

Effect of n-3 long-chain polyunsaturated fatty acids on wound healing using animal models – a review

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Abstract

The present review summarizes results of experiments, mostly performed on rodents, regarding the effects of fish oil (FO) and its biologically active constituents, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on the healing of cutaneous wounds, but also of selected other types of injury. Structure, metabolism and functions of EPA/DHA in an organism are briefly mentioned, with an emphasis on the ability of these long-chain polyunsaturated fatty acids to modulate inflammation. Wound healing as a complex programmed sequence of cellular and molecular processes including inflammation, cell migration, angiogenesis, synthesis of provisional matrix, collagen deposition and reepithelialisation is briefly described. Markers for evaluation of the healing process include planimetry indices, tensile strength, quantification of collagen synthesis including hydroxyproline determination, histopathology/immunohistochemistry and genomic/proteomic markers. As far as effects on wound healing are concerned, the main emphasis is put on the outcomes of experiments using a dietary FO/DHA/EPA administration, but the results of experiments with a parenteral application are also mentioned, together with selected relevant *in vitro* studies. An important conclusion from the above-mentioned studies is an inconsistency of FO/DHA/EPA effects on wound healing: decreased/increased collagen deposition; lower/higher counts of the inflammatory cells in the healing tissue; increased/decreased concentration of both pro- and anti-inflammatory cytokines; DHA accelerated/delayed wound healing process. Some experiments indicate superiority of DHA over EPA regarding wound healing.

Hydroxyproline, inflammation, interleukins, rodents, docosahexaenoic acid, fish oil

The present review deals with the effects of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) in animal (especially rodent) models, the results of these experiments being also relevant to human health. Positive effects of n-3 LC-PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on human health have been reported in cardiovascular diseases (Givens and Gibbs 2008), autoimmune diseases (Zárate et al. 2017), foetal and neonatal brain development (Ruxton et al. 2005), dementia (Barberger-Gateau et al. 2002), cognitive function (van Gelder et al. 2007) and wound healing (McDaniel et al. 2008). From these varied topics, the present review deals selectively with the potential of EPA and DHA (fish oil) to modulate particular phases of wound healing, especially (but not exclusively) cutaneous wound healing.

Long-chain polyunsaturated fatty acids n-3

Structure, metabolism, functions in an organism

In a mammalian organism, LC-PUFA n-3 (first double bond comes from the third carbon from the methyl end of the molecule), such as EPA (20:5 n-3), docosapentaenoic acid (DPA, 22:5 n-3) or DHA (22:6 n-3) can be synthesized from α -linolenic acid (ALA; 18:3 n-3) by the action of desaturases and elongases (Das 2006; Jump 2008). The LC-PUFA n-3 are important components of the cell membranes (Das 2006), where they influence

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membrane fluidity and behaviour of the integral membrane proteins and participate in regulation of many functions in the organism, including regulation of an inflammatory response (Schmitz and Ecker 2008), which is one of several overlapping phases of wound healing (Caetano et al. 2016).

Metabolism of n-3 PUFA in the mammalian organism should be considered together with metabolism of the n-6 group of PUFA originating in linoleic acid (LA; 18:2 n-6). Physiologically the most important metabolite of the n-6 group is arachidonic acid (AA; 20:4 n-6; Zárata et al. 2017). The key metabolites of both EPA and AA (together called eicosanoids) are endoperoxides, such as thromboxanes and prostaglandins produced under catalysis of cyclooxygenase on the one hand, and leukotrienes produced by an action of 5-lipoxygenase on the other hand (Nakamura et al. 2004; Das 2006; Jump 2008).

Eicosanoids produced from AA increase the tendency of thrombocytes to aggregate, act mostly as vasoconstrictors and have predominantly pro-inflammatory effects. EPA-derived eicosanoids have less pronounced and in many instances opposite effects in comparison with their AA-derived counterparts (Das 2006); inflammation modulating effect of EPA/DHA can make a difference especially in the initial phases of wound healing (McDaniel et al. 2008).

