The Effects of Parenteral Fish Oil Supplemented Nutrition on The Levels of DHA, EPA, Cytokines, and Wound Healing in Rats with Full-Thickness Burn Injury

Hassan SK[a], Sharif AS[a], Mohd Zaini RH[a], Wan Muhd Shukeri WF[a*]

[a]Department of Anaesthesiology and Intensive Care, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, Kelantan, Malaysia

Corresponding Author: Assoc. Prof. Dr. Wan Fadzlina Wan Muhd Shukeri
Department of Anaesthesiology and Intensive Care, School of Medical Sciences, Health Campus, Universiti Sains Malaysia, Kelantan, Malaysia
Email: wfadzlina@usm.my

ABSTRACT

INTRODUCTION: The present study aimed to examine the impact of fish oil supplementation on the concentrations of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), cytokines (interleukin-10 and -12), and wound healing in rats with full-thickness burn injury (FTBI). **MATERIALS AND METHODS**: Thirty male Sprague-Dawley rats weighing between 350 to 400 grams subjected to FTBI were randomized into either the experimental group (n=15), which received a standard diet supplemented with fish oil intravenously (which provided 400 mg/kg/d of DHA and 275 mg/kg/d of EPA) for five days, or the control group (n=15), which received a standard diet only. **RESULTS:** Compared to the control group, rats in the experimental group had a greater increase in mean blood DHA (P=0.002) and EPA (P<0.001) levels from baseline to day 21; a higher increase of mean IL-10 from baseline to day 10 (P=0.018); and a greater decrease in mean IL-12 from baseline to day 10 (P=0.04). When the burn wound tissues were examined, the experimental group showed greater blood vessel growth and a reduced presence of neutrophils compared to the control group. **CONCLUSION:** In summary, giving fish oil through injection raised the levels of DHA and EPA in the blood, reduced the activity of the pro-inflammatory cytokine IL-12, and increased the activity of the anti-inflammatory cytokine IL-10, which helps improve the healing of wounds in rats with FTBI.

KEYWORDS: fish oil, burns, cytokines, inflammation, wound healing

INTRODUCTION

Burn injury is a clear example of the most intense and prolonged inflammatory response. The inflammatory response triggered by burn injury is marked by the activation of inflammatory pathways and the production of cytokines.[1] Cytokines are potent endogenous mediators secreted by various cell types and are involved in multiple biological processes. These cytokines may exert pro-inflammatory, anti-inflammatory, or dual actions, contingent upon the affected area. Major pro-inflammatory cytokines include tumour necrosis factorα; interleukin IL-1, IL-8 and IL-12; and interferon-γ, while those of anti-inflammatory include IL-1 receptor antagonist, IL-4 and IL-10 (2). Notably, IL-6 has both pro- and anti-inflammatory properties depending on the physiological environment and stage of inflammation.[2] In this complex setting, the balance between pro- and anti-inflammatory cytokines is essential for proper regulation of inflammation. The process of wound healing after a burn injury is intricate and sequential, comprising at least three overlapping stages, with the initial step being inflammation.[3] Therefore, an extended or intensified inflammatory response results in a postponement of the succeeding stages of effective wound healing.[4]

Robust data indicates that pro-inflammatory cytokines contribute to the up-regulation of inflammatory responses, whereas effective down-regulation of pro-inflammatory cytokines levels[5] facilitates rapid wound healing. Further evidence, however, suggests otherwise, whereby diminished production of pro-inflammatory cytokines in the initial stage of wound healing was associated with impaired wound healing.[6] Nevertheless, it can be generally asserted that modulating pro-inflammatory cytokine production during the inflammatory phase may facilitate the process of wound healing.

The use of immune-enhancing nutrients is becoming more frequently investigated as single nutrients in addition to nutrition support, including fish oil. Fish oil is a rich source of omega-3 polyunsaturated fatty acids (PUFAs). While several forms of omega-3 PUFAs exist, the two that are crucial for human physiology and commonly examined in scientific studies are docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Multiple studies have demonstrated that omega-3 PUFAs are efficacious in treating or preventing conditions like cardiovascular disease, cancer, and inflammatory disorders, among others.[7] Prior research has indicated that omega-3 PUFAs can protect against the negative effects of stress during critical illness.[7] Nonetheless, the literature about fish oil supplementation in the burn injury group is quite sparse.

