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Olive oil promotes wound healing of mice pressure injuries through NOS-2 and Nrf2

Fernanda Seabra Schanuel [#], Bianca Oliveira Saguie [&], Andréa Monte-Alto-Costa [*]

Author´s affiliation:

Rio de Janeiro State University (UERJ)

Histology and Embryology Department - Tissue Repair Laboratory

Av. Marechal Rondon, 381/HLA

20950-003 Rio de Janeiro, RJ, Brazil

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[*] **Corresponding author:**

Dr. Andréa Monte-Alto-Costa

Histology and Embryology Department - Tissue Repair Laboratory

Av. Marechal Rondon, 381/HLA

20950-003 Rio de Janeiro, RJ, Brazil.

Phone:+55 21 2334 2421

E-mail address: amacosta.uerj@gmail.com, amacosta@uerj.br

Author´s contact information:

[#]nandasschanuel@gmail.com; [&]bsaguie@live.com

ABSTRACT

The pressure injury environment is characterized by overproduction of reactive oxygen species and exacerbated inflammation, which impair the healing of these lesions. Mediterranean-like diet may be a good intervention to improve the healing of pressure injury due to its anti-inflammatory and antioxidant components. So, this study evaluated the hypothesis that olive oil, as a main source of lipid in Mediterranean diet, could improve cutaneous wound healing of pressure injury in mice. Male Swiss mice were randomly divided into standard, olive oil or soybean oil plus olive oil groups and fat represented 10% of total calories in all groups. Four weeks after the beginning of diet administration, two cycles of ischemia-reperfusion (IR) by external application of two magnets disks were performed in the dorsal skin to induce pressure injury formation. Fourteen days after the end of the second IR cycle, olive oil based diet reduced neutrophils cells and cyclooxygenase-2 protein expression, increased nitric oxide synthase-2 and protein and lipid oxidation. Olive oil based diet also increased nuclear factor erythroid 2-related factor 2 protein expression and collagen type I precursor protein expression. In addition, olive oil based diet administration promoted wound closure 7, 10 and 14 days after the end of the second IR cycle. These findings support the hypothesis that olive oil based diet improves cutaneous wound healing of pressure injury in mice through the reduction of inflammation and stimulation of redox equilibrium.

Keywords: olive oil; pressure lesion; skin; mouse; wound healing; diet.

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1. Introduction

Pressure injury is a damage localized between the skin and underlying soft tissue, and a significant factor that collaborates to morbidity and mortality among patients with chronic conditions (Salcido et al. 2007; Edsberg et al. 2016). The treatment of this type of wound is often complex and costly to the health care system (Brem et al. 2010). Some risk factors associated with increased pressure injury incidence include immobility, spinal cord injury, elderly and poor nutrition (Allman 1989; Allman et al. 1995; Dorner et al. 2009; Taylor 2017). The etiology of pressure injury remains unclear, but increasing evidence demonstrates that the cycles of ischemia-reperfusion (IR)-induced injury may be a factor in the formation of these types of wounds (Salcido et al. 1995; Peirce et al. 2000). Previous studies in animals have demonstrated that the repeated IR cycles increases the synthesis of reactive oxygen species (ROS) which exceed the capacity of endogenous defense mechanisms, leading to an elevated inflammatory response and thus to skin necrosis (Peirce et al. 2000; Reid et al. 2004; Mustoe et al. 2006).

An environment rich in ROS as observed in pressure ulcers, have deleterious effects on lipids, proteins and nucleic acids of cells involved in skin repair, hence, cause tissue damage (Schafer and Werner 2008). However, low levels of ROS and nitric oxide (NO) are essential mediators of cellular signaling and plays an important role in skin wound healing, mainly in initiation of inflammatory phase, angiogenesis and matrix deposition (Soneja et al. 2005; Schafer and Werner 2008; Sen and Roy 2008). So, a strategy to maintain this delicate balance in biological systems and prevent oxidative damage to the cells

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may include a treatment with antioxidants, thus, improving the healing process (Soneja et al. 2005).

The Mediterranean diet has been associated with good health (Willett et al. 1995; Lopez-Miranda et al. 2010), it is characterized as a dietary pattern rich in plant foods, moderate intake of fish, dairy products, poultry and eggs and low consumption of red meat; and olive oil as the main source of fat (Lopez-Miranda et al. 2010). This diet presents low content of saturated fatty acids and high content of monounsaturated fatty acids (MUFAs), primarily derived from olive oil (Lopez-Miranda et al. 2010; Bach-Faig et al. 2011). The beneficial health effects of olive oil are due to its high content of MUFAs and phenolic compounds (mainly hydroxytyrosol and oleuropein), which have anti-inflammatory and antioxidant properties (Dell'Agli et al. 2006; Fito et al. 2007). Using *in vitro* studies, it was shown that olive oil or oleic acid modulates the production of inflammatory and oxidative mediators by neutrophils, macrophages and fibroblasts, important cells involved in wound healing process (Yaqoob and Calder 1995; Hatanaka et al. 2006; de la Puerta et al. 2009; Magdalon et al. 2012; Hatanaka et al. 2013). It has already been shown that dietary supplementation with olive oil or its main fatty acid, oleic acid, can be beneficial to healing process in animals, through reduction of inflammatory response and oxidative damage and increase in collagen deposition (Rodrigues et al. 2012; Rosa A dos et al. 2014; Donato-Trancoso et al. 2016), but there is no data about the effects of olive oil consumption, as a regular diet component, on wound healing. Considering that a balanced diet can improve the quality of life, especially for people who do not have access to supplementation, it would be interesting to evaluate if the

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beneficial effects of olive oil supplementation could be achieved by its regular consumption through diet.

Thus, we hypothesized that previous consumption of olive oil as the main source of dietary lipid (mimicking a Mediterranean diet) contribute to improved wound healing of pressure injury in mice through the reduction of inflammation and stimulation of redox equilibrium.

2. Materials and methods

2.1 Animals and diets

All procedures were carried out in accordance with Brazilian legislation for experimentation with animals. In addition, Ethical Committee for Animal Use of Rio de Janeiro State University approved this study (CEUA/043/2018).

Twenty-four male Swiss mice (12-week-old) were randomly divided into 3 groups (n=8 per group) and subjected to different dietary regimens: standard (SC – 10% fat from soybean oil, as proposed by American Institute of Nutrition (AIN-93M) (Reeves et al. 1993)), olive oil (OO – 10% fat from extra virgin olive oil) and soybean oil plus olive oil (SOOO – 2,5% fat from soybean oil + 7,5% fat from olive oil). Fatty acid composition of oils used are presented in table 1; SC, SO and SOOO diets presented 2.3%, 7.3% and 6% respectively of total energy from MUFAs. Diet components, besides fat, were the same for all chows (Table 2). Diets were manufactured by PragSoluções (Jaú, São Paulo, Brazil), and were administered over a 6 week period. Body mass was measured at the beginning and weekly until the end of the experiment.

2.2 IR-induced injury model

Mice had free access to their respective diets and water and food intake was monitored daily. Four weeks after the beginning of diet administration, all animals were intraperitoneally anesthetized with ketamine (150mg/kg) and xylazine (15mg/kg) and the dorsal skin was gently pulled up (including epidermis, dermis and hypodermis) and placed between a pair of neodymium magnets disks (8mm diameter and 3mm thickness) (G.A.M. Equipamentos Eletromagnéticos, São Bernardo do Campo, SP, Brazil) (Peirce et al. 2000; Stadler et al. 2004; Assis de Brito et al. 2014). Two consecutive cycles of ischemia-reperfusion (IR) consisting of 16h of pressure, induced by magnets (ischemia period), followed by a release period of 8h (reperfusion period) were performed to develop pressure injury. After the second IR cycle, all animals developed two circular lesions separated by a bridge of normal skin, and this point was considered day 0. The diets were administrated until the end of experimental period.

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2.3 Evaluation of wound closure

To evaluate the wound closure, wound area was recorded as previously mentioned (Nascimento and Costa 2006), 0 and 3, 7, 10 or 14 days after wounding.

2.4 Oral glucose tolerance test (OGTT)

Twelve days after the end of the second IR cycle, following a 12 h fast, glucose concentration from the tail tip was determined after glucose gavage in six randomly selected animals per group and oral glucose tolerance calculated as previously described (Otranto et al. 2013).