Functions of EPA/DHA on a molecular level are based (among others) on modulation of the signalling pathways mediated by transcription factors nuclear factor kappa B (NF- κ B; Kostadinova et al. 2005), peroxisome proliferator-activated receptors (PPARs; Arai et al. 2009) and sterol response-element-binding protein (SREBP; Nakamura et al. 2004; Jump 2008). Especially NF- κ B and PPAR γ are involved in signalling during the inflammation phase of wound healing. Inflammation is a relatively short part of the wound healing process, but its proper course is decisive from the viewpoint of a successful tissue remodelling and scar tissue formation (Otranto et al. 2010). Therefore the next part of the review is devoted to the evaluation of an EPA/DHA capability to modulate inflammation.

LC-PUFA n-3 as inflammation modulators

Anti-inflammatory effects of EPA and DHA are based, among others, on the competition with AA in eicosanoid synthesis (Schmitz and Ecker 2008) and on modulation of signalling pathways mediated by transcription factors PPAR α , PPAR γ and NF- κ B (Komprda 2012).

EPA and DHA are endogenous ligands of PPAR γ , whose activation increases the amount of the adipose tissue-derived anti-inflammatory hormone adiponectin (Siriwardhana et al. 2013). The EPA/DHA anti-inflammatory effect is further mediated by GPR120, a G-protein coupled receptor, whose activation leads to a repression of the macrophage-induced inflammation (Flock et al. 2013). According to Oliver et al. (2010), EPA/DHA decreases plasma levels of pro-inflammatory markers interleukin 6 (IL-6), tumour necrosis factor alpha (TNF- α) or interferon γ (IFN- γ), and increases the concentration of anti-inflammatory markers IL-10 and transforming growth factor beta (TGF- β).

During the acute inflammatory response, neutrophils are first to arrive at the site of inflammation and play an important protective role in innate immunity and host defence; however, excessive accumulation of neutrophils within the tissue can lead to tissue damage and amplification of the inflammatory response (Arita et al. 2005). Lipid mediators, such as prostaglandins and leukotrienes play pivotal roles in the initiation of acute inflammation, whereas resolvins and protectins, inflammation mediators derived from LC-PUFA n-3 (Serhan et al. 2015), promote and stimulate the active resolution of inflammation (Serhan and Savill 2005). The control of neutrophil infiltration is of wide interest in this situation, which underlines the importance of the above-mentioned resolvins and protectins (Dinarello 2010); resolution of acute inflammation is a central component of host defence and the return of tissue to homeostasis (Serhan et al. 2015). As an example, EPA-derived

resolvin E1 reduced leukocyte infiltration in a mouse peritonitis model and protected against the development of induced colitis, including decreased leukocyte infiltration and pro-inflammatory gene expression in a study by Arita et al. (2005).

As far as *in vitro* experiments are concerned, EPA and DHA decreased lipopolysaccharide (LPS)-induced pro-inflammatory IL-1 β expression in 3T3-L1 adipocytes (Cranmer-Byng et al. 2015); Romacho et al. (2015) reported prevention of NF- κ B activation (induced by TNF- α) in human primary adipocytes by EPA/DHA; DHA decreased degree of monocyte chemoattractant protein-1 and IL-6 secreted from murine adipocytes in an experiment of De Boer et al. (2014).

Regarding *in vivo* experiments, fish oil (rich source of EPA) tended to reduce IL-1 β and IL-12 production in LPS-challenged pigs (Liu et al. 2003), and n-3 PUFA intervention in LPS-challenged mice decreased levels of pro-inflammatory cytokines, including TNF- α (Liu et al. 2015). However, Hall et al. (2012) reported no substantial effect of fish oil (EPA and DHA) on pro-inflammatory cytokines in rodents. Similarly, Vigerust et al. (2013) found no significant differences between fish oil-fed and control animals in the hepatic concentrations of IFN- γ , IL-1 β , IL-2 or IL-6; and the content of pro-inflammatory IL-17 was even higher in the fish oil group.

