Oil mixes omega 9, 6 and 3, enriched with seaweed, promoted reduction of thermal burned modulating NF-kB and Ki-67[1]

Ana Paula Bomfim Soares Campelo[I], Márcio Wilker Soares Campelo[I], Gerly Anne de Castro Brito[II], Francisco Vagnaldo Fechine Jamacaru[III], Renata Ferreira de Carvalho Leitão[IV], Paulo Roberto Leitão de Vasconcelos[V]

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[I]Fellow PhD degree, Postgraduate Program in Surgery, Department of Surgery, Federal University of Ceara (UFC), Fortaleza-CE, Brazil. Scientific and intellectual content of the study, technical procedures, acquisition and interpretation of data, histological examinations, manuscript writing.
[II]PhD, Full Professor, Department of Morphology, UFC, Fortaleza-CE, Brazil. Acquisition and interpretation of data, histological examinations, critical revision.
[III]MD, Department of Pharmacology, UFC, Fortaleza-CE, Brazil. Analysis and interpretation of data, critical revision.
[IV]PhD, Associate Professor, Department of Morphology, UFC, Fortaleza-CE, Brazil. Acquisition and interpretation of data, histological examinations.
[V]PhD, Head and Full Professor, Researcher 1A-CNPq, Postgraduate Program in Surgery, Department of Surgery, UFC, Fortaleza-CE, Brazil. Scientific and intellectual content of the study, interpretation of data, manuscript writing, critical revision.

ABSTRACT

PURPOSE: To examine the effects of the oil mixes (ω-9, ω-6 and ω-3) in rats subjected to thermal burn. It was also aimed to assess whether the sources of ω3 would interfere with the effect of such mixes on the thermal injury.

METHODS: Thirty-six rats distributed into five groups: burned + water, burned + isolipid mix, burned + oil mix 1 (ALA), burned + oil mix 2 (ALA + EPA + DHA of fish) and burned + oil mix 3 (ALA + DHA from seaweed). The thermal injury was involving total thickness of skin. After the burns animals received the oil mixes for seven days. The lesions were evaluated by immunohistochemistry.

RESULTS: Animals receiving mix 3 showed a smaller extension of the thermal injury as compared to those that were supplemented with other oils mixes. Expression of Ki-67 in the receiving Mix 3 increased as compared to all the other groups. Animals supplemented with mix 3 were able to inhibit NF-κB in injured tissue.

CONCLUSION: Rats received oil mix in which the source of ω3 (ALA+DHA of seaweed) showed inhibition of NF-κB, increase in cell proliferation, and reduction the extension of thermal lesion.

Key words: Burn. Fatty Acids. Seaweed. Docosahexenoic Acid. alpha-Linolenic Acid. Rats.

Introduction

Burns are one of the most widespread injuries in accidents and remain a global public health issue[1]. The burn wound is a continuous and severe threat against the rest of the body due to invasion of infectious agents, antigen challenge, and repeated additional trauma caused by wound cleaning[2-4].

The sequence of events repairing the wound is categorized into four overlapping phases: inflammation, proliferation, tissue remodeling and scar maturation[5]. Burn wound healing involves a sequence of molecular and cellular events including inflammation, cell migration, angiogenesis, extracellular matrix synthesis, and re-epithelialization[6].

Nutrition is a hallmark of burn care because of large wound burden and severe catabolic state that accompanies severe burn injuries. The importance of the role of nutrition in wound healing is an area that has been widely explored over the last decade[7-10].

Currently, the dietary balance of lipid has motivated several investigations. In patients with the metabolic disorders, the balance between dietary lipids may modulate oxidative stress response. The relationship between the types of polyunsaturated fatty acids feeding[11] affects the synthesis of eicosanoids that act as intermediate messengers of growth factors, controlling growth and differentiation of epithelial cells[12].

Several experimental studies in rats have been carried out using polyunsaturated fatty acids, highlighting benefits of dietary supplementation after ultraviolet light exposure[13] or hot liquid[14-15].