This work aimed to fill a gap in the literature about the possible links between elevated DHA and EPA from fish oil supplementation, cytokine production, and wound healing in rat models with full thickness burn injury (FTBI). We hypothesised that rats receiving parenteral fish oil supplementation for five days post-FTBI would exhibit significantly elevated blood concentrations of DHA and EPA, reduced levels of proinflammatory cytokines, increased levels of anti-inflammatory cytokines, and improved wound healing compared to the control group.

MATERIALS AND METHODS

Study design

This was a preclinical, randomised and controlled trial conducted at the Animal Research and Service Centre at one of the university-affiliated hospitals in Malaysia. The protocol of the trial was approved by the Institutional Animal Care and Use Committee (Approval No.: USM/ Animal Ethics Approval/ 2011/(69)(315). The trial complied with the National Institutes of Health guide for the care and use of laboratory animals.[8]

Experimental animals

30 male Sprague-Dawley rats, weighing 350 to 400 g and aged nine to eleven weeks, were utilised in the investigation. All rats were healthy and exhibited no signs of illness or sickness. Every rat underwent acclimatisation for a minimum of five days to mitigate stress and familiarise them with human interaction. The subjects were accommodated in separate, sanitised cages inside the animal housing facility, subjected to a 12-hour light-dark cycle, and had unrestricted access to a standard feed and water before the commencement of testing.

Intervention

The rats were randomly allocated into two groups using simple randomisation. The control group received a standard diet only. The experimental group received a standard diet and parenteral administration of fish oil at a dose of 2.5 mL/kg/day for five consecutive days. The intravenous fish oil provided approximately 400 mg/kg/day of DHA and 275 mg/kg/day of EPA.

Full-thickness burn wound creations

On the day of injury, the rats were positioned ventrally and immobilised on their abdomens. Immediately before the procedure, the rats were anaesthetised via intramuscular injection of ketamine at a dosage of 35 mg/kg and xylazine at 5 mg/kg in the gluteal region. When fully anesthetised, the back of the rats was shaved and cleaned with povidone-iodine, 70% alcohol, and Hibiscrub®. A full-thickness burn wound was induced on the dorsal region of the rats, between the thoracic vertebrae and the sacrum, using hot metal applied perpendicularly for 30 seconds under sterile conditions. The area of the wound was approximately 1 cm x 1 cm in size. Following completion of the wounding, the rats were monitored for spontaneous breathing effort and movement. Every rat was accommodated in a solitary cage within a room and had unrestricted access to normal feed and water. The burn wound was covered with Aquacel® dressing and bandage to avoid self-inflicted biting.

Assessment of DHA, EPA and cytokine levels

For this assessment, 0.25 ml (about 1% of the total blood volume of rats) of whole blood was taken from each rat via the tail vein (caudal vein) at day 1 post-wounding (baseline) and sent for analysis. Subsequently, five rats were used for each group at every single follow-up time point (day 5, 10 and 21 post-wounding) for the blood marker analysis. We measured DHA and EPA by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and cytokine levels by enzyme-linked immunosorbent assay (ELISA). We chose to measure IL-10 as the anti-inflammatory cytokine[9] and IL-12 as the pro-inflammatory cytokine.[10]

Assessment of wound healing

The burn wound was assessed histologically at day 5, 10, and 21 post-wounding. For this assessment, five rats were used for each group at every follow-up day. To perform the histological examination, the rats were euthanized using intraperitoneal injection of phenobarbitone sodium 5 mg. Tissue biopsies from their wound were retrieved. The samples were cut into 2 cm x 2 cm strips parallel to the long axis of their bodies with sharp blades. The strips were subsequently sectioned, stained with haematoxylin and eosin (H&E), and analysed under a light microscope.

Wound healing was evaluated based on (1) the degree of neutrophil infiltration and (2) the extent of new capillary formation within the burn wound. These parameters were assessed semi-quantitatively using a scoring system ranging from 0 to 3 (Table I). All histological assessments were performed by an investigator blinded to the group allocation.