An equivocal effect of fish oil on inflammatory markers was confirmed by Komprda et al. (2018) who reported a higher concentration of both anti-inflammatory IL-4 and pro-inflammatory TNF- α in plasma of fish oil-fed LPS-challenged pigs in comparison with the palm oil-fed controls. A diet rich in DHA did not affect the amount of the nuclear (i.e. active) fraction of NF- κ B, but nevertheless increased the plasma level of anti-inflammatory TGF- β 1 in rats with an induced state of a low-grade inflammation (Komprda et al. 2016). This part of the review can be concluded by stating that LC-PUFA n-3 have an equivocal effect on inflammation and thus on the subsequent stages of wound healing.

Wound healing

Definition and phases

Wound healing is generally defined as the process by which a body tissue (usually skin) repairs itself after trauma, a dynamic process aimed at restoring the structure of the injured tissue (Caetano et al. 2016). It is a sequential process that occurs in three overlapping stages: inflammation, cell proliferation and tissue remodelling (Gurtner et al. 2008), resulting in scar tissue formation (Otranto et al. 2010). Some authors divide wound healing into four phases: inflammation, coagulation, tissue formation and tissue remodelling (Caetano et al. 2016). This complex programmed sequence of cellular and molecular processes includes inflammation, cell migration, angiogenesis, synthesis of provisional matrix, collagen deposition and reepithelialisation (Gercek et al. 2007). From the viewpoint of regenerative veterinary/human medicine and a new field of tissue engineering, the process of wound healing can be divided into two major phases: the early phase and the cellular phase: the early phase leads to haemostasis and formation of a makeshift extracellular matrix, the cellular phase involves an inflammatory response, synthesis of the granulation tissue and restoration of the epithelial layer (Nguyen et al. 2009).

Immediately after injury, platelets start to release cytokines and growth factors that recruit inflammatory cells and pro-inflammatory factors (serotonin, bradykinin, prostaglandins, thromboxanes, histamine) to stimulate local debridement, to degrade foreign particles and to provide provisional matrix for further proliferation of fibroblasts leading to formation of granulation tissue (Gurtner et al. 2008; Velnar et al. 2009).

The following phase is characterized by the formation of granulation tissue. Fibroblasts proliferate and migrate to the damaged tissue area in order to synthesize the new

extracellular matrix elements (Velnar et al. 2009; Guo and Dipietro 2010; Caetano et al. 2016), such as proteoglycans, glycosaminoglycans and collagen, which are deposited in the damaged area, where they replace the initial provisional matrix, comprised of fibrin (Campos et al. 2008; Velnar et al. 2009). The major structural component of granulation tissue, strengthening the extracellular matrix, is collagen, with an important constituent amino-acid proline, and hydroxyproline as a biochemical marker for collagen and an indicator of the progression of healing (Caetano et al. 2016).

When using animal (usually rodent) models for wound healing in humans, it is important to realize that while the closure of human wounds is primarily accomplished through proliferation and migration of cells at the wound edge, contraction is the driving force behind wound closure in rodents (Pensalfini et al. 2018). Nevertheless, the major cellular and molecular processes that occur during healing are conserved between both species and allow for rodent wounds to serve as a model for human wound repair (Galiano et al. 2004; Gurtner et al. 2008).

Markers for evaluation of the healing process

Planimetry (rate of healing)

To quantitate wound contraction and reepithelialization, a transparent plastic sheet is usually placed over the wound and its margins are traced (Nascimento and Costa 2006; Amadeu et al. 2007). After digitization, the wound area can be measured (Otranto et al. 2010). Nascimento and Costa (2006), and Otranto et al. (2010) estimated wound reepithelialization in rats by the difference between the total lesion area and the wound area still uncovered with epidermis. Similarly, dos Santos Rosa et al. (2014) evaluated wound contraction and reepithelialization in mice by tracing the margins of the total wound area and nonreepithelialized wound area using a transparent plastic sheet.

Scardino et al. (1999) traced (on sterile transparent polyethylene sheets) the total wound area, the area of epithelium on the wound and the area of unepithelialized granulation tissue in the wound centre in Beagle dogs on the day of surgery and several postoperative days, aiming to calculate the percentage of wound contraction, the percentage of epithelialization and the percentage of total wound healing compared to the original wound. The percentage of wound contraction can be measured using the formula of Ramanathan et al. (2017): wound contraction (%) = $[\text{wound area (day 0)} - \text{wound area (day n)}] / \text{wound area (day 0)} \times 100 - 3$. McDaniel et al. (2008) defined wound healing as the advance of the wound margins toward the wound centre and measured a daily area yet to be healed by a noncontact method using an orientation card of known dimensions placed next to the wound sites, single digital camera photogrammetry (SCP), and a wound measurement software.

