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n-3 Fatty acid supplementation and regular moderate exercise: differential effects of a combined intervention on neutrophil function

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CVD is associated with a cellular inflammatory/immune response. *n*-3 PUFA and moderate aerobic exercise independently alter cytokine production and leucocyte function. There is limited evidence for the combined effect of these treatments on immune function, particularly in patients with risk factors for CVD. We hypothesised that exercise would enhance the anti-inflammatory effects of *n*-3 PUFA. In a randomised, placebo-controlled study, fifty volunteers were allocated double-blind to consume either sunflower oil (6 g/d, placebo) or DHA-rich fish oil (6 g/d; about 2 g *n*-3 PUFA; 1·6 g DHA /d) for 12 weeks. Volunteers were further randomised to undertake regular exercise (walking 3 d/week for 45 min at 75 % of maximum heart rate) or maintain their usual physical activity for 12 weeks. Immune functions were assessed in blood taken initially and after 12 weeks. There was no effect on cytokine production by T cells and monocytes. Superoxide anion production from stimulated blood neutrophils was decreased by fish oil (19·5 (SEM 8·5) %, $P=0·016$) but not by exercise, and this change was negatively correlated with the incorporation of DHA into erythrocytes ($r-0·385$, $P=0·047$). Participation in regular exercise maintained neutrophil bactericidal activity, which decreased in non-exercising subjects (2·9 (SEM 0·7) %, $P=0·013$). Neutrophil chemotaxis and adherence were not significantly affected by exercise, oil, or the combination of the two. Thus the combination of moderate exercise and fish-oil supplementation, which reduces cardiovascular risk, may also help to counteract inflammation.

***n*-3 PUFA: Exercise: Neutrophils: T cells: Monocytes: Cytokines**

Fish oils, which are rich in *n*-3 PUFA, particularly DHA (22 : 6*n*-3) and EPA (20 : 5*n*-3), have been shown to suppress a range of components of inflammatory/immune reaction[1]. The anti-inflammatory nature of *n*-3 PUFA has generally been attributed to the inhibitory effects of EPA on the synthesis of eicosanoids from the *n*-6 PUFA, arachidonic acid (AA). EPA prevents the mobilisation of AA from cell membranes by phospholipase A_2[2] and competitively inhibits the oxygenation of AA by cyclo-oxygenase and 5-lipoxygenase[3]. Eicosanoids synthesised from EPA have substantially less inflammatory activity than those derived from AA[4]. Furthermore, supplementation with dietary fish oil has been reported to reduce the production of inflammatory cytokines such as IL-1, IL-6, and TNF-α[5–8], and to suppress the expression of vascular cell adhesion molecule-1[9]. *n*-3 PUFA may exert their effects on immune cells by altering membrane fluidity[10], or cell signalling pathways[1] and this may also explain the ability of fish oil to alter the function of leucocytes, particularly neutrophils[10].

While neutrophils are important mediators of host defences, they also contribute to tissue damage[11] and have been implicated as a cause of injury during myocardial ischemia-reperfusion[12]. Previous studies have reported decreases in neutrophil chemotaxis[3,13], adhesion[14,15], and superoxide anion production with *n*-3 supplementation[16–19], but other reports have found no effect on any of these measures of neutrophil function[20–23]. It is possible that this disparity is due to differences in fish-oil dose and the relative concentrations of EPA and DHA. Most studies have evaluated the effects of supplementation with EPA-rich oils rather than DHA-rich oils[24].

The anti-inflammatory effects of exercise have also been investigated and several studies have shown a reduction in inflammatory markers involved in the pathogenesis of atherosclerosis, including C-reactive protein, interferon γ (IFNγ), TNF-α, IL-1 and IL-6[25–27]. Furthermore, population studies demonstrate an inverse relationship between physical activity and fitness, and markers of inflammation, particularly C-reactive protein[28–30]. The effect of exercise on neutrophil function is less clear[31] and limited to the effects of an acute exercise bout; however these reports are inconsistent and likely to be confounded by factors such as training status,

Abbreviations: AA, arachidonic acid; FO, fish oil-consuming group; FOX, fish oil and exercise group; IFNγ, interferon γ; MNL, mononuclear leucocytes; PKC, protein kinase C; SO, sunflower oil-consuming group; SOX, sunflower oil and exercise group.

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exercise intensity and population differences[31,32]. Fewer studies have investigated the effect of regular exercise training on neutrophil function, although there is some evidence to suggest that it may reduce neutrophil respiratory burst activity. In light of these findings, exercise may have important implications in the prevention of vascular and inflammatory diseases.

