the main factors that seems to be involved in the wound healing process. However, the data presented in the present paper only partially confirm our idea of the role of peroxides in skin reparative events. In fact, based in our hypothesis, all the ozonated oils should have had the same effect on WH, since they have the same amount of peroxide.

One reason of the different effect on wound healing from the ozonated oils used could be their different composition in PUFA. In fact, there is a clear difference in the level of n-6 and n-3 fatty acids among the three oils, with almost 3 times more n-6 in the sesame oil compared to the OO and LO (Table 1). Different rheological behaviours due to inherent differences in the molecular configuration of unsaturated acyl chains after ozonation could provide further explanation for the differences observed on the wound parameters. It has been demonstrated that ozone easily reacts with PUFA of the vegetable oils firstly forming the trioxolane moiety that when an ozonated oil is applied on the wound containing exudates it decomposes releasing its derivative products such as aldehydes and H_2O_2 (Travagli et al., 2009, 2010). In our study we have shown that the levels of 4-HNE in the skin treated with ozSO was higher compared to the other treatments and this could be the consequence of the higher level of n-6 present in SO compared to OO and LO (Crews et al., 2006; European Pharmacopoeia, 2010, 2011). In fact, 4-HNE derives mainly from the oxidation of n-6 PUFA, essentially arachidonic and linoleic acid (Poli et al., 2008). 4-HNE is an unusual compound containing three functional groups that in many cases act in concert explaining its high reactivity and because of its electrophilic nature, 4-HNE can form adducts with cellular protein nucleophiles. Indeed, the reactivity of 4-HNE explains its potential involvement in the modulation of enzymes activity, signal transduction and gene expression (Poli et al., 2008). 4-HNE is also an efficient cell signalling molecule able to modulate the expression of several genes and therefore it may influence important cellular functions such as cell growth, differentiation, and angiogenesis all process involved in wound healing (Leonarduzzi et al., 2004). An increasing bulk of literature indicates that 4-HNE, depending on its concentration, can have opposite effects: activating or inhibiting stress response mechanisms, such as mitogen-activated protein kinases (MAPKs), modulating redox-sensitive transcription factors such as NFκB (Amma et al., 2005; Lee et al., 2008). The activation of NFκB observed in the ozSO treated skin is most likely a consequence of the increased levels of 4-HNE. Among its several function, NFκB has been shown to be critical in modulating the healing process in injury (Sen and Roy, 2008). Fakhrzadeh et al. (2004) have reported that overexpression of superoxide dismutase not only prevents O_3 related changes in bronchoalveolar lavage fluid protein, macrophage number and hydroxyalkenal levels but also O_3-dependent activation of NFκB. These results associate therefore O_3 exposure to NFκB activation and parallel with our pioneering work where we have demonstrated that ozone exposure is able to activate NFκB in cutaneous tissue using the same animal model (SKH1 mice) (Valacchi et al., 2004). It should be mentioned that

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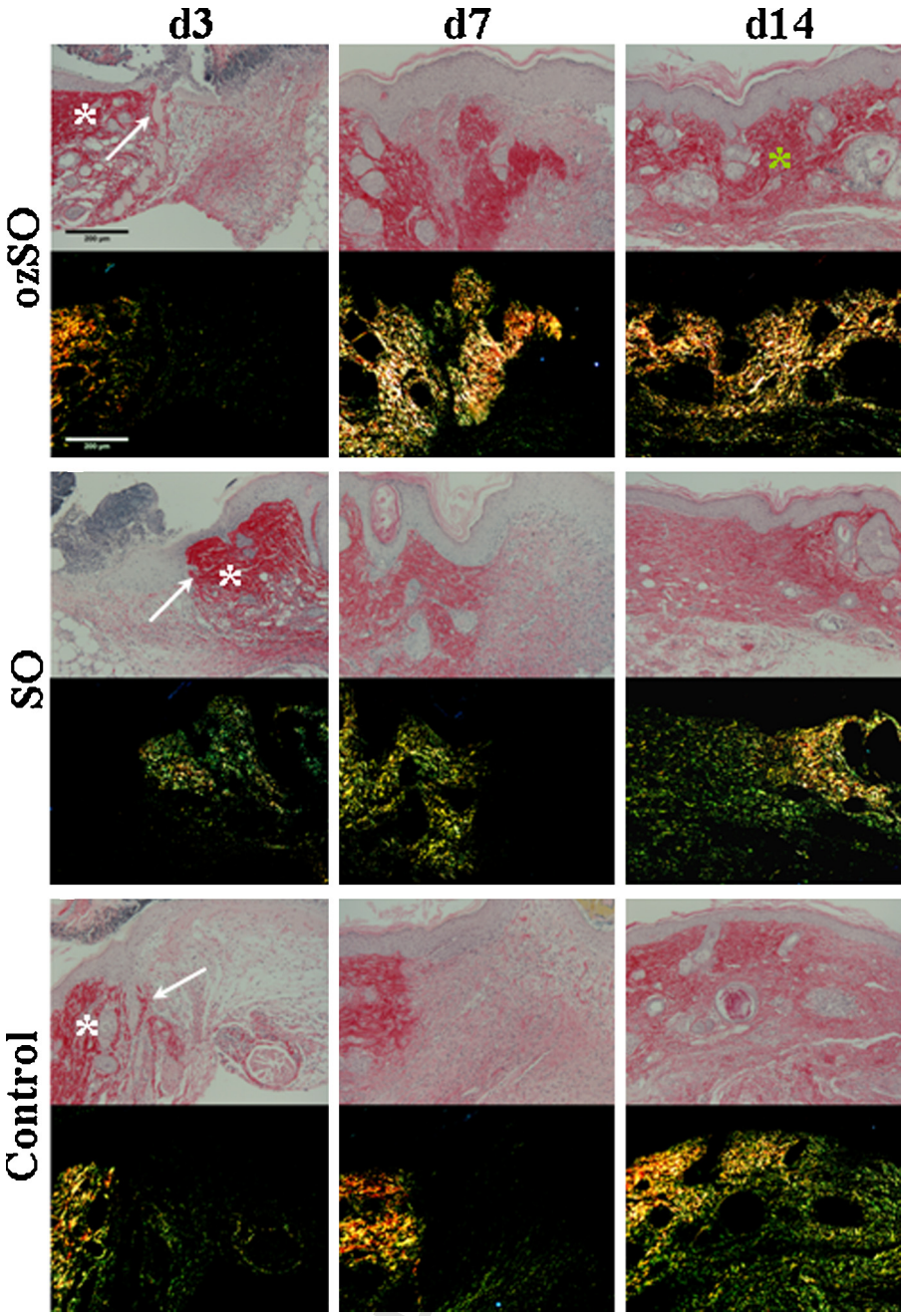