Amadeu et al. (2003) elaborated a stereological method of Baddeley et al. (1986) and Gundersen et al. (1988), which enables to obtain information about three-dimensional structures based on observations made in two dimensional structures. The authors (Amadeu et al. 2003) calibrated on the monitor the test system with cycloids, the minor axes of cycloids being arranged in parallel with the defined vertical axis. Skin fields were analysed in papillary and reticular dermis for surface density, length density and volume density for vessels and myofibroblasts using videomicroscopic system.

Tensile strength

For tensile strength measurements, not an excision, but an incision model of wound is usually used (Sathyanarayanan et al. 2017). The healed wound tissues are removed, harvested tissues are trimmed into strips of suitable length and width (with the original wound lying lengthwise in the centre of the sample), and apart from tensile strength (in MPa), a percentage of elongation at break (%) is usually measured (Ramanathan et al.

2017). Tensile strength increment indicates improved wound healing (Sathyanarayanan et al. 2017).

Local strain analysis at a physiological level of tension using a multiscale mechanics approach to the characterization of murine excisional wounds subjected to uniaxial tensile loading showed the presence of two distinct regions within the wound in an experiment of Pensalfini et al. (2018): a very compliant peripheral cushion and a core area undergoing modest deformation. The authors reported negligible engagement of collagen located in the centre of a 7-day old wound with the compliant cushion at the wound periphery protecting the newly-formed tissue from excessive deformation at this phase; the early remodelling phase was characterized by a restored mechanical connection between the far field and the wound centre.

Assessment of collagen synthesis

The standard procedure for an estimation of the extent of collagenesis is based on the determination of hydroxyproline (Caetano et al. 2016). Hydroxyproline is produced post-translationally by hydroxylation of the amino acid proline and since this hydroxylation is almost entirely specific to the collagen protein, L-hydroxyproline (especially T4L-hydroxyproline) is an important marker for directly measuring the content of collagen in biological samples (Watanabe et al. 2015). Hydroxyproline comprises approximately 13.5% of the mammalian collagen (Gorres and Raines 2010).

Hydroxyproline is usually determined spectrophotometrically after the acid hydrolysis of the sample (Otranto et al. 2010; dos Santos Rosa et al. 2014). Morphometric analysis by Caetano et al. (2016) can also be used as a simple, rapid and low-cost technology for evaluating total collagen in cutaneous wound specimens. Nevertheless, a biochemical hydroxyproline assay is still frequently used (Gercek et al. 2007; Ramanathan et al. 2017; Sathyanarayanan et al. 2017).

The method of Lin and Kuan (2010) includes chromophore formation without solvent transfers that allows the analysis of multiple specimens with excellent sensitivity, high specificity at low cost and shorter analysis time. The procedure of Colgrave et al. (2008) utilizes a highly selective and sensitive method of multiple reaction monitoring by mass spectrometry. Watanabe et al. (2015) developed a procedure using coupling systems with metabolic enzymes of the T4L- and T3L-hydroxyproline pathways from microorganisms and reported a successful hydroxyproline estimation within a broad range of wavelengths using a spectrophotometric assay, the results being consistent with those determined by high performance liquid chromatography.

It should be concluded however, that the hydroxyproline content reported in different experiments evaluating the effect of the same active substance (EPA, DHA) in a comparable phase of wound healing and applying various above-mentioned methods of hydroxyproline determination sometimes differs as much as by one order of magnitude and a comparison of the data obtained by different methods should therefore be taken with caution.

