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# Immunobiology

# Oleic acid modulation of the immune response in wound healing: A new approach for skin repair

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## ARTICLE INFO

## ABSTRACT

Injury triggers inflammatory responses and tissue repair. Several treatments are currently in use to accelerate healing; however, more efficient formulations are still needed for specific injuries. Since unsaturated fatty acids modulate immune responses, we aimed to evaluate their therapeutic effects on wound healing. Skin wounds were induced in BALB/c mice and treated for 5 days with n-3, n-9 fatty acids or vehicle (control). n-9 treated mice presented smaller wounds than control and n-3 at 120 h post-surgery (p.s.). Collagen III mRNA, TIMP1 and MMP9 were significantly elevated in n-9 group compared to n-3 or vehicle at 120 h p.s. Among the inflammatory mediators studied we found that IL-10, TNF-$\alpha$ and IL-17 were also higher in n-9 treated group compared to n-3 or vehicle at 120 h p.s. Interestingly, COX2 had decreased expression on wound tissue treated with n-9. Inflammatory infiltrate analysis revealed diminished frequency of CD4$^+$, CD8$^+$ and CD11b$^+$ cells in n-9 wounds at 24 and 120 h p.s., which was not related to cell death, since in vitro apoptosis experiments did not show any cell damage after fatty acids administration. These results suggested that unsaturated fatty acids, specifically n-9, modulate the inflammation in the wound and enhance reparative response in vivo. n-9 may be a useful tool in the treatment of cutaneous wounds.

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## Introduction

Skin serves as a protective barrier against the environment and loss of cutaneous integrity, in the absence of adequate repair, can lead to major disability or even death. Wound healing is a complex process which involves inflammation, formation of granulation tissue, reepithelialization, matrix formation and remodeling (Cardoso et al. 2004). Usually, injury activates local and systemic immune response with inflammatory reaction and complex interactions among cells and soluble mediators (Schaffer and Barbul 1998). Blood components are also released activating the clotting cascade which induces hemostasis and provides a matrix for the influx of macrophages, neutrophils and lymphocytes (Cardoso et al. 2004; Martin and Leibovich 2005). Besides leukocyte recruitment and production of mediators, the synthesis and remodeling of extracellular matrix (ECM) during wound repair are regulated by molecules like growth factors, cytokines, metalloproteinases (MMP) and their inhibitors (TIMPs) (Moali and Hulmes 2009). MMPs eliminate damaged proteins, destroy the provisional ECM, facilitate cell migration to the centre of the wound, remodel the granulation tissue and regulate the activity of some growth factors (Armstrong and Jude 2002; Birkedal-Hansen 1995; Moali and Hulmes 2009; Rayment et al. 2008).

Along with growth factors, cytokines, and chemokines, the establishment of the inflammatory reaction also involves the release of arachidonic acid metabolites as a relevant step at the beginning of the wound repair. Polyunsaturated fatty acids are the primary precursors of many of these lipoic mediators with crucial functions in inflammation (Bohmig et al. 1997; Bouwens et al. 2009; Calder et al. 2002; Yaqoob 1998). While n-3 and n-6 participate in the biosynthesis of several lipoic mediators, they may also act as structural components, together with n-9 monounsaturated, for the synthesis of membrane phospholipids, thus contributing to the cell membrane physiology in mechanisms such as signaling transduction and cell proliferation (Ziboh et al. 2000).

Since the primary objectives of the treatment of wounds are rapid wound closure with functional and aesthetically satisfactory scar tissue, the utilization of drugs that modulate the inflammatory

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response and the development of more efficient formulations are of major importance in several disease conditions. Thus, the aim of this work was to evaluate the potential therapeutic effects of the unsaturated fatty acids n-3 and n-9 on the inflammatory response of cutaneous wound healing.

## Materials and methods

### Animal procedures

Approximately 200 BALB/c mice were divided in groups of 3–5 animals. Mice were aged (6–8 weeks old) and gender matched, housed in individual cages with water and food *ad libitum* in 12-h dark–light cycles. Each group was used as a unique data point in our experimental design; each data point was performed in duplicates or triplicates, depending on the experiment. Animals were anesthetized with tribromoethanol and shaved at the wounding site. A previously outlined elliptical area of approximately 20 mm$^2$ of skin was surgically removed from mice dorsal region and the wounds were immediately treated with topical application of 30 μM of each fatty acid (n-3, n-9 or vehicle), in a volume of 50 μl. This treatment was repeated daily for 5 days, as described previously (Cardoso et al. 2004). Since events in the initial inflammatory stage of repair can influence subsequent stages (Cardoso et al. 2004), animals were euthanized at time points 0 h (control non-wounded skin), 3 h, 6 h, 24 h and 120 h post-surgery (p.s.). Skin samples corresponding to the whole area of the healing wounds were carefully removed along with a 2 mm surgical margin at the wound borders. The scared tissue was dissected until reach the suprafascial areolar plan of the long dorsal muscle. The surgical procedure was standardized and performed in the same way in all animals. Technical replicates were performed and collected samples were used for gene expression analysis and phenotyping of infiltrating inflammatory cells. Protocols were approved by the School of Medicine of Ribeirão Preto Institutional Animal Care and Use Committee.