2.5 Intraperitoneal insulin tolerance test (IPITT)

Fourteen days after the end of the second IR cycle, following a 12h fast, glucose concentration from tail tip was determined after intraperitoneally insulin injection in six randomly selected animals per group and intraperitoneal insulin tolerance calculated, as previously described (Otranto et al. 2013).

2.6 Sacrifice, blood sample collection, tissue harvesting and histologic analyses

Fourteen days after the end of the second IR cycle, animals were sacrificed by decapitation, under anesthesia and blood samples were collected. Serum alanine transaminase (ALT) was determined using Bioclin kit (Quibasa Química Básica, Belo Horizonte, Brazil).

Also, one lesion of each animal (n=8) was collected and frozen at -80°C for biochemical analyses, the other lesions and normal adjacent skin were collected, formalin fixed and paraffin-embedded. The sections were stained with Sirius Red and analyzed under polarized light to evaluate collagen fiber organization. Some sections were immunostained as mentioned below.

The retroperitoneal and epididymal fat were carefully dissected out, after the animals were sacrificed, and weighed. The results are expressed as retroperitoneal plus epididymal fat (g).

2.7 Immunohistochemistry and quantification

To evaluate inflammatory cells amount macrophages and neutrophils were quantified. For this, sections (n=5/group) were immunolabelled with a rat monoclonal antibody F4/80 (Serotec; Raleigh, United Kingdom) (macrophage

marker) or a rat monoclonal antibody myeloperoxidase-neutrophil marker (Santa Cruz; Santa Cruz, CA) as previously described (Assis de Brito et al. 2014). After labeling, slides were digitalized using a Pannoramic MIDI slide scanner (3DHistech Kft; Budapest, Hungary). To quantify F4/80-positive macrophages, 5 random fields per animal (32950 μm^2) were captured through image analysis software Panoramic Viewer (3DHistech Kft) and evaluated. Data are presented as cells per square millimeter. The amount of neutrophils was quantified using a stereological tool (point counting) as previously described (Baddeley et al. 1986; Gundersen et al. 1988; Romana-Souza et al. 2009). Data are presented as volume density of neutrophils (Vv [neutrophils] %). Analysis were performed blindly and repeated without difference between the replicates.

The amount of myofibroblasts and blood vessels with alpha-smooth muscle actin (α-SM actin) positive pericytes were evaluated in mouse tissue immunolabeled with a mouse monoclonal antibody raised against alpha-smooth muscle actin (α-SM actin) (DAKO; Carpinteria, CA), as previously described (Cardoso et al. 2007). After labeling, slides were digitalized and positive vessels and myofibroblasts were quantified as mentioned above. Data are presented as blood vessels per square millimeter and volume density of myofibroblasts (Vv [myofibroblasts] %).

2.8 Immunoblotting

Proteins of wound lysate (30 µg) were resolved by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and were transferred to nitrocellulose membranes. Immunoblotting was performed as previously described (Donato-Trancoso et al. 2016) for the following proteins: rabbit polyclonal anti-type I

collagen (col I) (Millipore, Temecula, CA), mouse monoclonal anti-type III collagen (col III) (Millipore), goat polyclonal anti-cyclooxygenase-2 (COX-2) (Santa Cruz), rabbit monoclonal anti-total nuclear factor kappa B (NF-κB p65) (Cell Signaling, Danvers, MA), rabbit polyclonal anti-phosphorylated nuclear factor kappa B (pNF-κB p65) (Cell Signaling), rabbit polyclonal anti- nitric oxide synthase inducible (NOS-2) (Santa Cruz), mouse monoclonal anti-nitrotyrosine (Santa Cruz Biotechnology) or rabbit polyclonal anti- nuclear factor erythroid 2-related factor 2 (Nrf2) (Santa Cruz) mouse monoclonal anti-β-actin (Sigma-Aldrich, São Paulo, Brazil) antibodies. The β-actin was used as a control of protein loading. The bands were quantified by densitometry analysis, which was performed using Image J software (National Institutes of Health). The results are expressed as arbitrary units (a.u.).

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2.9 Expression of carbonylated proteins by immunoblotting (post-electrophoresis derivatization)

To evaluate protein oxidation, carbonylated protein expression was determined by pos-electrophoresis derivatization as already described (Conrad et al. 2000), The β-actin was used as a control of loading protein and the results are expressed as arbitrary units (a.u.).

2.10 Biochemical analysis

To evaluate lipid peroxidation, the levels of lipid hydroperoxides were measured in wound lysate using the ferrous oxidation in xylenol orange method as described (Jiang et al. 1991). The results are expressed as nM of lipid hydroperoxides per mg total protein.

2.11 Statistical analysis

All data are presented as the mean ± standard error of mean (mean± SEM) and were analyzed by one-way analysis of variance with a Tukey's posttest or by a nonparametric Kruskal-Wallis with Dunn's posttest using GraphPad Prism software (version 6.0, GraphPad Software Inc, San Diego, CA). Values of $p<0.05$ were considered statistically significant for all tests.

3. Results

3.1 Olive oil based diet did not cause any change on murinometric values, glucose metabolism or liver toxicity

The mean body mass of the groups did not present difference throughout the experimental period (Fig.1A). The amount of food intake (g/animal/day) was the same in all groups (SC: 5.2 ± 0.2; OO: 5.5 ± 0.2; SOOO: 5.3 ± 0.2). Considering energy intake (kcal/animal/day) there was no difference between groups neither (SC: 20.0 ± 0.8; OO: 21.0 ± 0.9; SOOO: 20.0 ± 0.8). The amount of fat/animal mass (g/kg) was for 4.8, 4.8 and 4.7 for SC, OO and SOOO groups respectively.

The retroperitoneal plus epididymal fat mass (g) was lower in SOOO group when compared to SC ($p<0.05$) and OO ($p<0.05$) groups, respectively (Fig. 1B).

There was no difference between the average fasting blood glucose between groups (time 0, data not shown). The time course of glucose clearance was the same in all groups. The areas under the curve (AUCs) between 0 and 120 minutes for the glucose tolerance test did not present any difference between

the groups; indicating that the animals were not glucose intolerant (Fig. 1C). The IPITT measurement did not show any difference between the groups at the beginning of the test (time 0, data not shown), and glucose levels decreased after insulin administration during the test. The AUCs between 0 and 20 minutes for the insulin tolerance test did not present any difference between the groups, showing that the animals did not present insulin resistance (Fig. 1D).

The serum level of ALT is used as a clinical marker of liver injury (Amacher 1998). All diets did not induce an alteration in the expression of ALT, thereafter; there was no liver injury between groups (Fig. 1E).

3.2 Wound closure is improved by olive oil based diet in mice pressure injuries

Olive oil supplementation increased wound closure of chronically stressed and on pressure ulcers of mice (Rosa A dos et al. 2014; Donato-Trancoso et al. 2016). To evaluate the effect of olive oil from diet in dermal reconstruction of pressure injuries, wound areas were measured. Olive oil (OO) group presented increased wound contraction of pressure injuries when compared to SC group 7 days after the end of the second IR cycle ($p<0.05$; Fig. 2A). In olive oil containing groups (OO and SOOO), there was an increase in wound contraction when compared to SC group 10 and 14 days after the end of the second IR cycle ($p<0.05$ SC x OO and $p<0.01$ SC x SOOO; Fig. 2A).

Figure 2B shows representative pictures with the macroscopic aspect of cutaneous lesions 3, 7, 10 and 14 days after the end of the second IR cycle. The first signs of re-epithelialization were seen 7 days after the end of the second IR cycle and were more evident in OO group.

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3.3 Olive oil based diet modulates inflammatory signalization and oxidative damage

The wound bed of pressure ulcers presents infiltration of inflammatory cells, mainly neutrophils (Diegelmann 2003). The oral administration of olive oil or its abundant component oleic acid, accelerates the onset of inflammation and also its resolution in acute cutaneous lesions and in pressure ulcers of mice (Rodrigues et al. 2012; Donato-Trancoso et al. 2016). To characterize the type of inflammatory cells present in wounds, sections were immunolabelled. The volume density of neutrophils was lower in OO group when compared to SC group ($p<0.01$ Fig. 3A) 14 days after the end of the second IR cycle. The number of macrophages did not present any difference between the groups 14 days after the end of the second IR cycle (Fig. 3B).