However, this appears to be the first work that involves the simultaneous administration of omega 9 (ω-9), omega 6 (ω-6) and omega 3 (ω-3), with high ratios of ω-9:ω-6 and low ratios of ω-6:ω-3, as well as three different sources of ω-3 (ALA (alpha linolenic acid), EPA (eicosapentaenoic acid) + DHA (docosahexenoic acid) from fish oil, and DHA from algaes), to rats subjected to burning by thermal conduction.

In the present study the mixtures of oils were offered at nutraceutical concentrations with low ratio of ω-6:ω-3 and high ratio ω-9:ω-6 as presented in other biological situations[16-18].

The hypothesis of this study are that the use of combinations containing different proportions of omega 3, 6 and 9 amino acids may have a effect in the process of cell proliferation (re-epithelialization) and oxidative stress of skin burned. It was also aimed to assess whether not only the proposed oil mixes, but what source of ω-3 would interfere in the effect.

Methods

All surgical procedures and animal handling were conducted in accordance with Council for International organization of Medical Sciences (CIOMS) and the Guide for the Care and Use of Laboratory Animals from the Brazilian College of Animal Experimentation, after approval by the local ethics committee (UFC) (protocol #37/10). The study was designed to minimize the number of animals required for the experiments. The animals were housed in polypropylene cages at ambient temperature of 24ºC on a 12 h light-dark cycle.

Male Wistar rats (*Rattus norvegius albinus*), weighing 250-270g were under standard housing conditions with free access to water and chow, and were randomly assigned to five groups of six animals each: Burn-water (B+W); Burn-Isolipidic Mix (B+I); Burn-Mix 1 (B+M1); Burn-Mix 2 (B+M2); Burn-Mix 3 (B+M3). The groups B+M1, B+M2 and B+M3 received oily mixtures of ω-6/ω-3 (1.4:1 ratio) and ω-9/ω-6 (3.4:1 ratio), differing only in the source of ω-3: Mix 1 (ω-3 alpha-linolenic acid), Mix 2 (ω-3 alpha-linolenic, docosahexaenoic and eicosapentaenoic acids) and Mix 3 (ω-3 alpha-linolenic and docosahexaenoic acids). The B+I group received isolipidic mixture of ω-6/ω-3 (8:1 ratio) and ω-9/ω-6 (0.4:1 ratio). Mixtures are detailed on Table 1. The administered dose of each mixture corresponded to 1.2 g of oil/Kg of animal body, by orogastric administration, daily for seven consecutive days.

TABLE 1 - Division of groups and the mixtures composition (oils and ratio) with their respective sources of ω-3.

Groups	***Composition***	***ω-3 sources***	***Ratio***	
			ω-6:ω-3	ω-9:ω-6
Burn-Water (B-W)	Water	-	-	-
Burn-Isolipidic(B-I)	Isolipidic (corn and soybean)		8:1	0.4:1
Burn-Mix 1 (B-M1)	ω-9 (olive)+ω-6(canola)+ω-3	ALA(linseed)	1.4:1	3.4:1
Burn-Mix 2 (B-M2)	ω-9 (olive)+ω-6(canola)+ω-3	ALA+EPA+DHA (fish)	1.4:1	3.4:1
Burn-Mix 3 (B-M3)	ω-9 (olive)+ω-6(canola)+ω-3	ALA + DHA (seaweed)	1.4:1	3.4:1

Note: Mix=mixture; ALA=alpha-linolenic acid; DHA=docosahexaenoic acid and EPA=eicosapentaenoic acids.