### Fatty acids preparation

Linolenic (n-3) and oleic (n-9) acids (Sigma Chemical Co., St. Louis, MO) were prepared, daily, at 30 μM, in a solution of 50% glycerol in 0.02 M Tris–HCl, pH 7.4 (vehicle) for use in animal model of wound healing. We also used ethanol as diluent for *in vitro* assays. All preparations were made at the moment of use, to avoid oxidation.

### Macroscopic analysis of wound closure

The elliptical wounds were measured daily with a caliper of 0.01 mm precision (Vernier Caliper, Mitutoyo, Japan) and the area ($S$) was calculated as $S = \pi ab$, where $a$ and $b$ corresponded to one-half of the largest and one-half of the smallest diameter (measured from the edges of the original skin incision), respectively. All measurements were performed directly on the animals, by the same examiner. Wound closure was defined as percentage of reduction in the area of the original wounds. The macroscopic closure was also accompanied by photographic documentation, as shown in "Results".

### Gene expression analysis

Total RNA from skin wounds was extracted using Promega RNA extraction kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized with 1 μg of total RNA in a reverse transcription reaction using M-MLV reverse transcriptase (Promega, Madison, WI, USA).

**Table 1**
Primers sequences used in real-time PCR assays.

| Primers | | Sequences |
|---|---|---|
| β-actin | Sense | AGC TGC GTT TTA CAC CCT TT |
| | Antisense | AAG CCA TGC CAA TGT TGT CT |
| Cyclooxygenase-2 | Sense | AGC CTT CTC CAA CCT CTC CTA |
| | Antisense | CAC CTC TCC ACC AAT GAC CT |
| Collagen III | Sense | CTG CTG CCA TTG CTG GAG TTG |
| | Antisense | TGC AGC CTT GGT TAG GAT CAA |
| IFN-γ | Sense | GCA TCT TGG CTT TGC AGC T |
| | Antisense | CCT TTT TCG CCT TGC TGT TG |
| TNF-α | Sense | TGT GCT CAG AGC TTT CAA CAA |
| | Antisense | CTT GAT GGT GGT GCA TGA GA |
| IL-10 | Sense | TGG ACA ACA TAC TGC TAA CC |
| | Antisense | GGA TCA TTT CCG ATA AGG CT |
| TGF-β | Sense | GCT GAA CCA AGG AGA CGG AAT |
| | Antisense | GCT GAT CCC GTT GAT TTC CA |
| IL-17 | Sense | TGC CCT CCA CAA TGA AAA GA |
| | Antisense | AAC ACG AAG CAG TTT GGG AC |
| MMP2 | Sense | CGG AGA TCT GCA AAC AGG ACA |
| | Antisense | CGC CAA ATA AAC CGG TCC TT |
| MMP9 | Sense | GCG TGT CTG GAG ATT CGA CTT |
| | Antisense | TAT CCA CGC GAA TGA CGC T |
| TIMP1 | Sense | CTA TCC CTT GCA AAC TGG AGA |
| | Antisense | ACC TGA TCC GTC CAC AAA CA |

MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP.

Real-time PCR analyses were performed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Warrington, UK) using SYBR Green Platinum PCR Super Mix (Invitrogen, Carlsbad, USA). The standard PCR conditions were 95 °C/10 min, 40 cycles for 1 min/94 °C, 56 °C (1 min), and 72 °C (2 min), followed by the standard melting curve. PCR primers were used in a final concentration of 100 nM. Primers' sequences are listed in Table 1. Threshold for positivity of real-time PCR was determined based on negative controls. The results represent mRNA expression changes on n-3, n-9 or *vehicle treated* animals, relative to naïve non-wounded skins. $\Delta \Delta C_T$ calculation was based on the Applied Biosystems User's Bulletin #2 (P/N 4303859), in which the final data was the result of $2^{-\Delta \Delta C_T}$ calculations, by reference to the β-actin in each sample, using the cycle threshold ($C_T$) comparisons. The amount of targets was normalized to the endogenous reference β-actin and relative to the calibrators, which consisted of the medium of mRNA expression of naïve non-wounded skins, as described above. Negative controls without RNA and without reverse transcriptase were also performed. Results show one representative replicate of two, with 3–5 animals by group.