Only two studies have investigated the combined effect of regular exercise and *n*-3 PUFA supplementation on inflammation or immune function. Using a rat model, Robinson & Field[33] observed an increase in splenic natural killer cell cytotoxicity in sedentary but not exercise-trained animals supplemented with *n*-3 PUFA. In trained runners, *n*-3 PUFA supplementation failed to attenuate plasma levels of TNFα, IL-6 and transforming growth factor-β compared to placebo, following a strenuous exercise session[34]. Given the potential for *n*-3 PUFA and exercise to independently influence leucocyte function, we hypothesised that *n*-3 PUFA would enhance the anti-inflammatory effects of regular moderate exercise. Consequently, this 12-week intervention study evaluated the independent and combined effects of supplementation with a DHA-rich tuna fish oil and regular moderate exercise, on neutrophil function in overweight adults exhibiting cardiovascular risk factors.

Materials and methods

This study focused on changes in immune response as part of a larger trial investigating the effects of supplementation with DHA-rich tuna fish oil and regular aerobic exercise on cardiovascular and metabolic outcomes.

Subjects

Overweight and obese (BMI $>25\,kg/m^2$) adult (25–65 years of age) subjects who exhibited at least one of the following cardiovascular risk factors: mild hypertension (systolic blood pressure 140–160 mmHg or diastolic blood pressure 90–100 mmHg), elevated plasma TAG $>1{\cdot}6$ mmol/l or elevated total cholesterol $>5{\cdot}5$ mmol/l were recruited for a 12-week randomised, double-blind, parallel, placebo-controlled 2×2 factorial trial. Volunteers completed a diet and lifestyle questionnaire to determine initial suitability for the study. Exclusion criteria included smoking, lipid or blood pressure-lowering medications, diagnosed CVD or kidney disease, diabetes, prior consumption of fish-oil supplements and/or consuming two or more servings of oily fish per week, and regular participation in more than one physical activity session per week. To confirm eligibility, subjects attended a screening appointment to assess height, weight, blood pressure, and fasting TAG and total cholesterol concentrations in venous blood. Subjects also completed a medical screening, which included electrocardiogram monitoring (Nihon Kohden, Tokyo, Japan) during a graded exercise test to confirm their suitability for exercise training.

Fifty subjects, fourteen male and thirty-six female (age 50 (SEM 1) years), were enrolled in the study in two intakes, one in winter and one in summer, thus countering any potential seasonal variation in immune function. Ethics approval was obtained from both the University of South Australia and the University of Adelaide and written informed consent was obtained from all subjects prior to their participation.

Study design

Subjects were assigned to one of four intervention groups, which were matched for sex, BMI and TAG. These groups were then randomly allocated to consume six 1 g capsules of either DHA-rich tuna fish oil (Hi-DHA®; Nu-Mega Ingredients Pty Ltd, Brisbane, Australia), which provided 260 mg DHA and 60 mg EPA in each 1 g capsule, or sunflower oil, daily for 12 weeks. All capsules were identical in colour and shape. Peppermint essence was added to each capsule to ensure blinding to taste. Half of each of the fish oil and sunflower oil groups were further randomised to undertake exercise (FOX, SOX) or maintain their usual physical activity (FO, SO).

Subjects allocated to an exercise group (FOX and SOX) were required to run or walk for 45 minutes, three times per week at a heart rate corresponding to 75 % of their age-predicted maximum $(208-(0{\cdot}7\times age)$[35], i.e. a moderate exercise intensity. Subjects were asked to maintain their usual physical activity and diet with the exception of the exercise intervention and supplementation. Fasting blood samples were collected at Baseline (week 0), week 6 and week 12 of the intervention period. Subjects completed a 3-d physical activity diary (adapted from Bouchard[36]) and a weighed food record (analysed using Foodworks Professional Edition, Xyris Software, Version 3.02; Highgate Hill, Australia) on two weekdays and one weekend day prior to attending their scheduled clinic visits at weeks 0, 6 and 12.

Erythrocyte fatty acids

Erythrocyte fatty acid composition was determined at weeks 0, 6 and 12. Erythrocytes were isolated, washed with isotonic saline (0·9 %), frozen, thawed then lysed in hypotonic 0·01M Tris EDTA buffer, pH 7·4 and the membrane lipids were extracted and transmethylated according to the method of Lepage & Roy[37]. Fatty acid methyl esters were analysed using a Shimadzu gas chromatograph 20A (Shimadzu Corporation, Kyoto, Japan) fitted with a flame ionisation detector and a 50m BPX70 column (0·32 mm internal diameter and 0·25 μm film thickness; SGE Analytical Science, Ringwood, Australia). Individual fatty acids were identified by comparison with known fatty acid methyl ester standards (Nuchek Prep Inc., Elysian, USA) and expressed as a percentage of total fatty acids.