In Figure 3C there was an increase in the ratio pNF-κB p65/ NF-κB p65 between olive oil containing groups and SC group ($p<0.0001$ SC x OO and $p<0.05$ SC x SOOO), and between OO and SOOO groups ($p<0.01$) 14 days after the end of the second IR cycle. However, protein expression of NF-κB p65 (normalized by β-actin) did not present any difference between the groups. COX-2 protein expression was decreased when olive oil containing groups were compared to SC group ($p<0.05$ SC x OO and $p<0.0001$ SC x SOOO; Fig. 3D) and in SOOO compared to OO group ($p<0.01$) 14 days after the end of the second IR cycle (Fig. 3D).

NO and low levels of ROS are essential mediators of cellular signaling and play an important role in skin wound healing (Schafer and Werner 2008; Sen and Roy 2008). Recently, it was demonstrated that oleic acid stimulated ROS production by fibroblasts (Hatanaka et al. 2013). The olive oil group presented a

higher NOS-2 and nitrotyrosine protein levels when compared to SC group 14 days after the end of the second IR cycle ($p<0.01$; Fig. 3E and $p<0.05$; Fig. 3F, respectively). The carbonylated protein levels in OO group were augmented when compared to SC group 14 days after the end of the second IR cycle ($p< 0.01$; Fig. 3G and 3H). The levels of lipid hydroperoxides were higher in olive oil containing groups when compared to SC group 14 days after the end of the second IR cycle ($p<0.05$ SC x OO and $p<0.01$ SC x SOOO; Fig. 3I).

An environment rich in ROS, as observed in pressure injuries, may stimulate the activation of transcription factors, like Nrf2, which regulates diverse ROS-detoxifying enzymes, such as heme-oxygenase-1 (Schafer and Werner 2008). The OO group presented a higher Nrf2 protein expression when compared to SC ($p<0.01$) and SOOO ($p<0.05$) groups 14 days after the end of the second IR cycle (Fig. 3J).

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3.4 Olive oil based diet improves dermal reconstruction

The dietary supplementation with olive oil increases collagen deposition on chronically stressed and on pressure ulcers of mice (Rosa A dos et al. 2014; Donato-Trancoso et al. 2016). The effects of olive oil based diet on dermal reconstruction of pressure injuries was evaluated by myofibroblastic differentiation and collagen deposition and organization. The density of myofibroblasts and blood vessels number did not present any difference between the groups 14 days after the end of the second IR cycle (Fig. 4A and 4B).

Figure 4C presents collagen arrangement in granulation tissue, using Sirius red stained sections observed under polarization. The SC group showed, in superficial and deep areas of granulation tissue, reduced density and thin

yellow-greenish collagen fibers arranged parallel to surface. In superficial and deep areas of OO group reduced density and thin yellow-red collagen fibers were observed. In SOOO group showed an increased density of thin yellow-greenish collagen fibers when compared with SC and OO groups in deep and superficial areas of granulation tissue. Type III collagen protein expression was higher in SOOO group when compared to SC and OO groups ($p<0.0001$, Fig. 4D). The olive oil containing groups presented a higher type I collagen precursor when compared to SC group 14 days after the end of the second IR cycle ($p<0.001$ SC x OO and $p<0.05$ SC x SOOO; Fig. 4E).

4. Discussion

Pressure injury is a damage localized between the skin and underlying soft tissue, and a relevant problem that collaborates to morbidity and mortality among patients with chronic conditions (Salcido et al. 1995; Edsberg et al. 2016). One of the risk factors associated with increased pressure injury incidence is poor nutrition (Dorner et al. 2009; Taylor 2017). Obese and underweight patients can be considered as having poor nutrition since both have a diet that presents an imbalance of nutrients, and more vulnerable to develop pressure ulcer; and an improvement in nutrients balance may reduce the risk of developing pressure ulcers in these groups (Taylor 2017).

The Mediterranean diet has been associated with good health (Lopez-Miranda et al. 2010) and its principal fat source is olive oil (Bach-Faig et al. 2011); that has beneficial effects on blood lipids (Castaner et al. 2011) and has been associated to low cardiovascular mortality (de Lorgeril and Salen 2006; Covas 2007). In recent studies, it was demonstrated that olive oil supplementation

increased wound closure of chronically stressed and on pressure ulcers of mice (Rosa A dos et al. 2014; Donato-Trancoso et al. 2016), but the effects of olive oil as a main source of diet lipid in wound healing of pressure injury it is still undetermined. Although the use of dietary supplements is disseminated nowadays, it is possible and interesting to obtain several benefits through the adherence to a balanced diet. For this reason, we investigated if the use of olive oil as a main source of lipid, with previous administration (mimicking a Mediterranean diet), could be a good therapy to improve the cutaneous wound healing of pressure injuries due to its anti-inflammatory and antioxidant properties.

Several studies have demonstrated that adherence to Mediterranean diet regimen may reduce body weight and blood glucose levels (Tierney and Roche 2007; Estruch et al. 2016; Agnoli et al. 2018). However, in our study, the diet administration did not change these parameters. All experimental groups were fed with isoenergetic diets that differ only in source of fatty acids. The predominant fatty acid in soybean oil is linoleic acid (approximately 51%, polyunsaturated fatty acid) (Reeves et al. 1993). Olive oil is rich in oleic acid, a MUFA (Perez-Jimenez et al. 2007). In our study, the content of MUFA was 2.3% in SC, 7.3% in OO and 6% in SOOO groups. A meta-analysis demonstrated beneficial effects on weight and other parameters for obesity management only when the source of total energy was more than 12% from MUFAs and for a long period of consumption (Schwingshackl et al. 2011).

The serum level of ALT is an universal marker used to measure liver injury (Amacher 1998). When a liver injury occurs, hepatocytes release this enzyme into the extracellular space from where they enter the bloodstream, augmenting

its circulating expression. Since, in the present study, there was no difference in ALT levels among groups we can conclude that diets were not hepatotoxic.

Some studies have showed that supplementation with olive oil improves cutaneous wound healing in impaired lesions due to reduction of local inflammatory response, such as neutrophils and macrophage infiltration on wound bed (Rosa A dos et al. 2014; Donato-Trancoso et al. 2016). In our study olive oil containing groups showed a reduction in wound area and in neutrophils density; however we did not observe difference in the amount of macrophage cells between groups. Meanwhile, in those studies the olive oil was supplemented and the treatment started near wounding induction; in our, olive oil was used as main source of diet lipid and its administration started 4 weeks previously to the pressure injuries induction, and lasted until the end of experiment. The previous administration of olive oil was, probably, a key factor that affected positively the healing process.

In chronic pressure ulcers, the synthesis of COX-2 induce prostaglandin E_2 (PGE_2) synthesis and may contribute to chronic inflammation and cell death by apoptosis (Abd-El-Aleem et al. 2001). In the present study we showed a decrease in COX-2 protein levels in olive oil containing groups. A previous study showed a decreased in mRNA for COX-2 in mice treated with oleic acid, the main fatty acid presented in olive oil (Cardoso et al. 2011). Mammals lack the enzymatic machinery to convert n-9 into arachidonic acid (Arab 2003), a key point in prostaglandin synthesis. So, an increased supply of oleic acid might counteract arachidonic acid, by reducing the relative abundance of it, and reducing the generation of inflammatory mediators (Hostmark and Haug 2013).

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In wound healing process, low levels of reactive oxygen species (ROS) are essential mediators of cellular signaling (Schafer and Werner 2008). In the present study, there is an increase in oxidative damage (hydroperoxides lipids and carbonylated proteins) in olive oil group. These effects could be related to a stimulatory effect of fatty acids on nicotinamide adenine dinucleotide phosphatase oxidase (NADPH oxidase) activity, which is responsible for ROS generation (Schafer and Werner 2008). Recently, it was demonstrated that oleic acid stimulates ROS production by fibroblasts, via dose dependent activation of NADPH oxidase complex (Hatanaka et al. 2013), and it is also known that mice deficient in NADPH oxidase 4 present impaired repair, showing the important role of redox signaling in wound repair process (Levigne et al. 2016). Corroborating the importance of ROS in wound repair, Hatanaka et al (2006) have showed that neutrophils treated with oleic acid have an increment in ROS production, and it is associated with phagocyte defense that occurs mainly through the NADPH oxidase complex (Hatanaka et al. 2006).