Histopathology and immunohistochemistry (IHC) of cutaneous wound healing

Periodically collected samples of the granulation tissue from wound sites using predominantly a rodent model are usually evaluated (Nascimento and Costa 2006; Gercek et al. 2007; Otranto et al. 2010; dos Santos Rosa et al. 2014; Ramanathan et al. 2017; Sathyanarayanan et al. 2017; Zhou et al. 2017). As far as histological preparations are concerned, samples of the healing tissue including ca 2 mm of skin surrounding the wound are usually taken, fixed in 10% buffered formalin, dehydrated by a gradual alcohol series, cleared in xylene, embedded in paraffin blocks, sectioned into a size of (usually) 5 μm thickness, stained with haematoxylin-eosin (H&E) and observed

under light microscopy. The evaluated markers are usually inflammatory cell infiltration, neovascularization (angiogenesis), fibroblast proliferation and epidermal remodelling (Gercek et al. 2007; Sathyanarayanan et al. 2017). Apart from H&E staining, Sirius red and toluidine blue can be used for evaluation of collagen fibres and mast cells, respectively (Nascimento and Costa 2006).

Regarding IHC assessment, paraffin embedded tissue sections, xylene-rehydrated and treated in a series of ethanol solutions are usually used (Ramanathan et al. 2017). If the detection of the searched-for protein is based on a reaction catalysed by a peroxidase (conjugated with a secondary antibody), endogenous peroxidases must be quenched beforehand by submersing the treated sections in a solution of H_2O_2 in methanol (Gercek et al. 2007). Rabbit polyclonal IgG (Ramanathan et al. 2017) or mouse monoclonal antibody (Otranto et al. 2010; dos Santos Rosa et al. 2014) are usually used as a primary antibody for immunostaining. Goat anti-rabbit antibody (Zhou et al. 2017) and biotinylated anti-rat antibody (dos Santos Rosa et al. 2014) are frequently applied secondary antibodies in this context. The last mentioned authors (dos Santos Rosa et al. 2014) also used an anti-mouse secondary antibody conjugated with Alexa Fluor 647 for the detection of epithelial cadherin (E-cadherin) when evaluating cutaneous wound healing in mice.

The following antigenic markers are most frequently detected by the semi-quantitative IHC procedures: alpha smooth muscle actin (α -SMA) (Zhou et al. 2017), e.g. in the blood vessel wall (Otranto et al. 2010) or in myofibroblasts (Nascimento and Costa 2006; dos Santos Rosa et al. 2014); myeloperoxidase-positive neutrophils, F4/80-positive macrophages, 4-hydroxynoneal-positive cells and E-cadherin (dos Santos Rosa et al. 2014; see above). Other proteins, detected by IHC, with an important informative value regarding evaluation of cutaneous wound healing, include cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS; Ramanathan et al. 2017) or transforming growth factor beta (TGF- β) and platelet-derived growth factor (PDGF; Gercek et al. 2007).

Genomic and proteomic markers of wound healing

Expression of the genes that are active during the process of wound healing is usually evaluated by quantitative polymerase chain reaction (q-RT-PCR) after extraction of total RNA by (usually) guanidinium thiocyanate-phenol-chloroform extraction (TRIzol reagent; Figueroa et al. 2012) and a reverse transcription of messenger RNA (mRNA) to complementary DNA (cDNA; Turk et al. 2013; Ramanathan et al. 2017).

Expression of the following genes was mostly quantified in experiments evaluating the effect of EPA/DHA (fish oil) on wound healing: vascular endothelial growth factor (*VEGF*), epidermal growth factor (*EGF*) and transforming growth factor beta (*TGFB*; Ramanathan et al. 2017); *TGFBI*, cyclooxygenase 2 (*COX2*) and α -SMA (Zhou et al. 2017); protein kinase B (*Akt*) and cyclic AMP responsive element binding protein (*CREB*; Figueroa et al. 2012); *ACTA* (coding for alpha skeletal muscle actin), *COL1A1* (type I collagen), *VIM* (coding for vimentin, type III intermediate filament protein, marker of mesenchymal derived cells), *CDH* (coding for E-cadherin, cell adhesion molecule enabling formation of adherens junctions to bind cells with each other) and *Snail* (coding for zinc finger protein SNAIL, transcription factor promoting repression of E-cadherin to regulate epithelial-to-mesenchymal transition; Pastor-Clerigues et al. 2014); G-protein coupled receptor GPR120 (Arantes et al. 2016).