Fig. 5. Wound repair was analyzed by using Picrosirius staining. Under polarization microscopy, type I fibres are radiant yellow/orange while type III fibres are green. In the ozSO at d3, as well as at d7, the extent of injury (the area starting from the arrow) is less than in controls (SO and control), wound healing occurring more rapidly in ozSO (the white star symbol indicates the normal dermis, in which collagen fibres are stained in red by Picrosirius observed under light microscopy). At d14, in ozSO the dermis returns to be normal (green star symbol), with well-oriented Picrosirius red-stained collagen fibres. Instead, in the control the reparative phenomena are still evident, there are less collagen fibres than in ozSO (original magnification, ×100; scale bar: 200 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the dose-dependent effect relationship between level of oxidative stress (4-HNE) and NFκB exhibits a biphasic profile, consistent with the hormesis dose–response model (Calabrese, 2013): while a moderate levels of α, β-unsaturated aldehydes can activate NFκB through an IκB kinase independent mechanism, extremely high levels of α, β-unsaturated aldehydes have been shown to inhibit NFκB activation by blocking IκBα phosphorylation (Byun et al., 2002; Valacchi et al., 2005).

This could be the case for the delayed effect of highly ozSO (peroxidation levels of circa 3000), where the high levels of peroxidation had an inhibitory effect on the wound healing process. Such an effect could be a consequence of NFκB inhibition (Valacchi et al., 2011). This hypothesis would parallel previous work where exposure of aged mice to ozone would delay wound healing process because the high oxidative levels derived from both ozone exposure and the increased physiological oxidative stress present in the old animals (Lim et al., 2006).

NFκB activation has been also associated with VEGF and PCNA induction and this explains the increased levels of both proteins in the tissue treated with ozSO. In general, the events that characterize the wound healing process can be summarized as the inflammation stage (Phase I); proliferation with synthesis of extracellular matrix

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(Phase II) and the remodelling stage (Phase III) in which the scar is transformed to the healed wound. During this well coordinated process there is the appearance of several cell types that follow a specific order (platelets, neutrophils, macrophages, lymphocytes, fibroblasts and angiogenesis) and it has been shown that VEGF plays a critical role the regulation of all these events (Bates and Jones, 2003; Bao et al., 2009). Interestingly, although only the treatment with ozSO had a significant effect on the wound closure rate, VEGF was up-regulated also by ozOO treatment. This is in line with the previous work by Kim et al. (2009) where ozOO was able to clearly induce VEGF together with other growth factors (PDGF, TGFβ and FGF). It is possible that, in our experimental procedure, the treatment with ozOO is not able to activate all the pathways involved in the wound healing process. In addition, the increased level of VEGF did not parallel the eventual new vessels formation in the tissue, since there was not any difference in terms of angiogenesis with the control group. This discrepancy could be a consequence of the low level n-6 present in olive oil, and therefore the lower level of the "bioactive products" such as 4-HNE involved in this process. In fact, only the treatment with ozSO was able to significantly activate the proliferative biomarker PCNA. Re-epithelialization is a process of restoring the epidermis and consists of proliferation and migration of cutaneous cells to the wound edge to resurface epidermal defects (Li et al., 2007) and therefore the induction of PCNA explains the increased wound healing rate observed in the animal treated with ozonated sesame oil. In addition, it has been shown a direct correlation between NFκB activation and PCNA regulation and this is in support of our findings where we have detected an increased NFκB nuclear translocation in the tissue treated with ozSO.

In conclusion, these results demonstrate that application of ozonated oils, especially the ozonated sesame oil with a peroxidation value around 1500, can improve acute cutaneous wound repair in the SKH1 murine model by affecting the early phases of the process. Taken together, the data within presented suggest that topical application of specific vegetable matrices in the form of ozone derivatives may be considered as an alternative therapeutic modality to enhance cutaneous wound healing.

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