Leucocyte preparation

Blood samples were collected in heparinised tubes at weeks 0 and 12, and separated into mononuclear leucocytes (MNL) and neutrophils, >96 % purity and >99 % viable, by centrifugation on Hypaque-Ficoll of density 1·44[38]. This was prepared by mixing Ficoll 400 (Amersham Pharmacia, Uppsala, Sweden) with sodium diatrozoate (Sigma Aldrich, St Louis, USA) and Angiografin (Schering pty Ltd, Sydney, Australia). Leucocyte functions were conducted under conditions accredited by the National Association of Testing Authorities of

Australia (NATA). This ensures a limited degree of variability between testing conducted at different times.

Neutrophil functions

Superoxide anion production was measured spectrophotometrically using the nitroblue tetrazolium reduction test[39]. Briefly, 100 μl neutrophils (5×10^6/ml) in Hanks' balanced salt solution (HBSS) were treated with 250 μl phorbol myristate acetate (PMA) (0·4 μg/ml), or diluent (250 μl HBSS). 500 μl nitroblue tetrazolium solution (Sigma Aldrich, St Louis, USA) was added to each tube and allowed to incubate for 20 min at 37 °C. The tubes were then centrifuged (600 ***g*** for 5 min) and neutrophils resuspended in 200 μl of ethanol–PBS (1 : 1, v/v) to lyse cells. Following incubating at room temperature for 30 min, the 200 μl suspensions were transferred to wells in a microtitre tray. Absorbance was measured at 570 nm using a Dynatech MR 7000 plate reader (Dynatech Laboratories, Chantilly, USA).

Neutrophil chemotaxis was determined by measuring neutrophil migration under agarose using the chemotactic agent f-met-leu-phe (Sigma), as previously described[40]. Bactericidal activity was assessed by determining viability of bacteria at 0 and 60 min, following incubation of neutrophils with complement treated *Staphylococcus aureus* (strain 6571; National Collection of Type Cultures, Oxford, England), as described previously[41]. Neutrophil adherence was measured by adherence to plasma-coated plates, according to the method of Powell[42].

Cytokine production from stimulated mononuclear leucocytes

MNL were cultured in the presence or absence of the T lymphocyte mitogens, phytohaemagglutinin (2 μg/ml; Murex Diagnostics, Dartford, England) or Concanavalin A (40 μg/ml; Sigma)[43] for 72 h at 37°C in CO_2–air (5 %, v/v) and high humidity. Cytokine production (lymphotoxin, IFNγ and IL-2) was measured in the culture supernatants by ELISA using cytokine-specific monoclonal antibodies, as described by Costabile[43]. Cytokine production by monocytes (TNFα, IL-1β, IL-6) was examined by stimulating MNL with bacterial lipopolysaccharide (1 μg/ml) for 24 h as above[44]. Absorbance at 450/750 nm was read using a Dynatech MR 7000 plate reader (Dynatech Laboratories). The limit of detection for all cytokines was approximately 0·02 ng/ml. The inter-assay CV were <8 % for lymphotoxin, IL-1β, IL-2 and IL-6, and <16 % for IFNγ and TNFα. The intra-assay CV were <6 % for all cytokine assays.

Statistical analysis

This study was a sub-study of a larger trial where sample size was determined based on a metabolic-related primary outcome measure. Data were analysed using Statistica for Windows (Version 5·1, StatSoft Inc., Tulsa, USA). Differences at baseline (week 0) were determined by one-way ANOVA. Treatment effects and their interactions over time were assessed by 3-factor ANOVA with repeated measures with oil treatment (fish oil or sunflower oil) and exercise treatment (exercise or non-exercise) being the between-group factors and time (weeks 0 and 12) being the within-subject repeated measurement. Where a significant difference was detected at baseline, data were analysed by 3-factor ANCOVA (oil × exercise × time), which assessed changes in select parameters with the baseline values included as a covariate. Where ANOVA showed a significant main effect, differences between means were identified *post hoc* using Tukey's procedure. Pearson product-moment correlation coefficients were used to describe the relationship between two different variables. A probability value of $P<0·05$ was considered statistically significant. Values are expressed as means with their standard errors.