Due to the fact that inflammation is an important phase of wound healing, some authors quantified expression of the genes coding for pertinent cytokines/chemokines: interleukin (IL)-1 β , IL-4, IL-6, IL-10, IL-13, IL-22, TGF- β , interferon gamma (IFN γ), tumour necrosis factor alpha (TNF α) and monocyte chemoattractant protein 1 (MCP1; Turk et al. 2013); TNF- α , IL-1 β and IL-6 (Weldon et al. 2007). Glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) and β -actin are usually used as normalization (“housekeeping”) genes (Figueroa et al. 2012).

As far as expression on the protein level is concerned, Western blot procedure (including cell lysis by Triton X-100 and EDTA, inhibition of proteases, solubilisation with sodium dodecyl sulphate [SDS] and polyacrylamide gel electrophoresis [PAGE]; Turk et al. 2013) is usually applied (Chen et al. 2012; Chao et al. 2014; dos Santos Rosa et al. 2014).

Within experiments evaluating effect of PUFA n-3 on wound healing in general (not only skin lesions), following proteins were quantified using Western blot analysis: VEGF-A, VEGF receptor 2 (VEGFR2) and E-cadherin (dos Santos Rosa et al. 2014); COX-2, TGF- β 1 and SMAD2/3 (mothers against decapentaplegic homolog 2/3, signal transducers for TGF- β 1 receptors; Zhou et al. 2017); α -SMA, SMAD2/3, phospho-SMAD2/3, ERK (extracellular signal-related kinase), phospho-ERK and CD68 (Cluster of Differentiation 68, protein strongly expressed in monocytes and circulating macrophages, and also by tissue macrophages) and GGT (gamma-glutamyltransferase; Chen et al. 2012); EGFR (epidermal growth factor receptor), phospho-EGFR, PLC (phospholipase C) γ 1 and phospho-PLC γ 1 (Turk et al. 2013); ERK1/2, phospho-ERK1/2 and eNOS (endothelial NO synthase; Chao et al. 2014); phospho-SMAD3, phospho-ERK1/2, phospho-Akt and nuclear β -catenin (Pastor-Clerigues et al. 2014); cytoplasmic and nuclear p65 (nuclear factor NF-kappa B p65 subunit) and I κ B- α (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha, protein inhibiting the NF- κ B transcription factor; Weldon et al. 2007).

Fish oil (eicosapentaenoic and docosahexaenoic acid) in wound healing

Dietary administration

As far as cutaneous wound healing is concerned, high fat diet, irrespective of the type of fat/oil, delays wound contraction and reepithelialization, increases the inflammatory infiltrate, delays myofibroblastic differentiation, collagen deposition, epithelial and connective tissue cell proliferation and angiogenesis (Nascimento and Costa 2006); the authors reported negative effects of a high-fat diet on rat cutaneous wound healing especially due to the prolongation of the inflammatory phase.

Otranto et al. (2010) compared the effect of different edible oils (sunflower, linseed, fish) on cutaneous wound healing in rats and concluded that all edible oils delayed wound closure, and affected the inflammatory infiltrate and collagen deposition. However, in the fish oil group, more abundant inflammatory cells, high density of dilated blood vessels and high density of collagen fibres were found; the best results were achieved with sunflower oil. The authors (Otranto et al. 2010) are cautious regarding fish oil due to the increase in collagen synthesis possibly resulting in excessive scar tissue. Fish oil was also evaluated as a less suitable in comparison with olive oil in application to healing of excisional lesions in (rotationally) stressed mice in a study by dos Santos Rosa et al. (2014). Olive oil, but not fish oil, inhibited stress-induced reduction in wound contraction, reepithelialization, hydroxyproline levels and blood vessel density; fish oil (contrary to olive oil) was not able to reverse stress-induced increases in VEGF expression and number of macrophages and neutrophils.

On the other hand, Scardino et al. (1999), when comparing menhaden fish oil (with PUFA n-6/n-3 ratio of 0.3) and a control oil (PUFA n-6/n-3 ratio of 7.7) in wound healing in Beagle dogs, found in the fish oil group at five days post-surgery significantly less epithelialization of open wounds, less oedema in sutured wounds, and tendencies of less tissue perfusion, lower level of prostaglandin E2 and negative wound contraction in open wounds. Therefore, the authors concluded that PUFA n-3 does not appear to have an

outstanding long-term effect on wound healing due to (among other things) the lack of granulation tissue over which the epithelium would advance.