Table I: Scoring system for histological measurement of neutrophil infiltration and new capillary formation

Score	Number of structures
0	Not present
1	Low number
2	Moderate number
3	Present all over or relatively high number

Statistical analysis

Data for the levels of DHA, EPA, and cytokine are presented as mean (standard deviation [SD]). The differences between day 1 and days 5, 10, and 21 in the control and experimental groups were analysed using multivariate analysis of variance.

Data for the neutrophil infiltration score and new capillary formation score are presented as descriptive statistics using cross-tabulation.

All tests are two-sided, with p-values of less than 0.05 deemed statistically significant. All analyses were completed using SPSS, version 20 (IBM software).

Sample size calculation

The sample size was calculated based on detecting a difference between groups in the change of circulating IL-10 from baseline (day 1) to follow-up, as a key immunomodulatory outcome. In this study, different rats were assessed at each terminal time point (days 5, 10, and 21), with five rats per group per follow-up day.

Using the observed mean change in IL-10 from baseline to day 5 (experimental group: 0.10 ng/mL; control group: 0.02 ng/mL; expected difference, δ = 0.08 ng/mL) and assuming a conservative common standard deviation of 0.03 ng/mL, the required sample size for a two-sided comparison of two independent means was estimated using:

$$n = \frac{2\,(Z\alpha/2 + Z\beta)^2\,(\sigma)^2}{(\delta)_2}$$

With a two-sided α = 0.05 ($Z_{\alpha/2} = 1.96$) and 80% power ($Z_{\beta} = 0.84$), the minimum sample size was:

$$n = \frac{2\,(1.96 + 0.84)^2\,(0.03)^2}{(0.08)^2} \approx 2.2$$

Thus, at least 3 rats per group per time point were required. To allow for biological variability, multiple planned outcomes (fatty acids, cytokines, and histology), and potential attrition, the sample size was increased to 5 rats per group at each follow-up time point (days 5, 10, and 21), resulting in a total of 30 rats randomized equally to control and experimental groups.

RESULTS

At the completion of the study period, one rat from each group died from an infected burn wound; the rat from the control group died at day 14, while the rat from the experimental group died at day 18.

The effect of fish oil supplementation on blood DHA and EPA levels

The mean levels of blood DHA and EPA measured at day 1, 5, 10 and 21 in the two groups are shown in Figure 1. Overall, rats from both groups had similar levels of blood DHA and EPA at baseline. However, at the subsequent follow-up time points, rats from the experimental group had higher mean levels of blood DHA and EPA than the control group. The levels peaked at day 5, and decreased gradually thereafter, following completion of the fish oil administration for five days. By the end of study period at day 21, the mean levels of both DHA and EPA were still above the baseline values.

Comparison of the differences from day 1 to day 5, 10, and 21 of the blood DHA and EPA mean levels between the two groups are shown in Table II. There was an elevation from baseline to all follow-up time periods in both the mean levels of blood DHA and EPA, which was considerably greater in the experimental group than in the control group ($P<0.05$).