Results

Subjects and protocol compliance

Of the fifty subjects initially enrolled, five withdrew prior to beginning or during the trial: three subjects had a change in work or family circumstances, one did not consume the required number of daily capsules, and another was lost to follow up. Five subjects failed to comply with the exercise requirements and were excluded. None of the subjects experienced adverse effects of the treatment. Baseline (week 0) characteristics for subjects who completed the 12-week intervention (forty subjects) are listed according to treatment group in Table 1. There were no differences between groups

Table 1. Baseline (week 0) characteristics of study participants
(Mean values with their standard errors)

	Treatment group								
	FO (*n* 11)		FOX (*n* 10)		SO (*n* 11)		SOX (*n* 8)		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	*P**
Sex (male : female)	3 : 8		2 : 8		4 : 7		2 : 6		0·88
Age (years)	52	2	49	2	50	3	51	2	0·84
BMI (kg/m^2)	35	2	34	2	35	1	34	1	0·99
Body fat (%)	45	1	46	2	45	2	45	3	0·95
Systolic blood pressure (mmHg)	128	5	132	5	128	3	133	3	0·75
Diastolic blood pressure (mmHg)	70	3	77	3	72	2	78	3	0·17
TC (mmol/l)	6·8	0·5	6·1	0·5	6·3	0·6	6·9	0·6	0·70
TAG (mmol/l)	1·6	0·3	1·5	0·2	1·5	0·2	1·8	0·3	0·79

FO, fish oil; FOX, fish oil and exercise; SO, sunflower oil; SOX, sunflower oil and exercise; TC, total cholesterol.
*There were no significant differences for any variable.

at baseline. Analysis of physical activity and diet diaries showed good compliance with the dietary and physical activity requirements for the trial (data not shown). There was no change in energy intake (assessed by weighed food records) in any of the treatment groups and >85 % of exercising subjects completed the required number of exercise training sessions.

Effects on erythrocyte fatty acid concentrations

Due to mishandling of blood samples, some data were lost to analysis and consequently fatty acid profiles of erythrocyte membranes were only obtained from 27 subjects at weeks 0, 6 and 12 (Table 2). However, the data show little variation and therefore appear to be representative of the total sample. There were no differences in EPA or DHA concentrations between groups at week 0 (one-way ANOVA, $P=0{\cdot}13$ and $P=0{\cdot}57$, respectively). However, DPA concentration was significantly greater in the SOX *v*. FOX group at week 0 (Tukey's test, $P=0{\cdot}019$). One-way ANOVA also detected a significant difference between groups for total *n*-3 concentrations at week 0, and *post hoc* analysis indicated that the total long chain *n*-3 PUFA concentration in SOX was significantly greater than SO ($P=0{\cdot}033$).

The percentage of total long chain *n*-3 PUFA in erythrocytes rose progressively in the FO and FOX groups during the 12-week supplementation period: from 10·2 to 13 % in the FO group and from 10 to 13·3 % in the FOX group. This increase was statistically significant (ANCOVA oil × time interaction for total long chain *n*-3 PUFA, $P<0{\cdot}002$) and entirely attributed to the change in DHA, which increased by 80 % in FO and 85 % in FOX (ANOVA oil × time interaction for DHA, $P<0{\cdot}001$). Total long chain *n*-3 PUFA and DHA content remained unchanged in the SO and SOX groups. *Post hoc* analysis detected a marginal reduction in EPA for all subjects during the intervention (main effect of time, $P<0{\cdot}013$).

Effects on neutrophil functions

There were no significant differences between groups for any measure of neutrophil function at baseline (Table 3). ANOVA did not detect any 3-factor interactions for any of these measures. However, a significant 2-factor oil × time interaction was detected for superoxide production ($P=0{\cdot}016$). There was a significant decrease in superoxide production in the combined fish oil group (−19·5 (SEM 8·5)%, $P=0{\cdot}041$) with no change in the combined sunflower oil group (10·7 (SEM 14)%, $P=0{\cdot}82$). Superoxide production was negatively correlated with DHA concentration in erythrocyte membranes (r −0·385, $P=0{\cdot}047$). A significant 2-factor exercise × time interaction was detected for bactericidal activity ($P=0{\cdot}013$). Subjects who did not participate in the exercise programme had a significant reduction in bactericidal activity by week 12 (Tukey's test, $P=0{\cdot}002$), while the exercise group remained unchanged ($P=0{\cdot}99$). For all subjects, there was a main effect of time on adherence, which increased during the intervention (Table 2, $P<0{\cdot}05$).