### Flow cytometry

To characterize the inflammatory infiltrate in the lesions, the skin samples were dissociated with 50 μg/ml liberase CI enzyme solution (Roche Diagnostics GmbH, Mannheim, Germany) using a Medimachine (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Tissue homogenates were filtered, leukocytes viability evaluated by trypan blue exclusion and cells were used for immunolabeling assays. Cell suspensions were incubated with anti-CD16/CD32 mAb (Fc block, Clone 2.4G2-Pharmingen, San Jose, CA, USA) followed by incubation with anti-CD4 PerCP (BD Biosciences Pharmingen, San Jose, CA, USA), anti-CD8 FITC-conjugated (BD Biosciences) or anti-CD11b PE-conjugated antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Cell analysis was based on side/forward scatter gates for lymphocyte or monocyte enriched populations and then gated on CD4/CD8 or CD11b positive cells respectively (FlowJo software). The isotype controls used were rat IgG2a-PerCP, rat IgG2b-FITC and rat IgG1-PE (BD Biosciences PharMingen). Results demonstrated the mean ± SEM of the percentage of each antibody
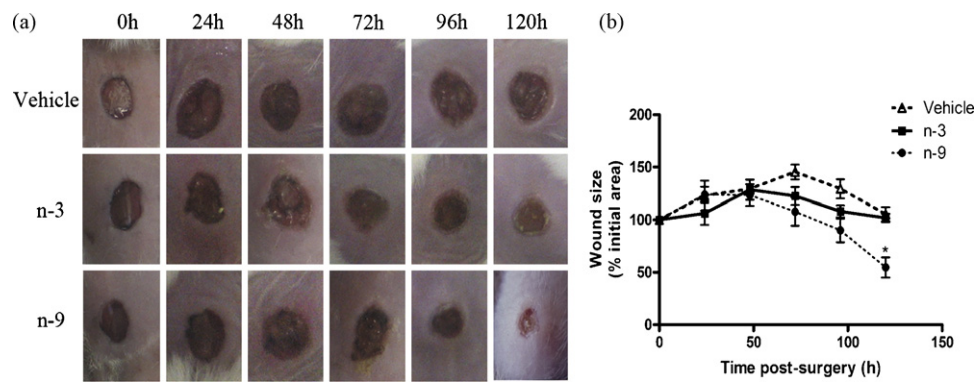
**Fig. 1.** n-9 fatty acids induced faster wound closure when compared to control and n-3 treated mice. Skin wounds were induced in the dorsal region of BALB/c mice and treated daily for 5 days with topical application of 30 μM of n-3, n-9 fatty acids or vehicle (control). Wounds were measured daily with a caliper and photographed. Macroscopic wound closure on 0 h, 24 h, 48 h, 96 h and 120 h post-surgery (p.s.) is shown in (a) and measurements of wound area along the experimental period of treatment in (b). Results are representative of three independent experiments. *$p < 0.05$ (n-3 vs. n-9 and n-9 vs. vehicle).

specific stained subpopulation within the gated cells. Cytometric analysis was performed with wound samples obtained from five animals per data point.

### Apoptosis assay

*In vitro* apoptosis assays were performed with mouse splenocytes. Briefly, cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 5% fetal calf serum (Sigma–Aldrich, St. Louis, MO, USA), $5 \times 10^{-5}$ M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mM non-essential amino acids, 2.2 M sodium pyruvate, 10 mM HEPES and 2.0 g/L sodium bicarbonate (all from Gibco), at 37 °C in a humidified atmosphere of 5% $CO_2$. The spleen cells were cultured in 6-well plates (Corning Inc. Life Sciences, Acton, MA, USA) at $5 \times 10^5$/well in 4 ml culture medium. Splenocytes were allowed to attach to the plate and were then incubated with ethanol (vehicle), n-3 or n-9 fatty acids at 30 μM. The final concentration of ethanol in the culture medium did not exceed 0.05%, a concentration known not to be toxic to the cells, as reported previously (Siddiqui et al. 2001). Cells cultured only with medium or saponin 0.01% were used as negative or positive controls, respectively. After 24 h of culture, cells were collected and stained with annexin V/7AAD for FACS acquisition and analysis of spleen cells apoptosis.

### Statistical analysis

Results were submitted to the one-way analysis of variance ANOVA, followed by Tukey's post-test. Values of $p < 0.05$ were considered statistically significant.

### Results

#### Fatty acids treatment induced differential wound closure

First, to verify the effects of fatty acids treatment on the cutaneous repair, we analyzed the lesions closure throughout the 5-day experimental period. n-9 fatty acids induced faster healing of the injured skin, as observed by macroscopic (Fig. 1a) and morphometric (Fig. 1b) analysis. These differences could be observed as soon as 72 h, 96 h post-surgery (p.s.) and were more pronounced on 120 h p.s., when observed differences were statistically significant (Fig. 1b, $p < 0.05$). n-3 treated lesions were significantly larger than n-9 at 120 h p.s. (Fig. 1b, $p < 0.05$) but not different from those that received the fatty acids vehicle as treatment (control). Additionally, n-9 treated mice presented less edema and a thinner fibrin

clot cover when compared to control and n-3 treated lesions, during the 120 h observation period (Fig. 1a).