Burns

In the first day, the rats were anesthetized with an intramuscular injection of ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg) and immediately before inflicting the burn, the area was shaved with a standard electric shaving machine to obtain a smooth surface and hairless skin, two 10 x 10 mm squares were outlined with a sterile surgical marker on each side and along the vertebral column using a prepared template (an X-ray film with a 10 x 10 mm grid) positioned between the thoracic and abdominal limbs. The dorsal skin was surgically prepared with successive applications of 2% chlorhexidine scrub. A previously described model[19-20] was used with some modifications. The original tip of an ordinary 40W soldering iron was replaced with tip and square 10 x 10 mm copper plate. An electronic temperature controller with a thermocouple type feedback sensor was connected 2 mm above the plate tip in order to allow precise optimal temperature monitoring at the tip of the instrument. Additionally, a digital multimeter with a K type thermocouple was fixed to the copper plate to assure real time optimal control of the temperature applied to the skin (surface counter). The desired stamp temperature (200°C) was reached 5 min after switching on the electric current. The device was positioned vertically under its own weight (85g) and applied to each skin burn site during nine seconds to inflict the burns as outlined. Immediately after each burn injury, the respective wound was cooled off during 1 min with gauze embedded in isotonic saline at 22°C, as described elsewhere[21]. The plate produced a burn area of approximately 1 cm^2/wound. Four burns were produced on each animal (4 cm^2/animal) leaving approximately 1 cm intact skin between burned areas. Following one hour of the procedure, the burns injuries were photographed (see macroscopic analysis for details) and the animals received water, or isolipidic oil mix, or one specific mix of oils for each respective group, and were returned to their individual cages for recovery with free access to rat chow and tap water. Analgesia was administered during 24h after burn injury by adding 30 mg codeine phosphate hemihydrate to 500 ml tap water.

Histopathology

At the end of the experiments (seventh day) all animals were killed by an overdose of anesthetics (ketamine+xylazine). Tissue samples were collected from the dorsum with a surgical blade, removing skin fragments (20 mm x 5 mm), including the central scar, adjacent healthy tissue and underlying panniculus carnosus muscle. Tissue samples were fixed in formalin for 24 hours before being transferred to 70% ethanol solution. Further processing included paraffin embedding and sectioning, to generate 5-µm-thick tissue coronal sections to be mounted on glass slides. The slides were stained using hematoxylin and eosin. The extent of skin damage was assessed by a pathologist, who was not aware of which treatment was applied to the animals, using a light microscope (Olympus, X100).

Immunohistochemistry

Immunohistochemistry for Ki-67 (cell proliferation), NF-κB p50 (NLS) and HNE-J (lipidic peroxidation) was performed on skin tissue using the streptavidin–biotin-peroxidase method in formalin-fixed, paraffin-embedded tissue sections (4 µm thick), mounted on poly-L-lysinecoated microscope slides. The sections were deparaffinized and rehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (vv-1) hydrogen peroxide and washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4ºC) with primary rabbit anti-Ki-67 and rabbit anti-NF-κB, mouse anti-HNE-J antibody (individual slide for each antibody) diluted 1:200 in PBS plus bovine serum albumin (PBS–BSA). The slides were then incubated with biotinylated goat anti-rabbit or anti-mouse (for each primary antibody respectively) diluted 1:400 in PBS–BSA. After washing, the slides were incubated with avidin–biotin-horseradish peroxidase conjugate (Strep ABC complex by Vectastain-ABC Reagent and peroxidase substrate solution) for 30 min, according to the Vectastain protocol. Ki-67 and NF-κB and HNE-J were visualized with the chromogen 3,3´diaminobenzidine (DAB). Negative control sections were processed simultaneously as described above but with the first antibody being replaced by PBS–BSA 5%. None of the negative controls showed immunoreactivity. Slides for HNE-J were counterstained with Harry's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene and cover slipped.

Acquisition and processing of the immunohistochemistry images

Digital images of histological samples were acquired in standard fashion using a light microscope (Olympus, x100) fitted with a digital camera. The procedure included an initial

scan of the histological preparation at x40 magnification in order to identify areas with increased density (hot spots) of the structures of interest (starting and ending edge of the burned zone containing the tissue of re-epithelialization). Three hot spots selected for each sample were then color-scanned at x100 magnification. Images were saved as Windows® bitmaps measuring 640 x 480 pixels.

Images were processed by Morphometric Analysis System (SAMM), a software developed specifically for this purpose[22]. The system was previously adjusted to recognize the color spectrum of the structures of interest, according to the staining technique employed. This step enabled the software to automatically identify and segment the structures of interest (separating them from the other components of the histological preparation) in both full images and user-defined regions of interest (ROI). However, interactive segmentation (making changes in segmentation parameters) remained an option whenever the automatic mode was deemed inadequate. Following segmentation, the software provided the quantification of the structure of interest in the image or ROI by calculating the area density, that is the quotient between the area occupied by the structure of interest and the total area analyzed.