Nitric oxide plays an important role in skin wound healing. A previous study showed that inhibition of NOS cause a delay in wound healing process (Amadeu and Costa 2006). The olive oil group also presented an increased in NOS-2 protein levels. Yaqoob et al (1995) had showed an augment in NO production in olive oil group, and suggested the effects of dietary lipids upon NOS might be regulated by fatty acids or by compounds derived from them (Yaqoob and Calder 1995). NO also stimulates the collagen deposition as saw in previous studies (Amadeu et al. 2007; Schanuel et al. 2015). Additionally, previous studies showed stimulation of collagen III mRNA expression in olive group (Cardoso et al. 2011) and an increased type I collagen protein deposition (Donato-Trancoso et al.

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2016). In the present study, the groups that had olive oil in their composition, showed an increased in collagen III (SOOO) and type I collagen precursor (OO and SOOO) protein levels and collagen fibers structure, although no difference in myofibroblast density was observed.

Although olive oil stimulate NO expression (Martins et al. 2010) which also led to an angiogenic effect (Rizk et al. 2004), there was no difference in blood vessels number between experimental groups. Other study also showed no difference in blood vessels number, and a decreased in NO production when oleic acid was topically applied, but in this study, the NO production was only detected until 48 hours and blood vessels were evaluated until 10 days after wounding (Cardoso et al. 2004). Our olive oil containing groups had an advanced wound healing process, and it is possible that blood vessels were already disappearing by apoptosis (Desmouliere et al. 1995).

Events such as inflammation and oxidative stress, as observed in pressure injuries, transmit signals to cells to trigger cytoprotective gene expression for preservation of endogenous redox balance, which are regulated by the transcription factor Nrf2 (Kobayashi and Yamamoto 2006; Battino et al. 2018). Nrf2 acts by stimulating transcription of phase II detoxification enzimes, such as antioxidant enzymes, hemeoxigenase-1, glutathione transferase and others (Battino et al. 2018). It has been reported that natural compounds may activate Nrf2 (Battino et al. 2018), in fact, in our study the olive-oil group showed a higher Nrf2 protein expression. Olive oil has been shown to be effective against oxidative stress and recent studies support that this effect is mainly related to its high content of phenolic compounds, like hydroxytyrosol and oleuropein (Visioli et al. 1998; Fito et al. 2007). Several studies have demonstrated an activation of Nrf2

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after hydroxytyrosol and oleuropein treatment (Martinez-Huelamo et al. 2017). It may seem controversial the presence of oxidative damage, as discussed above, and high expression of Nrf2, but it is important to remember that the presence of ROS is important to wound healing process and we suggest that the balance is obtained by both, expression of ROS and Nrf2.

Although the benefits of Mediterranean diet are well described (Lopez-Miranda et al. 2010) and have already been discussed in this article, it is important to emphasize that olive oil is the main source of fat but is not the only one. A correct balance in essential fatty acids n-6 (linoleic acid) and n-3 (linolenic) is desirable (Reeves et al. 1993). Three groups were included in this study, one with the recommended amounts of fatty acid – group SC (Reeves et al. 1993), one with only olive oil – group OO, and a group with olive and soybean oil – SOOO that aimed to approach the balance in fatty acids composition, mainly by including n-6 fatty acids. In fact some results (wound contraction, NF-κB activation, COX-2 expression, lipid peroxidation and type I collagen deposition) showed that the presence of soybean oil associated with olive oil can also improve the wound healing process. It is known that linoleic acid is particularly important for cutaneous barrier function (Schurer et al. 1999; Munoz-Garcia et al. 2014), and in a future study the effects of olive oil and olive oil associated with soybean oil need to be addressed.

In conclusion, olive oil containing diet promotes cutaneous wound healing of mice pressure injuries due to the increase of ROS and NO synthesis, promoting collagen deposition; reduction in inflammatory response due to the reduction of neutrophils and COX-2 protein synthesis; and stimulation of Nrf2 expression, probably by phenolic compounds.

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Conflicts of interest

The authors have no conflict of interest to declare.

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References

Abd-El-Aleem, S. A., Ferguson, M. W., Appleton, I., Bhowmick, A., McCollum, C. N. and Ireland, G. W. 2001. Expression of cyclooxygenase isoforms in normal human skin and chronic venous ulcers. J. Pathol. **195**(5): 616-623 doi: 10.1002/path.992.

Agnoli, C., Sieri, S., Ricceri, F., Giraudo, M. T., Masala, G., Assedi, M., et al. 2018. Adherence to a Mediterranean diet and long-term changes in weight and waist circumference in the EPIC-Italy cohort. Nutr. Diabetes, **8**(1): 22 doi: 10.1038/s41387-018-0023-3.

Allman, R. M. 1989. Pressure ulcers among the elderly. N. Engl. J. Med. **320**(13): 850-853 doi: 10.1056/NEJM198903303201307.

Allman, R. M., Goode, P. S., Patrick, M. M., Burst, N. and Bartolucci, A. A. 1995. Pressure ulcer risk factors among hospitalized patients with activity limitation. JAMA, **273**(11): 865-870.

Amacher, D. E. 1998. Serum transaminase elevations as indicators of hepatic injury following the administration of drugs. Regul. Toxicol. Pharmacol. **27**(2): 119-130 doi: 10.1006/rtph.1998.1201.

Amadeu, T. P. and Costa, A. M. 2006. Nitric oxide synthesis inhibition alters rat cutaneous wound healing. J. Cutan. Pathol. **33**(7): 465-473 doi: 10.1111/j.1600-0560.2006.00472.x.

Amadeu, T. P., Seabra, A. B., de Oliveira, M. G. and Costa, A. M. 2007. S-nitrosoglutathione-containing hydrogel accelerates rat cutaneous wound repair. J. Eur. Acad. Dermatol. Venereol. **21**(5): 629-637 doi: 10.1111/j.1468-3083.2006.02032.x.

Arab, L. 2003. Biomarkers of fat and fatty acid intake. J. Nutr. **133 Suppl 3**(3): 925S-932S doi: 10.1093/jn/133.3.925S.

Assis de Brito, T. L., Monte-Alto-Costa, A. and Romana-Souza, B. 2014. Propranolol impairs the closure of pressure ulcers in mice. Life Sci. **100**(2): 138-146 doi: 10.1016/j.lfs.2014.02.007.

Bach-Faig, A., Berry, E. M., Lairon, D., Reguant, J., Trichopoulou, A., Dernini, S., et al. 2011. Mediterranean diet pyramid today. Science and cultural updates. Public Health Nutr. **14**(12A): 2274-2284 doi: 10.1017/S1368980011002515.

Baddeley, A. J., Gundersen, H. J. and Cruz-Orive, L. M. 1986. Estimation of surface area from vertical sections. J. Microsc. **142**(Pt 3): 259-276.

Battino, M., Giampieri, F., Pistollato, F., Sureda, A., de Oliveira, M. R., Pittala, V., et al. 2018. Nrf2 as regulator of innate immunity: A molecular Swiss army knife! Biotechno.l Adv. **36**(2): 358-370 doi: 10.1016/j.biotechadv.2017.12.012.

Brem, H., Maggi, J., Nierman, D., Rolnitzky, L., Bell, D., Rennert, R., et al. 2010. High cost of stage IV pressure ulcers. Am. J. Surg. **200**(4): 473-477 doi: 10.1016/j.amjsurg.2009.12.021.

Cardoso, C. R., Favoreto, S., Jr., Oliveira, L. L., Vancim, J. O., Barban, G. B., Ferraz, D. B., et al. 2011. Oleic acid modulation of the immune response in wound healing: a new approach for skin repair. Immunobiology, **216**(3): 409-415 doi: 10.1016/j.imbio.2010.06.007.

Cardoso, C. R., Souza, M. A., Ferro, E. A., Favoreto, S., Jr. and Pena, J. D. 2004. Influence of topical administration of n-3 and n-6 essential and n-9 nonessential fatty acids on the healing of cutaneous wounds. Wound Repair Regen. **12**(2): 235-243 doi: 10.1111/j.1067-1927.2004.012216.x.

Cardoso, J. F., Souza, B. R., Amadeu, T. P., Valenca, S. S., Porto, L. C. and Costa, A. M. 2007. Effects of cigarette smoke in mice wound healing is strain dependent. Toxicol. Pathol. **35**(7): 890-896 doi: 10.1080/01926230701459986.