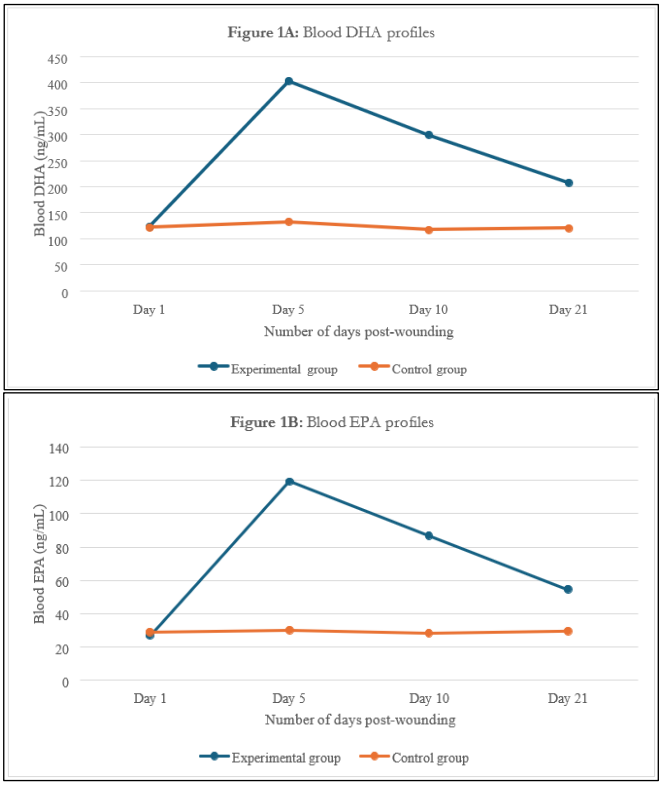

Figure 1. 1A: Blood DHA profiles and **1B:** Blood EPA profiles (in ng/mL) at day 1 (baseline), 5, 10 and 21. Data are expressed as mean.

The effect of fish oil supplementation on blood IL-10 and IL-12 levels

The mean levels of blood IL-10 and IL-12 measured at day 1, 5, 10, and 21 in the two groups are shown in Figure 2. Overall, rats from both groups had similar levels of blood IL-10 and 1L-12 at baseline. However, at

the subsequent follow-up time points, rats from the experimental group had higher mean levels of blood IL-10 (Figure 2A), but lower levels of IL-12 (Figure 2B), compared to the rats from the control group.

Comparison of the differences from day 1 to day 5, 10, and 21 of the blood IL-10 and IL-12 mean levels between the two groups is shown in Table III. For IL-10, there was an increase from baseline to all follow up time points in the experimental group, which was significantly different from the control group at day 5 and day 10. For IL-12, there was a decrease from baseline to day 5 and to day 10 in the experimental group, which was significantly different from the control group. There was no statistically significant difference in the mean changes of both IL-10 and IL-12 at day 21 between the two groups.

Table II: Differences of blood DHA and EPA mean levels (in ng/mL) from day 1 (baseline) to day 5, 10, and 21 between the experimental group and control group

		Mean (95% CI)	F-statistic	p-value [a]
Blood DHA levels (ng/mL)				
Day 5 – Day 1	Control group	2.20 (-42.34, 46.74)	102.23	<0.001
	Experimental group	278.38 (233.84, 322.92)		
Day 10 – Day 1	Control group	0.56 (-21.27, 22.39)	180.08	<0.001
	Experimental group	180.26 (158.42, 202.09)		
Day 21 – Day 1	Control group	2.35 (-23.09, 27.79)	29.92	0.002
	Experimental group	82.80 (57.35, 108.24)		
Blood EPA levels (ng/mL)				
Day 5 – Day 1	Control group	1.10 (-13.01, 15.21)	111.32	<0.001
	Experimental group	92.42 (78.30, 106.53)		
Day 10 – Day 1	Control group	0.68 (-2.50, 3.86)	833.50	<0.001
	Experimental group	57.12 (53.93, 60.30)		
Day 21 – Day 1	Control group	1.00 (-2.80, 4.80)	141.73	<0.001
	Experimental group	27.17 (23.37, 30.97)		