Effect on T cell and monocyte function

Concanavalin A and phytohaemagglutanin were used to examine any effects on the ability of T cells in the MNL fraction to produce cytokines (lymphotoxin, IFN-γ, IL-2)

Table 2. Erythrocyte fatty acid composition (% of total fatty acids) at week 0 and after 6 and 12 weeks of intervention with fish oil (FO) and exercise (FOX) or sunflower oil (SO) and exercise (SOX)

(Mean values with their standard errors)

		Treatment group							
		FOX		FOX		SO		SOX	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
EPA*†	week 0	1·75	0·07	1·57	0·07	1·46	0·11	1·73	0·11
	week 6	1·71	0·05	1·49	0·05	1·39	0·11	1·62	0·06
	week 12	1·62	0·07	1·49	0·06	1·36	0·12	1·63	0·07
	n	7		9		4		7	
DPA†	week 0	4·66	0·15	4·50[a]	0·11	4·49	0·11	5·30[b]	0·30
	week 6	4·49	0·12	4·33	0·13	4·11	0·28	4·92	0·12
	week 12	4·48	0·13	4·48	0·10	4·00	0·24	4·99	0·16
	n	7		9		4		7	
DHA*‡	week 0	3·83	0·19	3·97	0·34	3·40	0·15	3·53	0·32
	week 6	6·12	0·16	6·30	0·29	3·53	0·33	4·19	0·22
	week 12	6·89	0·17	7·35	0·33	3·62	0·14	4·20	0·17
	n	7		9		4		7	
Total *n*-3 PUFA*‡	week 0	10·24	0·25	10·04	0·27	9·34[a]	0·16	10·56[b]	0·20
	week 6	12·32	0·22	12·13	0·32	9·02	0·52	10·73	0·35
	week 12	13·00	0·22	13·33	0·33	8·98	0·38	10·82	0·34
	n	7		9		4		7	

DPA, docosapentaenoic acid.

Mean values within a row with different superscript letters are significantly different (1-factor ANOVA, $P<0{\cdot}05$). No 3-factor interactions were detected.

* Significant main effect of time, $P<0{\cdot}05$.

† Significant 2-factor oil × exercise interaction, $P<0{\cdot}05$.

‡ Significant 2-factor oil × time interaction, $P<0{\cdot}05$.

Table 3. Neutrophil functions at weeks 0 and 12 of intervention*
(Mean values with their standard errors)

		Treatment group							
		FO		FOX		SO		SOX	
		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Superoxide production‡ (OD_{570nm})	week 0	0·14	0·02	0·15	0·02	0·12	0·02	0·13	0·01
	week 12	0·11	0·02	0·10	0·02	0·14	0·03	0·13	0·02
	change	−0·03	0·02	−0·05	0·02	0·02	0·02	0·01	0·02
	n	11		10		11		8	
Bactericidal activity§ (% killing)	week 0	96	1	96	1	96	1	93	1
	week 12	93	1	95	1	94	1	96	1
	change	−3	1	−1	1	−1	2	2	2
	n	10‖		10		10‖		7‖	
Chemotaxis (mm/90min)	week 0	2·15	0·05	2·17	0·07	2·16	0·06	2·07	0·07
	week 12	2·03	0·08	2·23	0·07	1·98	0·09	2·11	0·10
	change	−0·13	0·11	0·06	0·11	−0·18	0·11	0·04	0·12
	n	11		10		11		8	
Adherence† (OD_{570nm})	week 0	0·17	0·04	0·23	0·05	0·25	0·04	0·21	0·02
	week 12	0·31	0·05	0·32	0·04	0·26	0·04	0·27	0·03
	change	0·15	0·06	0·09	0·05	0·01	0·06	0·06	0·02
	n	10‖		10		10‖		8	

FO, fish oil; FOX, fish oil and exercise; SO, sunflower oil; SOX, sunflower oil and exercise; TC, OD, optical density.
* Data were analysed with a 3-factor repeated measures ANOVA with interaction for oil, exercise and time, with $P<0·05$ considered significant. No 3-factor interactions were detected.
† Significant main effect of time, $P<0·05$.
‡ Significant oil × time interaction, $P<0·05$.
§ Significant exercise × time interaction, $P<0·05$.
‖ Subjects excluded due to insufficient harvesting of cells or technical problem with assay.

and lipopolysaccharide to stimulate monocyte cytokines (IL-1β, TNF and IL-6) in the MNL. The production of cytokines from concanavalin A, phytohaemagglutanin and lipopolysaccharide stimulated MNL did not differ between groups at baseline, and remained unchanged following the 12-week intervention, although ANOVA detected a significant main effect of time for several of these cytokines (Table 4).