Several authors evaluated the efficiency of PUFA n-3 in healing of injuries different than cutaneous excisions/incisions. In cholestatic liver injury (induced by bile duct ligation, BLD) in rats, chronic DHA supplementation alleviated BLD-induced increase of TGF- β 1, IL-1 β , connective tissue growth factor and collagen expression (Chen et al. 2012); DHA had an antifibrotic effect: decreased α -SMA-positive matrix producing cells and Smad 2/3 activity (i.e. fibrogenic potential of TGF- β 1). DHA also decreased leukocyte accumulation and NF- κ B activation. The authors concluded that DHA shows multifactorial hepatoprotective, anti-oxidative, anti-inflammatory and anti-fibrotic effect.

According to Coelho de Castilho et al. (2015), preoperative supplementation with PUFA n-3 in rats was associated with increased collagen deposition of the type I fibres in colonic anastomoses on the 5th postoperative day; no differences in the tensile strength or collagen maturation index in comparison with control were found. Dietary supplementation with PUFA n-3 (in combination with ascorbic acid) improved (by additive action) the healing of ischemic colonic anastomoses in rats in a study by Ekçi et al. (2011). On the other hand, Drzymała-Czyż et al. (2012) reported higher intensity of inflammation and tissue expression of IL-1 α and IL-10 in the DHA-fed group of rats that underwent restorative proctocolectomy (induced pouchitis) in comparison with control.

Based on the results of Figueroa et al. (2012), DHA protected and functionally improved the spinal cord injury in rats. A DHA pretreatment increased the percentage of white matter sparing (axonal preservation), and increased the survival of NG2⁺, APC⁺ and NeuN⁺ cells in the ventrolateral funiculus, dorsal corticospinal tract and ventral horns, despite the lack of inhibition of inflammatory markers for monocytes/macrophages and astrocytes. DHA also increased the levels of Akt and CREB mRNA and protein. Figueroa et al. (2012) therefore concluded that DHA-mediated activation of pro-survival/anti-apoptotic pathways may be independent of its anti-inflammatory effects.

DHA decreased inflammation and joint destruction in mice with collagen-induced arthritis in an experiment of Olson et al. (2013); DHA, but not a mixture of DHA/EPA, decreased arthritis severity and joint damage, decreased level of the anti-collagen (CII) antibodies, downregulated IL-1 β , IFN- γ and upregulated protective IL-10. According to the authors, the dietary administration of DHA is a useful intervention strategy against inflammatory arthritis.

Parenteral application

Arantes et al. (2016) applied a 30 μ M DHA solution once a day as a topical treatment of a cutaneous excision in male Wistar rats. DHA significantly accelerated wound healing, promoted a reduction of IL-1 β expression and increased expression of IL-6 and TGF- β ; involved in this process was the molecular activation of GPR 120. However, in comparison with control, fish oil emulsion applied in rats on cutaneous wound healing showed a lower hydroxyproline level, shallower wounds, worse histologic score and lower expression score of TGF- β and PDGF-AA (Gerçek et al. 2007); nevertheless, according to the authors, PUFA n-3 does not seem to have adverse effects on wound healing.

Hall et al. (2012) applied EPA or DHA intravenously in rats that underwent compression spinal cord injury (SCI). DHA, but not EPA, decreased neutrophil numbers in some areas of the injured epicentre and decreased plasma level of C-reactive protein. However, neither DHA nor EPA reversed the inflammatory response in the liver caused by the SCI, and neither was able to return to the control values the increased levels of IL-6, IL-1 β and TNF- α in the SCI epicentre at 4 h after injury; therefore, the neuroprotective effects of LC PUFA n-3 in rat compression SCI can only partly be attributed to the reduction of early inflammatory events after injury.