[a]Multivariate analysis of variance test

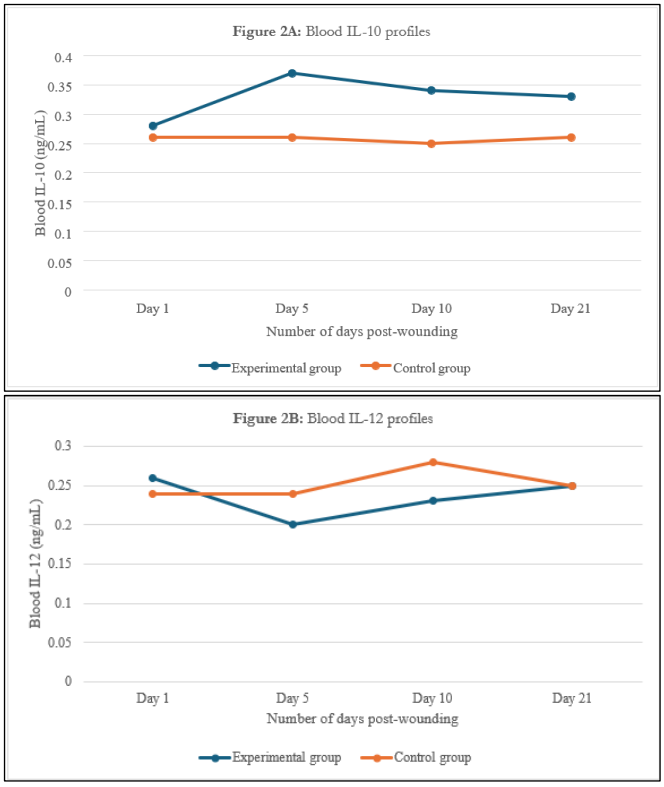

Figure 2. 2A: Blood IL-10 profiles and 2B: Blood IL-12 profiles (in ng/mL) at day 1(baseline), 5, 10 and 21. Data are expressed as mean.

Table III: Differences of mean blood IL-10 and IL-12 levels (in ng/mL) from day 1(baseline) to day 5, 10 and 21 between experimental group and control group

		Mean (95% CI)	F-statistic	p-value [a]
Blood IL-10 levels (ng/mL)				
Day 5 – Day 1	Control group	0.02 (-0.03, 0.03)	33.81	<0.001
	Experimental group	0.10 (0.07, 0.12)		
Day 10 – Day 1	Control group	-0.01 (-0.04, 0.03)	8.87	0.018
	Experimental group	0.06 (0.03, 0.10)		
Day 21 – Day 1	Control group	0.01 (-0.02, 0.03)	1.37	0.286
	Experimental group	0.02 (-0.01, 0.05)		
Blood IL-12 levels (ng/mL)				
Day 5 – Day 1	Control group	0.04 (-0.02, 0.03)	25.52	0.001
	Experimental group	-0.07 (-0.09, -0.04)		
Day 10 – Day 1	Control group	0.02 (-0.04, 0.09)	5.98	0.040
	Experimental group	-0.07 (-0.14, -0.01)		
Day 21 – Day 1	Control group	0.01 (-0.02, 0.02)	4.37	0.081
	Experimental group	0.03 (0.01, 0.05)		

[a]Multivariate analysis of variance test

The effect of fish oil supplementation on neutrophil infiltration and neovascularisation of burn wounds

The crosstab in Table IV shows the study groups by neutrophil infiltration score and new capillary formation score within each follow-up time point (day 5, 10, and 21). In terms of neutrophil infiltration, at day 5, a lower

proportion of rats from the experimental group (20%) displayed marked presence of neutrophil infiltration (neutrophil infiltration score =3) than rats from the control group (40%). By day 21, half of the rats from the experimental group no longer had neutrophil infiltration (neutrophil infiltration score =0) while the remaining half only had minimal neutrophil infiltration (neutrophil infiltration score =1) in their burn wound. In contrast, 75% of rats in the control group still had mild to moderate neutrophil infiltration (neutrophil infiltration score = 1 to 2) at day 21. In terms of neovascularisation, a higher proportion of rats from the experimental group (40 %) showed the presence of neovascularisation (new capillary formation score >1) than rats from the control group (20%). By day 21, the rats from both groups displayed a similar high degree of neovascularisation (new capillary formation score = 3).

Table IV: Cross-tabulation of study groups by neutrophil infiltration score and new capillary formation score

		Neutrophil infiltration score			
		0	1	2	3
Day 5	Control group (n = 5)		2 (40 %)	1 (20 %)	2 (40 %)
	Experimental group (n = 5)		2 (40 %)	2 (40 %)	1 (20 %)
Day 10	Control group (n = 5)		2 (40 %)	3 (60 %)	
	Experimental group (n = 5)		1 (20 %)	3 (60 %)	1 (20 %)
Day 21	Control group (n = 4)	1 (25%)	2 (50 %)	1 (25%)	
	Experimental group (n = 4)	2 (50%)	2 (50%)		
		New capillary formation score			
		0	1	2	3
Day 5	Control group (n = 5)	4 (80 %)	1 (20 %)		
	Experimental group (n = 5)	3 (60 %)	2 (40 %)		
Day 10	Control group (n = 5)		1 (20 %)	3 (60 %)	1 (20 %)
	Experimental group (n = 5)			1 (20 %)	4 (80 %)
Day 21	Control group (n = 4)				4 (100%)
	Experimental group (n = 4)				4 (100%)

DISCUSSION

In this randomised controlled preclinical trial, we showed that parenteral supplementation with fish oil (which provided 400 mg/kg/d of DHA and 275 mg/kg/d of EPA) leads to an increase in the blood levels of DHA and EPA among rats with FTBI. Blood DHA and EPA levels increased from baseline and reached maximum concentration at day 5 following administration of the fish oil, and gradually dropped thereafter. This trend was expected as the result coincides with the duration of administration of the fish oil, which was five days in total.