Statistical analysis

After a Kolmogorov–Smirnov test of normality, data were submitted to one-way analysis of variance followed by Tukey's multiple comparison test. Parametric data were expressed as mean ± SD. The level of statistical significance was set at 5%.

Results

Microscopic analysis

On the seventh day there was smaller extension of the lesion only in the burn-Mix 3 as compared to extension observed in the burn-water group ($p<0.05$). There was no difference between burn-water, burn-isolipidic, burn-mix 1 and burn-mix 2 group, see Figure 1.

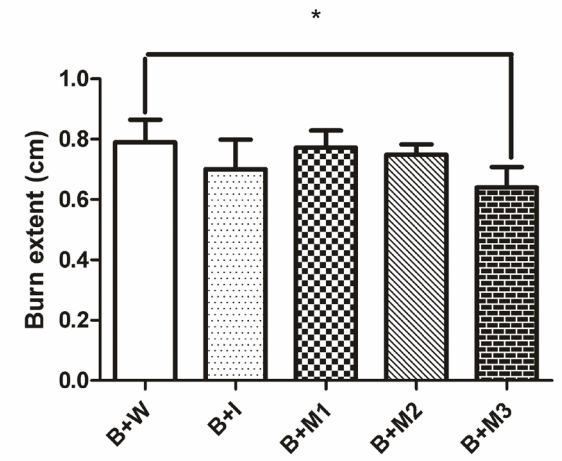


FIGURE 1 - Burn extension in cm on the seventh day for each group, visualized with optical microscopic (magnification x10). Results presented as mean and standard deviation. (n=6), *$p<0.05$. Abbreviations: B+W = group burned and administered water; B+I = group burned and administered isolipdic mixture; B+M1 = group burned and administered mixture 1 of oils; B+M2 = group burned and administered mixture 2 of oils; B+M3 = group burned and managed mixture 3 of oils.

Quantitative analysis of immunohistochemistry

There was a higher detection (positively stained) of antibody Ki-67 antibody in burn-mix 3 group than burn-water group ($p<0.05$). There was however no statistical significant differences when the burn-water group was compared to other groups, see Figure 2 A and B.

There was less detection (positively stained) of antibody NF-κB in the Mix 3 Group than burn-water group ($p<0.05$). There were no significant statistical differences when the burn-water group was compared to other groups, see Figure 3 A and B.

There were no significant statistical differences in detection (positive staining) of antibodies HNE-J between the groups ($p>0.05$).