Comparative results of the *in vitro* studies

DHA inhibited the development of non-small cell tumours (in the non-small cell lung cancer cell line A 459) through a ROS-mediated inactivation of the PI3K/Akt signalling pathway (Yin et al. 2017). When tested on the mouse immortalized colonocyte model, both EPA and DHA delayed (during the early response to intestinal wounding) the activation of key wound-healing processes in the colon (Turk et al. 2013) due to the reduced EGFR ligand-induced receptor activation, which was associated with a reduction in a downstream activation of cytoskeletal remodelling proteins. Chao et al. (2014) found that DHA inhibited VEGF-induced cell migration in a culture of umbilical vein endothelial cells, which implies that the effect of DHA on angiogenesis and wound repair is at least partly by virtue of its attenuation of cell migration.

The effect of a fish oil commercial emulsion on inflammatory and pro-fibrotic liver markers was tested in culture supernatants of monocytes using a liver epithelial-to-mesenchymal transition (EMT) induced by TGF- β 1 (Pastor-Clerigues et al. 2014). Monocytes stimulated *in vitro* with LPS induced a strong inflammatory response that was suppressed by a commercial emulsion containing 100% of fish oil. This emulsion also suppressed the TGF- β 1-induced EMT, contrary to the control soybean oil emulsion (which enhanced EMT).

Pretreatment with 100 μ M EPA or DHA decreased the amount of TNF- α , IL-1 β and IL-6 proteins in the THP-1 monocyte-derived macrophages stimulated with LPS (Weldon et al. 2007). Both EPA and DHA also decreased TNF- α , IL-1 β and IL-6 mRNA expression, but DHA was more potent. Moreover, both EPA and DHA downregulated LPS-induced NF- κ B/DNA binding in the THP-1 macrophages, but only DHA decreased macrophage nuclear p65 expression and increased cytoplasmic I κ B- α expression. The authors (Weldon et al. 2007) concluded that DHA was more effective than EPA in alleviating LPS-induced, partly NF- κ B-mediated, pro-inflammatory cytokine production in macrophages.

Summary

Based on the results of the pertinent experiments, effects of fish oil or its active constituents EPA/DHA on wound healing are very inconsistent. The amount of hydroxyproline and collagen deposition was increased in some experiments, but decreased in other ones. Regarding inflammatory cells, the numbers of macrophages and neutrophils were increased by fish oil, but the numbers of leukocytes were decreased by DHA. DHA either increased or decreased inflammation after injury; EPA/DHA were not able to reverse the inflammatory response and their protective effects seem to be only partly attributed to the reduction of the early inflammatory events after injury. As far as particular pro- and anti-inflammatory markers are concerned, DHA both increased and decreased TGF- β ; TGF- β was also decreased by fish oil. On the other hand, it seems that DHA consistently decreases IL-1 β and TNF- α , and is able to increase IL-10. Therefore, it is not surprising that DHA likely mediates the activation of pro-survival/anti-apoptotic pathways independently of its anti-inflammatory effects.

Inconsistency also regards the wound healing rate: DHA both accelerated and delayed the wound healing processes in similar experiments. Finally, it follows from the results of some experiments that DHA is more effective in wound healing than EPA.

Conclusions of the above-mentioned literature data, including recommendations for future experiments can be formulated as follows: 1) The effects of long-chain polyunsaturated fatty acids (LC-PUFA) n-3 on wound healing tested on rodents are very inconsistent. 2) Regarding mammalian skin morphology, an optimal model for wound healing in humans is a pig (Seaton et al. 2015); however, an application of LC-PUFA n-3 using a porcine model is not described in available literature. 3) Due to the unconvincing effects of dietary

LC-PUFA n-3 in physiological concentrations in *in vivo* experiments, it would be interesting to try to apply these fatty acids topically at higher concentrations in combination with nanoparticles; using nanoparticles in wound healing is extensive (Rajendran et al. 2018), but a description of their effects in combination with LC-PUFA n-3 is missing altogether in the given context. 4) A cutting-edge method of MALDI (matrix-assisted-laser-desorption-ionization) with a detection of time-of-flight (TOF) can be used e.g. in a quantification of collagen in the tissues (Nimptsch et al. 2011), but again, its application in the evaluation of cutaneous wound healing in a porcine model using a combination of LC-PUFA n-3 and nanoparticles would be completely original.

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