#### n-9 fatty acids down regulated cyclooxygenase-2 (COX2) and induced collagen III expression

Because n-9 treatment induced faster wound closure, we evaluated if unsaturated fatty acids could modulate inflammation and tissue repair by interfering with the mRNA expression of cyclooxygenase-2 enzyme and collagen III, which are important molecules for the production of pro-inflammatory mediators and extracellular matrix (ECM) deposition, respectively. We observed that COX2 mRNA was significantly increased in n-3 treated wounds at 24 h (Fig. 2a, n-3 vs. vehicle, $p < 0.05$) and 120 h p.s. In the same time points of observation, mRNA expression for COX2 was notably decreased in n-9 treated animals, with transcription levels lower than non-wounded control samples (Fig. 2a, n-3 vs. n-9, $p < 0.05$). On the other hand, collagen III expression was increased 120 h p.s., especially in n-9 treated group in comparison to n-3 treated mice, as shown in Fig. 2b (n-3 vs. n-9, $p < 0.05$).

#### n-9 fatty acids modulated cytokine expression in the wounds

To evaluate the modulation of the inflammatory/immune response by unsaturated fatty acids, real-time PCR was performed on healing skins to quantify the expression of cytokines related to wound repair. Although it is known that TGF-β is important in inflammation, angiogenesis, reepithelialization, and connective tissue regeneration in the repair process (Denton et al. 2009; Kane et al. 1991), our results did not point to differences in the expression of this cytokine (Fig. 3d) nor IFN-γ (Fig. 3a) among the groups at any time period analyzed. Even so, a trend towards higher expression of IFN-γ could be observed in later times independent of mice treatment (Fig. 3a). On the other hand, mice which received the topical treatment with the monounsaturated fatty acid n-9 showed increased gene transcription for TNF-α (Fig. 3b), IL-10 (Fig. 3c) and IL-17 (Fig. 3e) when compared to n-3 and vehicle, especially at 120 h p.s. ($p < 0.05$). Moreover, increased gene expression for IL-10 could also be observed in n-9 treated mice as early as 6 h p.s. (Fig. 3c, $p > 0.05$).

#### Fatty acids modified tissue repair by altering metalloproteinase balance

To understand the ECM remodeling by unsaturated fatty acids, real-time PCR assays for MMPs and its inhibitors were performed in treated and control skin samples at different time points p.s.
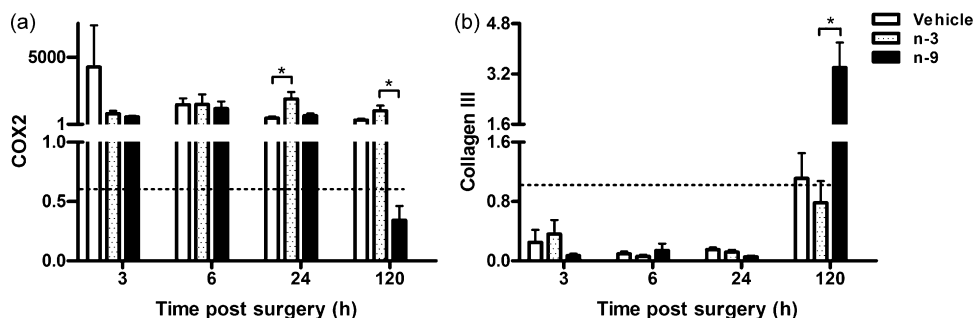
**Fig. 2.** n-9 fatty acids downregulated COX2 and induced collagen III expression. Cyclooxygenase (COX)-2 (a) and collagen III (b) mRNA was quantified by real-time PCR at different moments post-surgery, as described in "Materials and methods". Results were demonstrated as mRNA expression in the wounds relative to non-wounded skins. The relative levels of gene expression were calculated by reference to the β-actin in each sample, using the cycle threshold ($C_T$) method and were expressed as arbitrary units. Data (mean ± SEM) represent values from one experiment representative of two, with 3–5 mice by group. Dashed lines represent values of mRNA expression in control non-wounded skin samples. $*p < 0.05$.

A decrease was observed in MMP2 mRNA expression in n-3 and n-9 treated mice at 24 h p.s. (Fig. 4a, n-3 vs. vehicle and n-9 vs. vehicle, $p < 0.05$), which was followed by an increase in MMP9 and in the metalloproteinase inhibitor TIMP1 in n-9 group 120 h p.s. when compared to $n-3$ and control (Fig. 4b and c, n-9 vs. n-3 and n-9 vs. vehicle, $p < 0.05$), suggesting that fatty acids treatment of the wounds directly affects tissue remodeling.

*n-9 fatty acids reduced inflammatory infiltrate in the skin wounds*

To assess if fatty acids treatment, specifically n-9, induced faster wound closure by inhibiting leukocyte accumulation in the lesions, treated and control skin samples were collected and processed for FACS analysis. As demonstrated in Fig. 5a–c, treatment with n-9 reduced the number of macrophages (CD11b+ cells), CD4 and CD8 lymphocytes that infiltrated the wounds at 24 h p.s. The differences among n-9 and n-3 or vehicle treated mice were more prominent and statistically significant at 120 h p.s, when analysis revealed diminished detection of CD11b+ (Fig. 5d, n-9 vs. vehicle, $p < 0.05$), CD4+ (Fig. 5e, n-9 vs. vehicle and n-9 vs. n-3, $p < 0.05$) and CD8+

cells (n-9 vs. n-3, $p < 0.05$) in the lesions treated with oleic acid (n-9).