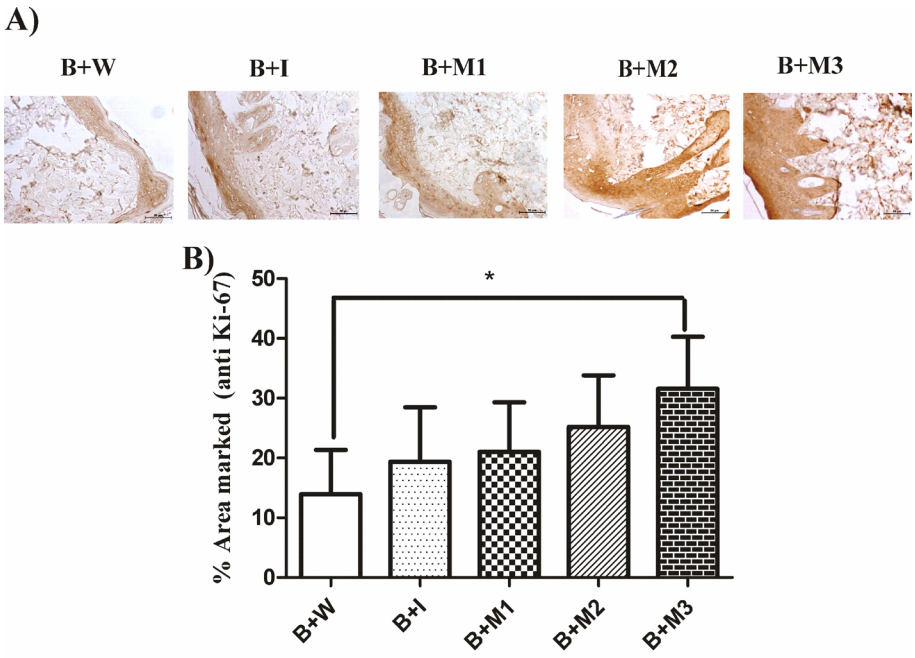


FIGURE 2 - A) Representative image of Immunohistochemical for anti-Ki-67 of the skin of an animal in each group. **B**) Percentage of area marked by anti-body Ki-67. Results presented as mean and standard deviation. (n=6), *p<0.05 (B+W vs. B+M3). Abbreviations: B+W = group burned and administered water; B+I = group burned and administered isolipdic mixture; B+M1 = group burned and administered mixture 1 of oils; B+M2 = group burned and administered mixture 2 of oils; B+M3 = group burned and managed mixture 3 of oils.

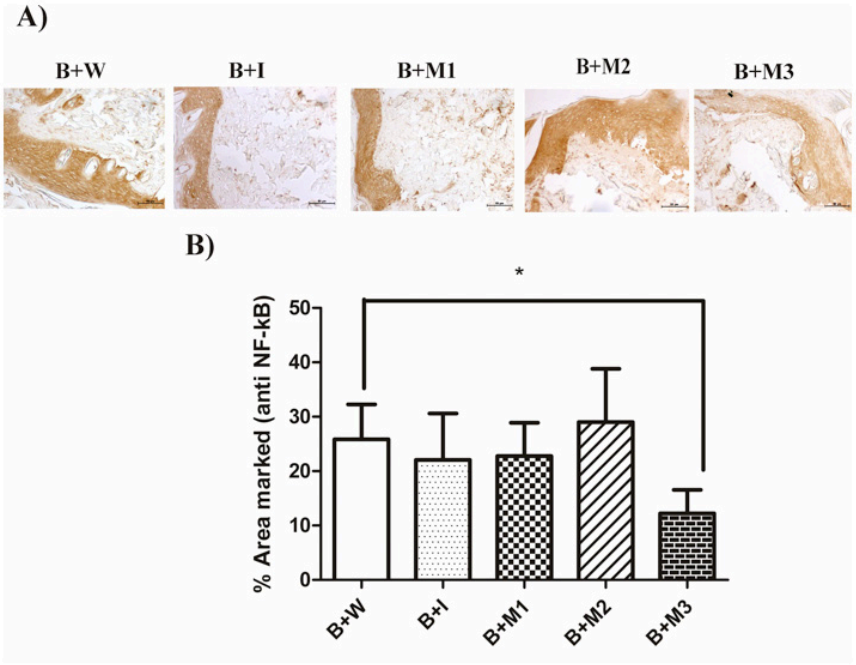


FIGURE 3 - A) Representative image of Immunohistochemical for anti- anti-NF-κB of the skin of an animal in each group. **B**) Percentage of area marked by anti-body anti-NF-κB. Results presented as mean and standard deviation. (n=6), *p<0.05 (B+W vs. B+M3). Abbreviations: B+W = group burned and administered water; B+I = group burned and administered isolipdic mixture; B+M1 = group burned and administered mixture 1 of oils; B+M2 = group burned and administered mixture 2 of oils; B+M3 = group burned and managed mixture 3 of oils.

Discussion

The present study was designed to examine the effects of oils mixtures ω-9, ω-6 and ω-3, as well as which type of the ω -3 source (ALA, EPA and DHA) may influence cell proliferation and stress oxidative in rat skin burned by direct conduction.

Experimental model of burns in animals have been an essential tool for the study of the pathophysiology of skin lesions

that may also occur in humans, which may help to develop new methods of treatment[24-25].

Burn lesions were induced by energy transfer using a copper plate in direct contact with the skin at continuous high temperature (200°C) electronically controlled, resulting in skin thermal injury of total thickness (epidermis, dermis, hypodermis and musculature) with lesion of the hair follicles and healing from the periphery to the center of the lesion. This method was used in this study due to its repeatability and uniformity[20].