Castaner, O., Fito, M., Lopez-Sabater, M. C., Poulsen, H. E., Nyyssonen, K., Schroder, H., et al. 2011. The effect of olive oil polyphenols on antibodies against oxidized LDL. A randomized clinical trial. Clin. Nutr. **30**(4): 490-493 doi: 10.1016/j.clnu.2011.01.013.

Conrad, C. C., Talent, J. M., Malakowsky, C. A. and Gracy, R. W. 2000. Post-Electrophoretic Identification of Oxidized Proteins. Biol. Proced. Online, **2**: 39-45 doi: 10.1251/bpo17.

Covas, M. I. 2007. Olive oil and the cardiovascular system. Pharmacol. Res. **55**(3): 175-186 doi: 10.1016/j.phrs.2007.01.010.

de la Puerta, R., Marquez-Martin, A., Fernandez-Arche, A. and Ruiz-Gutierrez, V. 2009. Influence of dietary fat on oxidative stress and inflammation in murine macrophages. Nutrition, **25**(5): 548-554 doi: 10.1016/j.nut.2008.10.010.

de Lorgeril, M. and Salen, P. 2006. The Mediterranean-style diet for the prevention of cardiovascular diseases. Public Health Nutr. **9**(1A): 118-123.

Dell'Agli, M., Fagnani, R., Mitro, N., Scurati, S., Masciadri, M., Mussoni, L., et al. 2006. Minor components of olive oil modulate proatherogenic adhesion molecules involved in endothelial activation. J. Agric. Food Chem. **54**(9): 3259-3264 doi: 10.1021/jf0529161.

Desmouliere, A., Redard, M., Darby, I. and Gabbiani, G. 1995. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. Am. J. Pathol. **146**(1): 56-66.

Diegelmann, R. F. 2003. Excessive neutrophils characterize chronic pressure ulcers. Wound Repair Regen. **11**(6): 490-495.

Draft

Donato-Trancoso, A., Monte-Alto-Costa, A. and Romana-Souza, B. 2016. Olive oil-induced reduction of oxidative damage and inflammation promotes wound healing of pressure ulcers in mice. J. Dermatol. Sci. **83**(1): 60-69 doi: 10.1016/j.jdermsci.2016.03.012.

Dorner, B., Posthauer, M. E. and Thomas, D. 2009. The role of nutrition in pressure ulcer prevention and treatment: National Pressure Ulcer Advisory Panel white paper. Adv. Skin Wound Care,**22**(5): 212-221 doi: 10.1097/01.ASW.0000350838.11854.0a.

Edsberg, L. E., Black, J. M., Goldberg, M., McNichol, L., Moore, L. and Sieggreen, M. 2016. Revised National Pressure Ulcer Advisory Panel Pressure Injury Staging System: Revised Pressure Injury Staging System. J. Wound Ostomy Continence Nurs. **43**(6): 585-597 doi: 10.1097/WON.0000000000000281.

Estruch, R., Martinez-Gonzalez, M. A., Corella, D., Salas-Salvado, J., Fito, M., Chiva-Blanch, G., et al. 2016. Effect of a high-fat Mediterranean diet on bodyweight and waist circumference: a prespecified secondary outcomes analysis of the PREDIMED randomised controlled trial. Lancet Diabetes Endocrinol. **4**(8): 666-676 doi: 10.1016/S2213-8587(16)30085-7.

Fito, M., de la Torre, R. and Covas, M. I. 2007. Olive oil and oxidative stress. Mol. Nutr. Food. Res. **51**(10): 1215-1224 doi: 10.1002/mnfr.200600308.

Gundersen, H. J., Bendtsen, T. F., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., et al. 1988. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS. **96**(5): 379-394.

Hatanaka, E., Dermargos, A., Hirata, A. E., Vinolo, M. A., Carpinelli, A. R., Newsholme, P., et al. 2013. Oleic, linoleic and linolenic acids increase ros production by fibroblasts via NADPH oxidase activation. PLoS One, **8**(4): e58626 doi: 10.1371/journal.pone.0058626.

Hatanaka, E., Levada-Pires, A. C., Pithon-Curi, T. C. and Curi, R. 2006. Systematic study on ROS production induced by oleic, linoleic, and gamma-linolenic acids in human and rat neutrophils. Free Radic. Biol. Med. **41**(7): 1124-1132 doi: 10.1016/j.freeradbiomed.2006.06.014.

Hostmark, A. T. and Haug, A. 2013. Percentage oleic acid is inversely related to percentage arachidonic acid in total lipids of rat serum. Lipids Health Dis. **12**: 40 doi: 10.1186/1476-511X-12-40.

Jiang, Z. Y., Woollard, A. C. and Wolff, S. P. 1991. Lipid hydroperoxide measurement by oxidation of Fe2+ in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. Lipids, **26**(10): 853-856.

Kobayashi, M. and Yamamoto, M. 2006. Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species. Adv. Enzyme. Regul. **46**: 113-140 doi: 10.1016/j.advenzreg.2006.01.007.

Levigne, D., Modarressi, A., Krause, K. H. and Pittet-Cuenod, B. 2016. NADPH oxidase 4 deficiency leads to impaired wound repair and reduced dityrosine-crosslinking, but does not affect myofibroblast formation. Free Radic Biol. Med. **96**: 374-384 doi: 10.1016/j.freeradbiomed.2016.04.194.

Lopez-Miranda, J., Perez-Jimenez, F., Ros, E., De Caterina, R., Badimon, L., Covas, M. I., et al. 2010. Olive oil and health: summary of the II international conference on olive oil and health consensus report, Jaen and Cordoba (Spain) 2008. Nutr. Metab. Cardiovasc. Dis. **20**(4): 284-294 doi: 10.1016/j.numecd.2009.12.007.

Magdalon, J., Vinolo, M. A., Rodrigues, H. G., Paschoal, V. A., Torres, R. P., Mancini-Filho, J., et al. 2012. Oral administration of oleic or linoleic acids modulates the production of inflammatory mediators by rat macrophages. Lipids, **47**(8): 803-812 doi: 10.1007/s11745-012-3687-9.

Martinez-Huelamo, M., Rodriguez-Morato, J., Boronat, A. and de la Torre, R. 2017. Modulation of Nrf2 by Olive Oil and Wine Polyphenols and Neuroprotection. Antioxidants, **6**(4): 73 doi: 10.3390/antiox6040073.

Martins, M. A., Catta-Preta, M., Mandarim-de-Lacerda, C. A., Aguila, M. B., Brunini, T. C. and Mendes-Ribeiro, A. C. 2010. High fat diets modulate nitric oxide biosynthesis and antioxidant defence in red blood cells from C57BL/6 mice. Arch. Biochem. Biophys. **499**(1-2): 56-61 doi: 10.1016/j.abb.2010.04.025.

Munoz-Garcia,, A., Thomas, C. P., Keeney, D. S., Zheng, Y. and Brash, A. R. 2014. The importance of the lipoxygenase-hepoxilin pathway in the mammalian epidermal barrier. Biochim. Biophys. Acta **1841**(3): 401-408 doi: 10.1016/j.bbalip.2013.08.020.

Mustoe, T. A., O'Shaughnessy, K. and Kloeters, O. 2006. Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. Plast. Reconstr. Surg. **117**(7 Suppl): 35S-41S doi: 10.1097/01.prs.0000225431.63010.1b.

Nascimento, A. P. and Costa, A. M. 2006. Overweight induced by high-fat diet delays rat cutaneous wound healing. Br. J. Nutr. **96**(6): 1069-1077.

Otranto, M., Nascimento, A. P. and Monte-Alto-Costa, A. 2013. Insulin resistance impairs cutaneous wound healing in mice. Wound Repair Regen **21**(3): 464-472 doi: 10.1111/wrr.12042.

Peirce, S. M., Skalak, T. C. and Rodeheaver, G. T. 2000. Ischemia-reperfusion injury in chronic pressure ulcer formation: a skin model in the rat. Wound Repair Regen. **8**(1): 68-76.

Perez-Jimenez, F., Ruano, J., Perez-Martinez, P., Lopez-Segura, F. and Lopez-Miranda, J. 2007. The influence of olive oil on human health: not a question of fat alone. Mol. Nutr. Food Res. **51**(10): 1199-1208 doi: 10.1002/mnfr.200600273.