*n-9 fatty acids treatment did not induce cell death*

To clarify whether diminished inflammatory infiltrate in the lesions could be related to cell death instead of modulatory effect of the fatty acids, we performed apoptosis assays in cultured splenocytes after n-3 or n-9 treatment. Results showed that *in vitro* treatment of splenocytes with the fatty acids or their diluent (ethanol), for 24 h did not induce any cell damage after their administration, as demonstrated by annexinV/7AAD staining of the samples (Fig. 6, apoptosis related to medium). Our results suggested that fatty acids treatment of the wounds was not toxic to leukocytes and reduced the inflammatory infiltrate in the lesions due to its immunomodulatory effects.

**Discussion**

Wound healing is a coordinated process that includes inflammatory reaction and formation of scar. A variety of therapies are
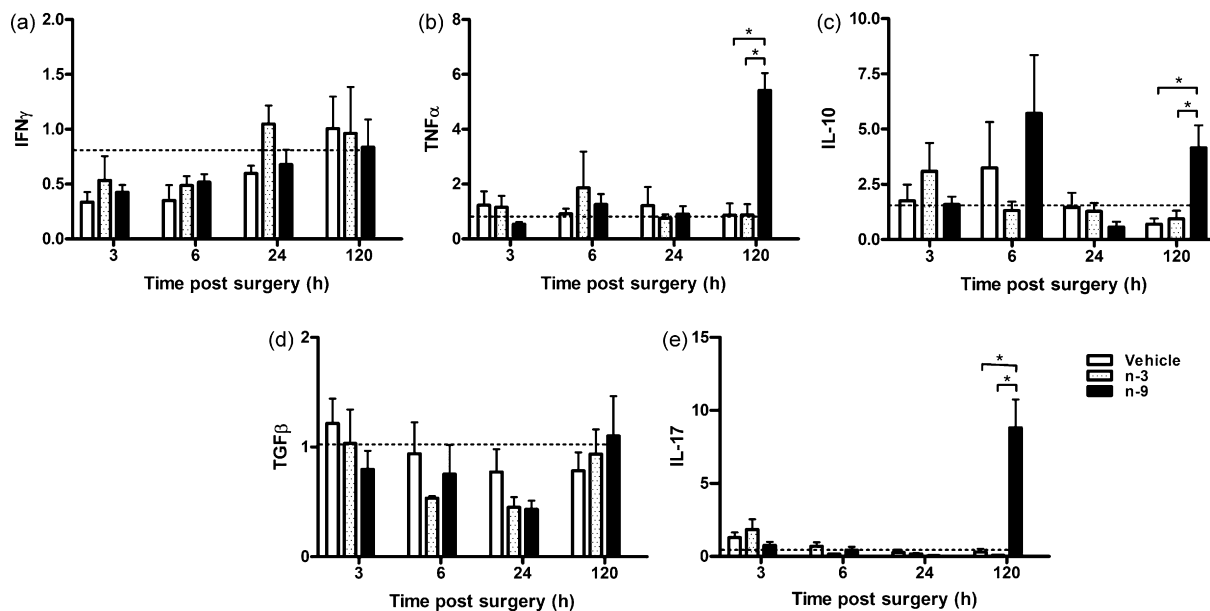