The Wistar rat was chosen because it is a small animal, enabling easy standardization with regard to age, weight, sex, housing, feeding, cleaning care and experimental manipulation. In addition, these animals have been used in various experimental models to evaluate treatment of burns[25-29].

The anesthetic method proved to be satisfactory by the relative ease induction and maintenance of the anesthetic plan, little volume administered and quick recovery. It was performed good analgesia with codeine in the water evidenced by the absence of respiratory complications or accidental deaths.

The site chosen for the accomplishment of the burns was the dorsal area of animals, in accordance with the observations of several researchers, who consider the skin of the back as the best place for experimental lesion, avoiding irritation on contact with saliva and self cannibalism[30-31].

Pharmaconutrients or "nutraceuticals" are isolated or combined nutrients that, at pharmacological doses, modify the biological response of the host. The most commonly used nutraceuticals are anti-inflammatory ω-3 fatty acids (EPA and DHA), glutamine, arginine and nucleotides[32].

The present study appears to represent the first demonstration that mixtures of oils containing low ratio of ω-6 to ω-3 and high ratio of ω-9 to ω-6 with nutraceutical concentrations, evaluated in a burn model by direct conduction in rats, resulted in increased re-epithelialization and lower marking of NF-κB stain, both events observed as omega 3 source type dependent.

Currently, there is solid evidence supporting the concept that the intake of nutrients with a specific focus may improve the final results by modulating the immune, metabolic and oxidative stress response[33-36].

Western societies have a diet with a ratio ω-6:ω-3 between 15:1 and 16,7:1[17]. In general, in an American diet, individuals consume fatty acids as follows: 89% is linoleic acid (AL) whereas only 9% is ALA. However the ideal ratio ω-6:ω-3 would vary between 1: 1 and 4:1[17,18,37,38].

Marine fish oils have high ω-3 content, mostly in the form EPA and DHA[33]. Studies show that a high ratio ω-6:ω-3, such as those mentioned in the above paragraph, promotes various diseases due to the increased production of NFk-B and inflammatory factors [16]. On the other hand, increased consumption ω-3 (i.e. with a decrease in the ratio of ω-6: ω-3) has opposite effects.

Vassiliou *et al*.[39] demonstrated in vitro that the oleic acid (ω-9) reduces in a dose-dependent way oxidative stress. The higher concentration of ω-9, the greater antioxidant effect.

It is known that the ω-3 fatty acid can come from different sources, therefore, in this work there were used three mixtures of oils with ω-3 different sources: Mixes 1 (ALA), mix 2 (ALA + EPA + DHA from fish), mix 3 (ALA + DHA from seaweed). A mixture of isocaloric oils, with ω-9:ω-6 ratio of 0.4:1 and ω-6:ω-3 ration of 8:1, was used as isolipidic neutral mix, as it should not promote neither anti-oxidant or anti-inflammatory action. There was also a control group that received only water (zero fat).

On the seventh day of the oil intake burned rats which received ω-3 from seaweed (M3 - DHA) showed a reduction in the lesion area as compared to those who received water, neutral isolipidic control, and other mixtures with different sources of ω-3 (Mix 1 - ALA, Mix 2 – ALA+ EPA + DHA from fish oil). The burn extension was considered edge-to-edge of re-epithelialization without interference of the crusts.

To study cellular proliferation, tissue staining of the nuclear protein marker Ki-67 was used. This protein is structurally associated with chromatin to develop function in cell proliferation and is present in the G1, S, G2 and M phases of the cell cycle, and absent in the cells during G0 phase. The monoclonal antibody against Ki-67 was obtained by Gerdes *et al.*[40]. It reacts with a proliferating cell nuclear antigen[41] and has previously been used in an experimental model of burn showing positive correlation between cell proliferation and wound healing in burned mice[42]. Mix 3 was the only group to show increased cell proliferation, as regard to Ki-67 tissue staining which is consistent with the results obtained by the microscopy analysis that demonstrated reduction in the burned extent.