Draft

Reeves, P. G., Nielsen, F. H. and Fahey, G. C., Jr. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J. Nutr. **123**(11): 1939-1951.

Reid, R. R., Sull, A. C., Mogford, J. E., Roy, N. and Mustoe, T. A. 2004. A novel murine model of cyclical cutaneous ischemia-reperfusion injury. J. Surg. Res. **116**(1): 172-180.

Rizk, M., Witte, M. B. and Barbul, A. 2004. Nitric oxide and wound healing. World J. Surg. **28**(3): 301-306 doi: 10.1007/s00268-003-7396-7.

Rodrigues, H. G., Vinolo, M. A., Magdalon, J., Vitzel, K., Nachbar, R. T., Pessoa, A. F., et al. 2012. Oral administration of oleic or linoleic acid accelerates the inflammatory phase of wound healing. J. Invest. Dermatol. **132**(1): 208-215 doi: 10.1038/jid.2011.265.

Romana-Souza, B., Santos, J. S. and Monte-Alto-Costa, A. 2009. beta-1 and beta-2, but not alpha-1 and alpha-2, adrenoceptor blockade delays rat cutaneous wound healing. Wound Repair Regen. **17**(2): 230-239 doi: 10.1111/j.1524-475X.2008.00453.x.

Rosa A dos, S., Bandeira, L. G., Monte-Alto-Costa, A. and Romana-Souza, B. 2014. Supplementation with olive oil, but not fish oil, improves cutaneous wound healing in stressed mice. Wound Repair Regen. **22**(4): 537-547 doi: 10.1111/wrr.12191.

Salcido, R., Fisher, S. B., Donofrio, J. C., Bieschke, M., Knapp, C., Liang, R., et al. 1995. An animal model and computer-controlled surface pressure delivery system for the production of pressure ulcers. J. Rehabil. Res. Dev. **32**(2): 149-161.

Salcido, R., Popescu, A. and Ahn, C. 2007. Animal models in pressure ulcer research. J. Spinal Cord Med. **30**(2): 107-116.

Schafer, M. and Werner, S. 2008. Oxidative stress in normal and impaired wound repair. Pharmacol. Res. **58**(2): 165-171 doi: 10.1016/j.phrs.2008.06.004.

Schanuel, F. S., Raggio Santos, K. S., Monte-Alto-Costa, A. and de Oliveira, M. G. 2015. Combined nitric oxide-releasing poly(vinyl alcohol) film/F127 hydrogel for accelerating wound healing. Colloids Surf. B Biointerfaces, **130**: 182-191 doi: 10.1016/j.colsurfb.2015.04.007.

Schurer, N. Y., Rippke, F., Vogelsang, K., Schliep, V. and Ruzicka, T. 1999. Fatty acid uptake by cultured human keratinocytes grown in medium deficient in or supplemented with essential fatty acids. Arch. Dermatol. Res. **291**(1): 47-53.

Schwingshackl, L., Strasser, B. and Hoffmann, G. 2011. Effects of monounsaturated fatty acids on cardiovascular risk factors: a systematic review and meta-analysis. Ann. Nutr. Metab. **59**(2-4): 176-186 doi: 10.1159/000334071.

Sen, C. K. and Roy, S. 2008. Redox signals in wound healing. Biochim. Biophys. Acta. **1780**(11): 1348-1361 doi: 10.1016/j.bbagen.2008.01.006.

Soneja, A., Drews, M. and Malinski, T. 2005. Role of nitric oxide, nitroxidative and oxidative stress in wound healing. Pharmacol. Rep. **57 Suppl**: 108-119.

Stadler, I., Zhang, R. Y., Oskoui, P., Whittaker, M. S. and Lanzafame, R. J. 2004. Development of a simple, noninvasive, clinically relevant model of pressure ulcers in the mouse. J. Invest. Surg. **17**(4): 221-227 doi: 10.1080/08941930490472046.

Taylor, C. 2017. Importance of nutrition in preventing and treating pressure ulcers. Nurs. Older People, **29**(6): 33-39 doi: 10.7748/nop.2017.e910.

Tierney, A. C. and Roche, H. M. 2007. The potential role of olive oil-derived MUFA in insulin sensitivity. Mol. Nutr. Food Res. **51**(10): 1235-1248 doi: 10.1002/mnfr.200700143.

Visioli, F., Bellomo, G. and Galli, C. 1998. Free radical-scavenging properties of olive oil polyphenols. Biochem. Biophys. Res. Commun. **247**(1): 60-64 doi: 10.1006/bbrc.1998.8735.

Willett, W. C., Sacks, F., Trichopoulou, A., Drescher, G., Ferro-Luzzi, A., Helsing, E., et al. 1995. Mediterranean diet pyramid: a cultural model for healthy eating. Am. J. Clin. Nutr. **61**(6 Suppl): 1402S-1406S doi: 10.1093/ajcn/61.6.1402S.

Yaqoob, P. and Calder, P. 1995. Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. Cell Immunol. **163**(1): 120-128 doi: 10.1006/cimm.1995.1106.

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Table 1. Fatty acid composition of soybean (SO) and olive (OO) oil

Fatty acid (%)	SO	OO
C16:0 (palmitic)	10.84	14.43
C16:1n7(palmitoleic)	0.07	2.10
C17:0 (margaric)	0.08	0.08
C17:1 (cis-10-heptadecanoic)	ND	0.21
C18:0 (stearic)	3.32	1.51
C18:1 n9 (oleic)	21.25	70.77
C18:1 (Cis-10-octadecenoic)	1.37	ND
C18:2 n6 (linoleic)	55.11	9.10
C18:3 n3 (α-linolenic)	4,79	0.95
C20:0 (arachidic)	0.35	0.36
C22:0 (behenic)	0.43	0.13
Total saturated (SFA)	15.02	16.51
Total monounsaturated (MUFA)	22.69	73.08
Total polyunsaturated (PUFA)	59.90	10.05
Total n-3 PUFA	4.79	0.95
Total n-6 PUFA	55.11	9.10

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Table 2. - Composition of standard (SC), olive oil (OO) and soybean plus olive oil (SOOO) chows

Content (g/Kg)	SC	OO	SOOO
Casein	140	140	140
Cornstarch	620,7	620,7	620,7
Sucrose	100	100	100
Soybean oil*	40		10
Olive oil*		40	30
Fiber	50	50	50
Vitamin Mix**	10	10	10
Mineral Mix**	35	35	35
L-cysteine	1,8	1,8	1,8
Choline	2,5	2,5	2,5
Antioxidant	0,008	0,008	0,008

*Extra virgin olive oil (Boriello) was supplied by Carla A.F.Rettuci (Contrafortes da Mantiqueira – Andradas - MG) and soybean oil (Liza) by Cargill Agrícola S.A. (Mairinque – SP - Brazil).
**Mineral and vitamin mixtures.
Energy content of all diets= 3800 Kcal/g;
Energy: 76% carbohydrates, 14% protein, 10% lipids;

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FIGURE CAPTIONS

Fig.1 Evaluation of murinometric values, glucose metabolism and liver toxicity of olive oil based diet. A) Body mass over 42 days on diets (One-way ANOVA). B) Epididymal plus retroperitoneal fat mass at the end of experiment period (One-way ANOVA). C) Area under the curve of oral glucose tolerance test (OGTT) (n=6/group), 12 days after the end of the second IR cycle (One-way ANOVA). D) Area under the curve of intraperitoneal insulin tolerance test (IPITT) (n=6/group), 14 days after the end of the second IR cycle (One-way ANOVA). E) Activity of alanine transaminase (ALT) in the serum of mice (n=7 or 8/group), 14 days after the end of the second IR cycle (One-way ANOVA). Values expressed as mean and standard error of mean (mean ± SEM). SC, standard chow; OO, olive oil chow; SOOO, soybean oil plus olive oil chow; AUC, area under the curve

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Fig.2 Effects of olive oil based diet on wound contraction of pressure injury in mice. A) Percentage of initial wound area 3 (d3), 7(d7), 10(d10) and 14(d14) days after the end of the second IR cycle (n=7 or 8/group/day) (Kruskal-Wallis test). B) Photographs of wounds 3 (d3), 7(d7), 10(d10) and 14(d14) days after the end of the second IR cycle. Dotted line represents lesion margins. Values expressed as mean and standard error of mean (mean ± SEM). *p<0.05; **p<0.01; comparison between SC, OO and SOOO groups. SC, standard chow; OO, olive oil chow; SOOO, soybean oil plus olive oil chow