**Fig. 3.** Fatty acids treatment modulated cytokine expression in the wounds. IFN-γ (a), TNF-α (b), IL-10 (c), TGF-β (d) and IL-17 (e) mRNA was quantified by real-time PCR at different moments post-surgery, as described in "Material and methods". Results were demonstrated as mRNA expression in the wounds relative to non-wounded skins. The relative levels of gene expression were calculated by reference to the β-actin in each sample, using the cycle threshold ($C_T$) method and were expressed as arbitrary units. Data (mean ± SEM) represent values from one experiment representative of two, with 3–5 mice by group. Dashed lines represent values of mRNA expression in control non-wounded skin samples. $*p < 0.05$.
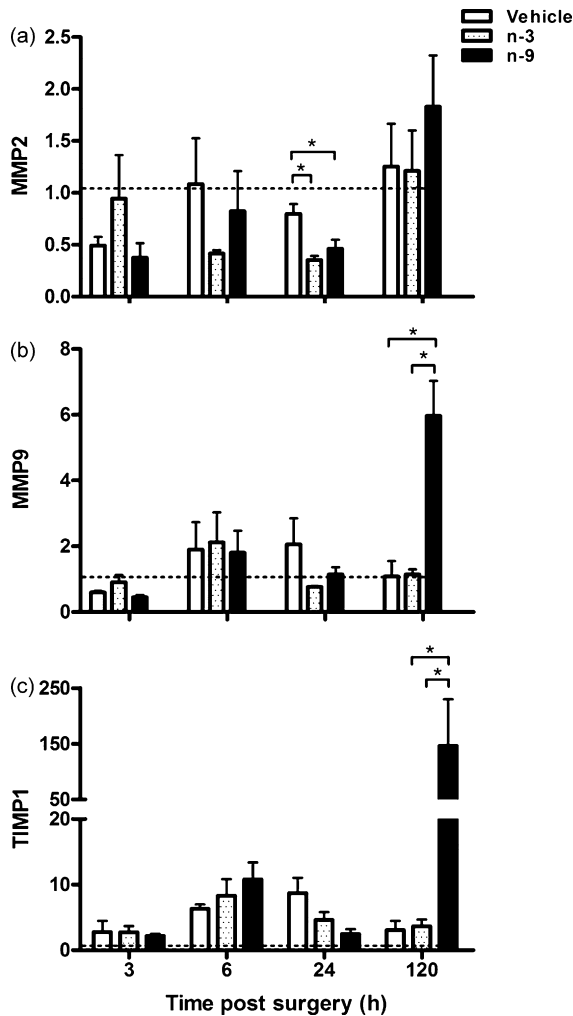
**Fig. 4.** Metalloproteinases (MMPs) and their inhibitors (TIMPs) are induced by n-9 fatty acids treatment. The levels of MMP2 (a), MMP9 (b) and TIMP1 (c) were quantified by real-time PCR at different moments post-surgery, as described in "Materials and methods". Results were demonstrated as mRNA expression in the wounds relative to non-wounded skins. The relative levels of gene expression were calculated by reference to the β-actin in each sample, using the cycle threshold ($C_T$) method and were expressed as arbitrary units. Data (mean ± SEM) represent values from one experiment representative of two, with 3–5 mice by group. Dashed lines represent values of mRNA expression in control non-wounded skin samples. $^*p < 0.05$.

currently in use to treat open and chronic wounds; however, more efficient formulations are still required in cases of unsuccessful or deficient repair. Therefore, in the present work, we evaluated the putative role of n-3 and n-9 unsaturated fatty acids in the modulation of the inflammatory and immune responses in the healing of cutaneous wounds.

It is known that unsaturated fatty acids can affect the gene expression of pro-inflammatory mediators (Grimm et al. 2002). In our study we found concomitant elevated expression of TNF-α and IL-17 in the oleic acid treated wounds. TNF-α is important in angiogenesis (Sainson et al. 2008), in the activation of endothelial cells and in the expression of adhesion molecules, thus contributing to the accumulation of phagocytes in the inflamed site (Cassatella 1999). In the present work, despite high TNF-α expression, we noted diminished leukocyte infiltration in oleic acid treated lesions. On the other hand, treatment of dry eye with n-3 is associated with a significant decrease in CD11b+ cells and diminished expression of corneal IL-1β, TNF-α, and conjunctival TNF-α (Rashid et al. 2008). In view of our results, we suggest that the kinetics of cytokine expression or the association between pro- and anti-inflammatory

molecules may be more decisive to the final wound closure than the production of a single cytokine. Moreover, IL-17 seems to be relevant in wound repair, since it was already associated with matrix deposition and fibrosis in a murine model of hypersensitivity pneumonitis/lung fibrosis (Simonian et al. 2009), besides being detected during bone fracture healing (Kokubu et al. 2008). In our study, the high levels of IL-17 was accompanied by increased collagen III expression at the final inflammatory phase of tissue repair, suggesting that the presence of this cytokine in n-9 treated lesions may be involved in accelerated wound healing. In addition, n-3 treated mice showed a trend towards decreased IL-17 expression in the wounds at 5 days p.s. compared to the initial times p.s., thus corroborating with the delay in wound repair in these mice in contrast to n-9 treated animals. On the other hand, since n-3 fatty acids are known to exert profound anti-inflammatory effects on many physiological processes, the apparent inhibition of the pro-inflammatory cytokine IL-17 in our model could be related to such function of these fatty acids, although it did not result in faster wound closure.

Collagen is a key constituent of granulation tissue synthesized by fibroblasts (Au and Ehrlich 2007). It was previously demonstrated that an increase in collagen type I and type III gene expression at 72 h post-injury in rabbit vocal folds coincides with the proliferative phase of wound repair in this model (Rousseau et al. 2008). In our previous work we showed decreased connective tissue fibers in the wounds of oleic acid treated mice (Cardoso et al. 2004). It is of note that this first study involved only histopathological analysis of all types of ECM fibers, without deeper investigation of mRNA or protein expression of the different connective tissue fibers implicated in wound healing. It is possible that, although the overall fiber deposition is smaller, n-9 fatty acids induced a selective expression of collagen III at 120 h p.s., which probably accounted for a specific collagen-driven wound closure in these mice. Moreover, it is also feasible that wounds may have closed by contraction driven by myofibroblast activity (Hinz 2007).