Production of anti-inflammatory cytokines to suppress the pro-inflammatory cytokines is important to reduce the inflammatory phase following burn injury.[5] IL-10 is an anti-inflammatory cytokine that is synthesised by monocytes, macrophages, and activated T- and B-cells.[9] Previous studies investigating the effects of fish oil supplementation on IL-10 secretion have reported mixed findings, including no significant effect on IL-10 production,[11] reduced IL-10 secretion,[12,13] or modulation of upstream inducers of IL-10.[14] These discrepancies may, in part, reflect differences in experimental models, timing of assessment, and the dynamic role of IL-10 during tissue repair. IL-10 is known to exert context- and time-dependent effects in wound healing, with early upregulation contributing to the resolution of excessive inflammation, while prolonged or sustained elevation may impair later stages of tissue regeneration. In the present study, fish oil

supplementation was associated with a transient increase in IL-10 levels during the early post-injury phase, followed by a subsequent decline, with no sustained differences observed at later time points. This temporal pattern suggests that fish oil may promote a timely anti-inflammatory response without prolonging IL-10 activity beyond the early inflammatory phase, which may be beneficial for effective wound healing.

IL-12 facilitates the activation and control of several cytotoxic immune cells, including macrophages, natural killer cells, and T-cells, hence categorising it as a pro-inflammatory cytokine.[15] The result of our study indicated that administration of parenteral fish oil is effective in decreasing the level of IL-12 in the bloodstream of rats following burn injury. This is, to our knowledge, the inaugural study on the influence of fish oil on IL-12 production under these circumstances. Puertollano et al (2005) documented the inhibitory impact of fish oil, indicating that IL-12 production in rats fed administered fish oil was markedly reduced at 72 and 96 hours' post-experimental infection with Listeria monocytogenes.[16] A study by Fritsche et al (2000) similarly showed that fish oil can lower the circulating level of IL-12.[17] The authors showed that fish oil inhibited two pro-inflammatory cytokines, interferon-γ and IL-12, in rats fed with omega-3 PUFAs.

Skin wound healing is a systemic process, traditionally including three overlapping classic phases: inflammation (neutrophils and mononuclear cell infiltration), proliferation (epithelisation, fibroplasia, neovascularisation, and formation of granulation tissue), and maturation (collagen deposit or scarring tissue formation).[3] We demonstrated that parenteral fish oil supplementation in rats with FTBI leads to reduced neutrophil infiltration at the burn site, resulting in the resolution of inflammation by the conclusion of the research period. It was probably this shortened inflammation phase that has consequently led to faster arrival of the proliferation phase, whereby rats that were supplemented with fish oil displayed more neovascularisation than the control group from the beginning of the study period.

CONCLUSION

Taken together, our results demonstrated that parenteral fish oil supplementation (400 mg/kg/d of DHA and 275 mg/kg/d of EPA) can increase the blood levels of DHA and EPA. Thus, supplementation with this nutrient inhibits a prolonged inflammatory response at the burn site, potentially through the down-regulation of the pro-inflammatory cytokine IL-12 and the corresponding up-regulation of the anti-inflammatory cytokine IL-10, which facilitates wound healing processes. Further translational studies are required to confirm or refute these findings and to explain the mechanism by which fish oil supplementation affects the regulation of IL-10 and IL-12.

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CONFLICT OF INTEREST

There were no conflicts of interest between the contributors of this research.

INSTITUTIONAL REVIEW BOARD (ETHICS COMMITTEE)

This study received ethical approval from Universiti Sains Malaysia Animal Care and Use Committee.

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