Fig.3 Effects of olive based diet on inflammatory signaling and oxidative damage 14 (d14) days after the end of the second IR cycle. A) Volume density of

neutrophils (Vv) (n=5/group) (Kruskal-Wallis test). B) Quantification data of F4/80 positive cells (cells/mm^2) (n=5/group) (Kruskal-Wallis test). C) The ratio between phosphorylated nuclear factor kappa B p65 (pNF-κB p65) and nuclear factor kappa B p65 (NF-κB p65) protein expression and nuclear factor kappa B p65 (NF-κB p65) protein expression (n=5/group) (One-way ANOVA). D) Cyclooxygenase-2 (COX-2) protein expression (n=5/group) (One-way ANOVA). E) Nitric oxide synthase inducible (NOS-2) protein expression (n=5/group) (Kruskal-Wallis test). F) Nitrotyrosine protein expression (n=5/group) (Kruskal-Wallis test). G) Oxidative damage in protein (n=5/group) (Kruskal-Wallis test). H) Representative image of carbonylated protein bands. I) Levels of lipid hydroperoxides in wound lysate (n=7 or 8/group) (One-way ANOVA). J) Nuclear factor erythroid 2-related factor 2 (Nrf2) protein expression (n=5/group) (Kruskal-Wallis test). The densitometry is expressed as arbitrary units (a.u.) for all immunoblottings. The β-actin was used as a loading control protein for all immunoblottings. Values expressed as mean and standard error of mean (mean ± SEM). *p<0.05; **p<0.01; ****p<0.0001 comparison between SC, OO and SOOO groups. SC, standard chow; OO, olive oil chow; SOOO, soybean oil plus olive oil chow

Fig.4 Effects of olive based diet on dermal reconstruction 14 (d14) days after the end of the second IR cycle. A) Volume density of myofibroblasts (Vv) (n=5/group) (Kruskal-Wallis test). B) Quantification data of blood vessels (cells/mm^2) (n=5/group) (One-way ANOVA). C) Representative images of Sirius red stained (scale bar =100μm). D) Type III collagen (col III) protein expression (n=5/group) (One-way ANOVA). E) Type I collagen precursor (col I precursor) protein

expression (n=5/group) (Kruskal-Wallis test). The densitometry is expressed as arbitrary units (a.u.) for all immunoblottings. The β-actin was used as a loading control protein for all immunoblottings. Values expressed as mean and standard error of mean (mean ± SEM). *p<0.05; ***p<0.001; ****p<0.0001 comparison between SC, OO and SOOO groups. SC, standard chow; OO, olive oil chow; SOOO, soybean oil plus olive oil chow

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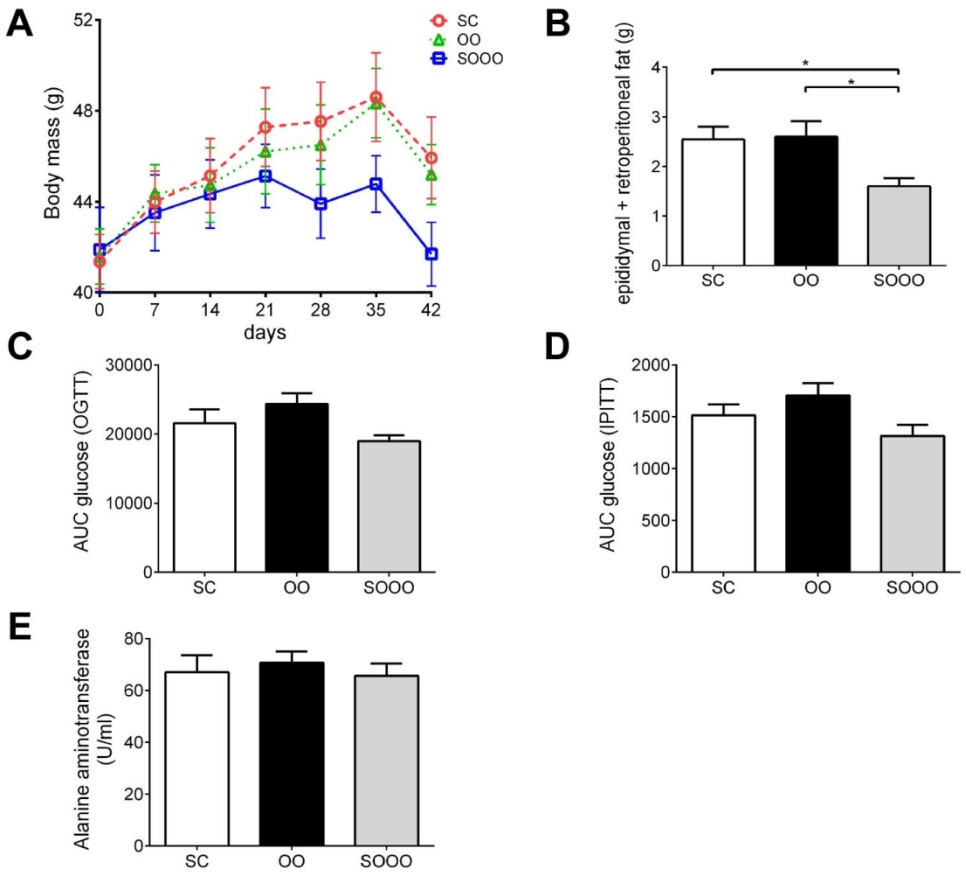

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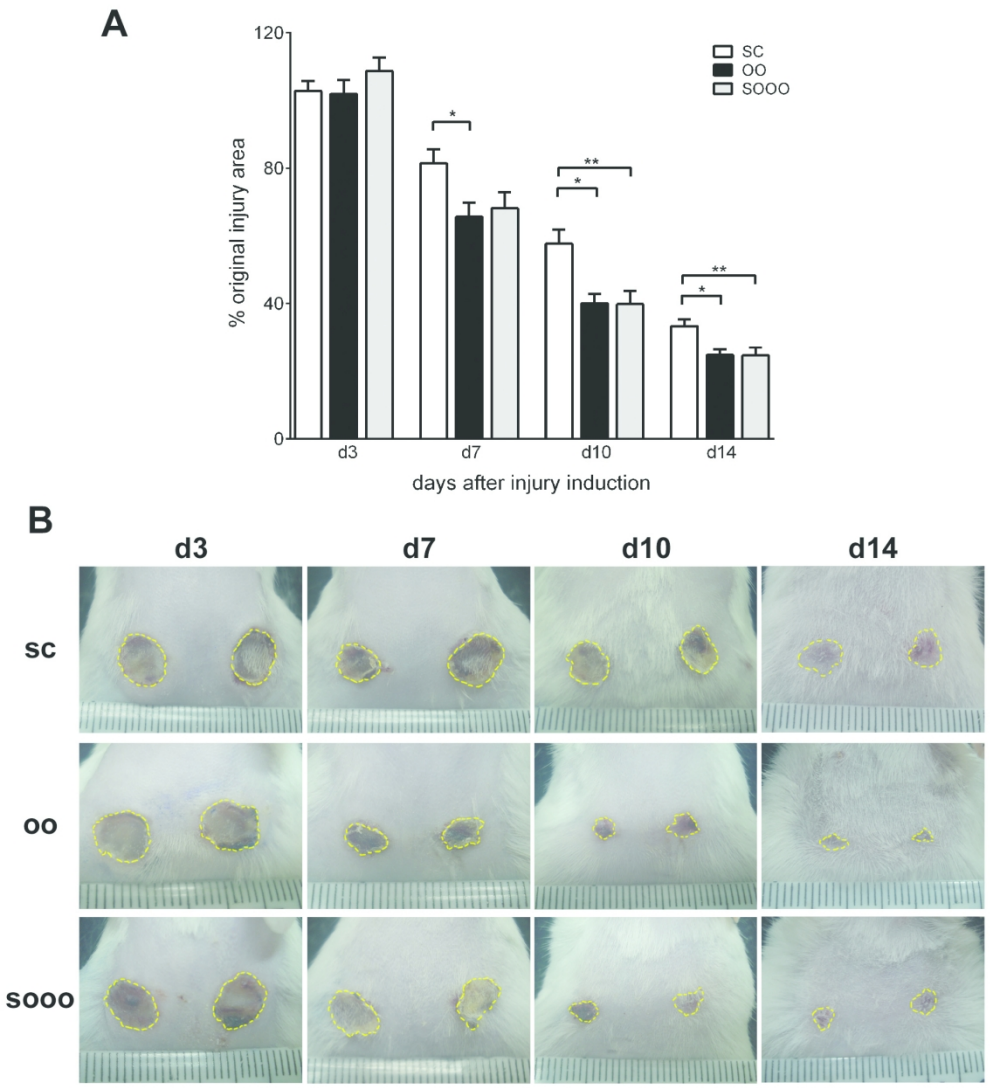