Besides the increase in collagen and pro-inflammatory cytokines in oleic acid treated mice, we also found elevated IL-10 and diminished COX2 gene expression in n-9 treated wounds. IL-10 has been classically described as an important modulator of the inflammatory process, inhibiting several pro-inflammatory pathways (Peranteau et al. 2008). Taken together, our data suggests that increase in IL-10 production may balance the effects of IL-17, thus modulating the inflammatory process in n-9 treated wounds and contributing for the accelerated wound healing. In addition, mammals lack the enzymatic machinery to convert n-9 into arachidonic acid. Therefore, oleic acid is not metabolized into arachidonic acid derived metabolites, which suggests that less pro-inflammatory lipid mediators were produced at the wound sites with the decline in COX2 gene expression. On the other hand, while COX2 is also involved in the production of scar tissue, the control of the amount of scar needed to heal a wound is essential for maintenance of tissue homeostasis and function (Wilgus et al. 2004). Therefore, the results presented here pointed to a relevant applicability of n-9 fatty acids in the modulation of the inflammatory process induced by COX2, collagen deposition and inflammatory mediators. Additionally, IL-10 may promote wound healing through modulation of pro-inflammatory mediators (Peranteau et al. 2008). Altogether, these data may explain the diminished inflammation and faster wound closure in n-9 treated mice. Another study showed a dose-dependent increase in vascular endothelial growth factor-alpha (VEGF-α) and IL-1β by neutrophils incubated in the presence of oleic and linoleic acids (Pereira et al. 2008), suggesting that oleic and linoleic acids stimulate other pro-inflammatory pathways, which may be critical for wound closure and tissue repair (Pereira et al. 2008). Considering our results, we suggest that oleic acid is able to modulate inflammatory responses, since pro- and anti-inflammatory mediators were modified by such treatment.
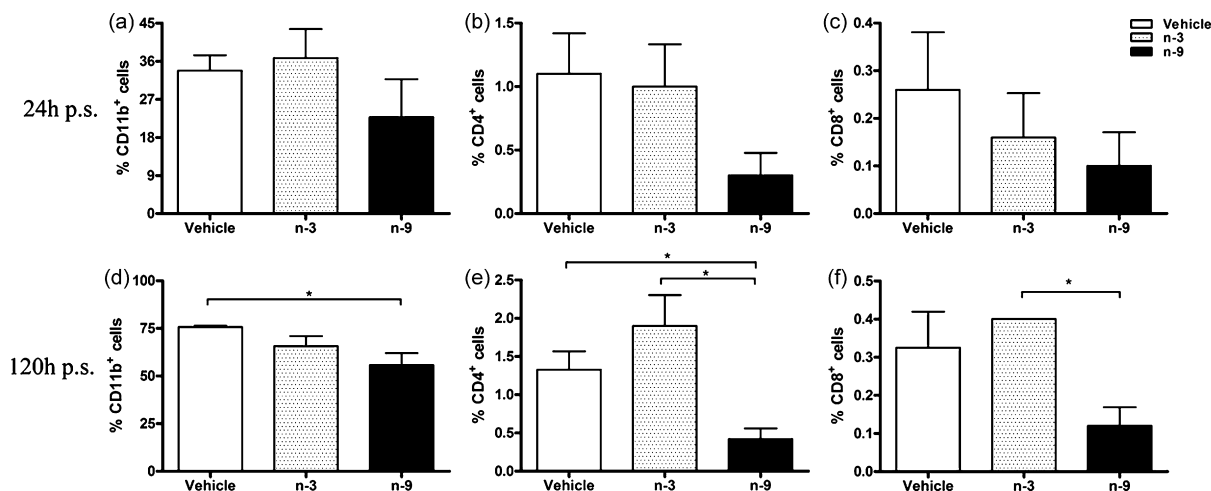
**Fig. 5.** n-9 fatty acids treatment reduced inflammatory infiltrate in the skin wounds. Leukocytes obtained from skin wounds of control or treated mice at 24 h (a–c) and 120 h (d–f) post-surgery were processed for FACS, as described in "Materials and methods". The percentage (mean ± SEM) of CD11b^{+} (a and d), CD4^{+} (b and e) and CD8^{+} (c and f) cells infiltrated in the wounds are shown as representative of two independent experiments. $*p < 0.05$.