Immunohistochemical assessment, in this study, was carried out quantitatively using the software developed specifically for morphometric study already used in other similar works[19-20]. This fully automated method allows absence of interference from the observer after the acquisition of the images as recommended by Dornelas[22].

The families of ω-3 and ω-6 use the same enzyme system, competing for enzymes in common. This competition alters the entire metabolism of the production of eicosanoids, such as PG, TX and LT. The eicosanoids from the metabolism of AGPIs ω-6 are pro-inflammatory mediators, while those from the metabolism of

ω-3 are anti-inflammatory. Some effects of AGPIs on immune and inflammatory systems are independent of eicosanoids generation. Studies emphasize that the series AGPIs ω-3 affect immune functions. These acids present a suppressor effect, inhibiting the proliferation of lymphocytes, antibody production, the production of pro-Inflammatory cytokines and others nuclear transcription molecules[11,43-45].

One of the most important links that self-perpetuating cycle of inflammation is NF-κB. It is a nuclear transcription factor (protein family) found in all cell types consisting of five subunits that act as heterodimers and homodimers that is involved in responses to stimuli such as stress, cytokines, free radicals, ultraviolet radiation and burns[43,46]. It acts basically as the "on/off" switch to the cell to generate inflammatory mediators. When activated, act as a transcription factor for a vast number of genes involved in immunoregulation, growth regulation and inflammation[47]. Activation of NF-kB is controlled by the inhibitor of kappa B (IkB) family of proteins. IkB proteins suppress NF-kB activity by binding to its protein subunits, preventing its translocation to the nucleus[48]. Once activated, NF-kB induces expression of inflammation-related signaling molecules, some of which are themselves responsible for NF-kB activation. Just a few examples of inflammation-related proteins whose expression is under control of NF-kB include cytokines.

Omega-3 fatty acids have been shown to inhibit NF-kB activation, and sometimes expression, in a number of cell lines and animal models. A study in HR-1 mice, a hairless mouse strain used to study skin disorders, showed that topical application of DHA reduces UVB radiation-induced IkB phosphorylation and subsequent NF-kB activation. The authors attributed this to a decrease in phosphorylation of mitogen and stress-activated kinase-1 (MSK1), a downstream target of the MAPK/ERK signaling pathway and known activator of NF-Kb[49,50].

The ω-3 fatty acids are able to stabilize and influence NF-κB signal transduction and gene expression by the level of EPA and DHA acquired by diet[44,45]. The EPA and DHA appear to help the resolution of inflammation and the production of potent anti-inflammatory molecules called resolvins. In the present study, only in M3 group, where omega-3 source was seaweed, there was observed lower immunohistochemical marking of NF-κB as compared to all other groups. Interestingly this source of ω-3 is rich in doxohexanoic acid.

To evaluate the antioxidant action of mixtures of oils with high ratio of ω-9:ω-6 and low ratio ω-6:ω-3 an immunohistochemical marker with the antibody anti-4-hydroxinonenal (Anti-HNE-J) was used. This antibody induces the production via lipid peroxidation of 4-hydroxinonenal, which is one of the mediators of cell death due to oxidative stress[51,52]. On the seventh day no difference was found among mixtures. Albeit in very different circumstances, the monounsaturated fatty acid oleic acid, present in the mixes studied, present theoretical anti-oxidant action, especially as regard to olive oil, which is rich in polyphenols. It may be possible that an anti-oxidant effect could occur in the early hours or days post-burn, and therefore future new experiments with earlier sampling should be performed.

Conclusion

Rats received oil mix in which the source of ω3 (ALA+DHA of seaweed) showed inhibition of NF-κB, increase in cell proliferation, and reduction the extension of thermal lesion.

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Correspondence:
Ana Paula Bomfim Soares Campelo
Departamento de Cirurgia
Universidade Federal do Ceará
Rua Professor Costa Mendes, 1608/3º andar
60430-140 Fortaleza – CE Brasil
Tel.: (55 85)3288-8063
Fax: (55 85)3288-8064
anapaulacampelo@bol.com.br
sbpana@bol.com.br

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