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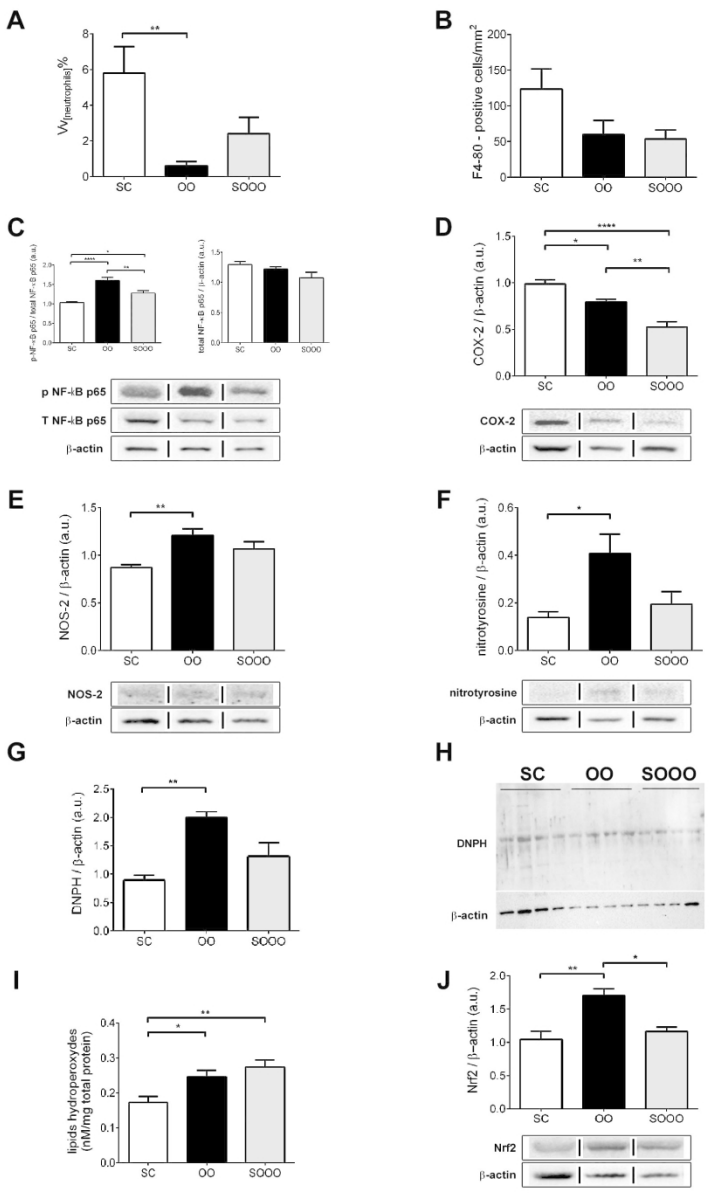

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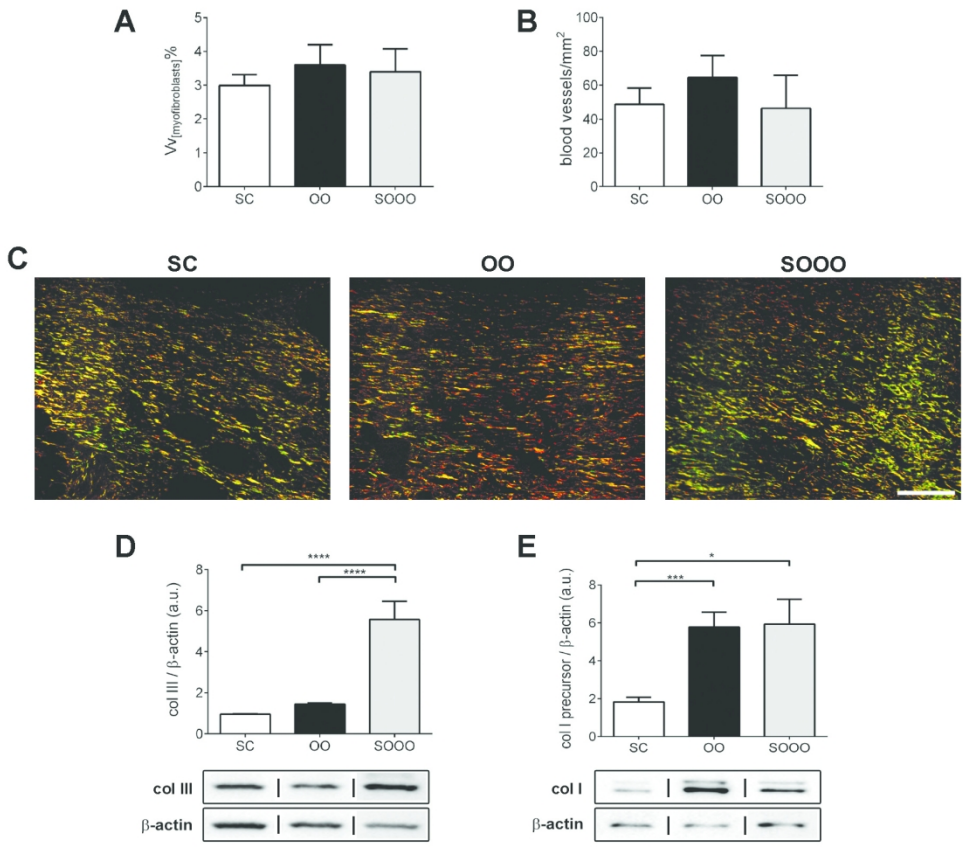
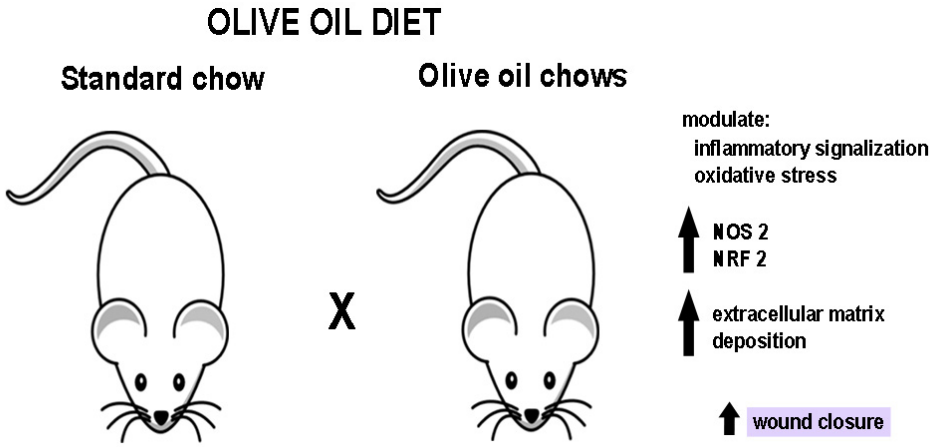


Fig.4 Effects of olive based diet on dermal reconstruction 14 (d14) days after the end of the second IR cycle. A) Volume density of myofibroblasts (Vv) (n=5/group). B) Quantification data of blood vessels (cells/mm2) (n=5/group). C) Representative images of Sirius red stained (scale bar =100μm). D) Type III collagen (col III) protein expression (n=5/group). E) Type I collagen precursor (col I precursor) protein expression (n=5/group). The densitometry is expressed as arbitrary units (a.u.) for all immunoblottings. The β-actin was used as a loading control protein for all immunoblottings. Values expressed as mean and standard error of mean (mean ± SEM). *p<0.05; ***p<0.001; ****p<0.0001 comparison between SC, OO and SOOO groups. SC, standard chow; OO, olive oil chow; SOOO, soybean oil plus olive oil chow

160x144mm (300 x 300 DPI)



85x39mm (300 x 300 DPI)