Fatty acids could also modify tissue repair by altering the balance of MMPs, which are key molecules in the healing process and tissue remodeling (Armstrong and Jude 2002). Usually, excessive levels of MMPs are present in chronic wounds preventing wound closure (Rayment et al. 2008). Although we did not examine the enzyme activity in the present work, the wounds which presented faster closure were those in which MMP and TIMP expression were higher, suggesting elevated tissue remodeling in mice treated with oleic acid. MMP2 and MMP9 are important modulators of immune responses (Gutierrez et al. 2008) and extracellular remodeling (Birkedal-Hansen 1995). Because TIMPs counter regulate MMPs, they prevent exacerbated injury during inflammation. In the current study, MMP9 and TIMP1 expression was significantly increased 5 days p.s. in n-9 treated mice, along with TNF-α and IL-17 mRNA expression. In accordance, IL-17 can induce the production of MMP9 in human macrophages (Jovanovic et al. 2000), which are also related to the pro-inflammatory cytokine TNF-α (Maione et al. 2009; Zhou et al. 2009) and TIMP1 (Jovanovic et al. 2001). Additionally, oleic acid presents cationic serine protease inhibitory activity and can be useful in the treatment of chronic inflammatory pathologies like pressure or diabetic wounds, where serine protease activity is increased to pathological levels (Edwards et al. 2007).
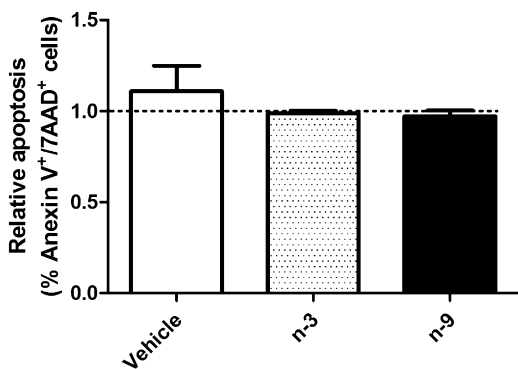


**Fig. 6.** Fatty acids treatment did not lead to cell death. Murine splenocytes were treated, *in vitro*, with 30 μM of n-3, n-9 fatty acids or ethanol (vehicle) and assayed for apoptosis markers 48 h later by flow cytometry. Graph shows the apoptosis ratio calculated by the percentage of annexinV[+]/7AAD^{+} cells relative to splenocytes cultured only in RPMI medium (control). Dashed line indicates the apoptosis in the cells from control medium.

Leukocytes are implicated in clearance of wound debris and skin defense; they are also involved in the inflammation and formation of new tissue during the repair process (Toulon et al. 2009). In the present study we pointed that n-9 fatty acids inhibit leukocyte accumulation in the lesions, which was not due to cell death, since no cell damage was observed in toxicity tests performed with splenocytes cultured with oleic or linolenic fatty acids at the same concentration used in the treatments *in vivo*. It is noteworthy that oleic and linoleic acids can induce apoptosis and necrosis in a human B-lymphocytes derived cell line when used at concentrations higher than that utilized in our study (Cury-Boaventura et al. 2004).

Fatty acids like oleic and linoleic acid, among others, can influence inflammation, lymphocyte function and cell-mediated immunity (Calder 2009; Calder et al. 2002). Unsaturated fatty acids like omega-3 can affect the gene expression of pro-inflammatory cytokines by altering cellular membrane fluidity, cell to cell signaling, mobility of cells, interaction of receptors with their agonist, membrane function and formation of secondary signals (Grimm et al. 2002). A recent publication has shown that electrophilic fatty acids derived from n-3 fatty acids are generated during inflammation by non-enzymatic reactions and can modulate inflammatory responses. These electrophilic fatty acids are generated by a COX2-catalyzed mechanism, specifically in activated macrophages and function as anti-inflammatory mediators by inhibiting pro-inflammatory cytokine and nitric oxide production as well as peroxisome proliferator-activated receptor-γ (PPARγ) activation. The authors suggest that electrophilic fatty acids may be involved in the systemic anti-inflammatory effect of n-3 rich diets. It is unknown whether n-9 fatty acids can produce similar monounsaturated types of compounds and which role these compounds would have in skin healing (Groeger et al. 2010).

Besides the beneficial anti-inflammatory actions of omega-3 polyunsaturated fatty acids in human diseases (Fedor and Kelley 2009; Lavie et al. 2009), in the present study we pointed to a more incisive role of omega-9 in wound repair. While essential dietary omega-3 lipids are the precursors of inflammatory mediators like eicosanoids, the non-essential monounsaturated omega-9 may interfere with the biological activities of essential lipids (De Lorgeril 2007). Moreover, omega-9 can compete with n-3 lipids to be incorporated into membrane phospholipids and disturb the generation of inflammatory mediators, raft formation and the fluidity of the cell membrane. This mechanism, along with the potential conversion into anti-inflammatory compounds, may be responsi-

ble for triggering the n-9 effects observed in our work. By evaluating the role of n-3 and also n-9 fatty acids in the treatment of cutaneous wounds, we showed that the unsaturated fatty acid n-9 but not n-3 caused marked changes in the healing process by presenting immunomodulatory effects on the skin of treated mice.

Finally, we proposed that oleic acid can modulate inflammatory and immune responses in skin lesions, which results in differential wound repair. We suggest that monounsaturated fatty acid n-9 may be a useful tool in the treatment of cutaneous wounds, especially in cases of skin burns, diabetic or pressure ulcers.

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