Discussion

The data presented demonstrate that there is no interactive effect of regular aerobic exercise training and *n*-3 PUFA supplementation on T cell and monocyte responses to phytohaemagglutanin/Concanavalin A and lipopolysaccharide respectively, in an overweight population with risk factors for CVD. The T cell responses were measured in terms of production of the T helper 1 cytokines, lymphotoxin, IFN-γ and IL-2, while for monocytes, IL-1β, TNF and IL-6 production was assessed. While the combined effects of these treatments on inflammation and immunological parameters have not previously been investigated, others have shown that both *n*-3 PUFA and regular exercise independently reduce risk factors for CVD and mortality[45,46] and modulate inflammatory markers[26,47], but these differences may be related to variations in dose and type of *n*-3 PUFA used[48].

In this study, subjects were supplemented with DHA-rich fish oil, providing a total *n*-3 PUFA intake of about 2·0 g/d, of which about 1·6 g/d was DHA. As expected, the concentration of total long chain *n*-3 PUFA in erythrocyte membranes increased progressively during the 12-week intervention. This change was entirely attributable to a substantial increase in DHA, the incorporation of which into erythrocyte membranes may have displaced EPA and could account for the observed reduction in EPA during the intervention. Additionally subjects may have reduced their consumption of fish and EPA from other sources, although it should be emphasised that baseline fish consumption was less than two servings of oily fish per week, and subjects taking fish- oil capsules were excluded. There is good evidence that the incorporation of *n*-3 PUFA into erythrocytes mimics the incorporation into other tissues[49]. Supplementation resulted in a near doubling of DHA in erythrocyte membranes. Comparable changes have been observed in platelets, mononuclear cells[50,51] and neutrophils[52], although there is some degree of variability in the time course for incorporation[50].

Unlike DHA, EPA competes directly with AA as a substrate for cyclo-oxygenase and 5-lipoxygenase[53], which leads to the production of less potent inflammatory eicosanoids than are produced from AA. In comparison, DHA may exert anti-inflammatory effects by (i) inhibiting gene expression of enzymes involved in eicosanoid synthesis[54];(ii) conversion into highly anti-inflammatory compounds[55]; or (iii) inhibiting expression of inflammatory mediators[1,56]. There is however limited data directly comparing the immunomodulatory properties of DHA and EPA. Supplementation with DHA but not EPA has been reported to suppress T lymphocyte activation[52], although neither EPA nor DHA affected neutrophil function, cytokine production by MNL or adhesion molecule expression. Howe[57] investigated the effect of EPA *v*. DHA-rich fish oils on inflammatory mediators during a 16-week double-blind crossover trial. They suggest that EPA-rich fish oil may be more effective than DHA-rich oil in suppressing IL-1β production, but this reduction was not significantly different from DHA. In an earlier trial, Meydani[8] reported a

Table 4. Cytokine production from stimulated neutrophils at baseline and following intervention with fish oil (FO) and exercise (FOX) or sunflower oil (SO) and exercise (SOX)*

(Mean values with their standard errors)

			Treatment group							
			FO		FOX		SO		SOX	
Cytokine production (ng/ml)	Stimulant		Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
LT†	Con A	week 0	8·52	2·13	13·41	6·62	7·28	1·00	10·43	2·26
		week 12	5·91	1·54	5·39	0·81	5·91	1·22	5·58	0·99
		change	−2·61	1·41	−2·10	1·36	−1·36	1·78	−4·85	2·24
		n	7		5‡		7		6	
LT†	PHA	week 0	30·26	4·30	30·04	6·53	36·71	6·96	32·37	7·27
		week 12	24·24	5·65	15·95	2·94	23·46	4·54	17·93	4·83
		change	−6·02	4·61	−14·10	6·15	−7·76	2·84	−14·44	8·01
		n	7		6		6[3]		6	
IFNγ	Con A	week 0	23·46	5·92	26·39	7·53	18·38	3·78	23·96	6·03
		week 12	21·52	5·80	31·52	1·13	23·31	6·04	26·59	6·47
		change	−1·94	2·95	5·14	8·10	4·93	6·01	2·63	6·59
		n	7		6		7		6	
IFNγ	PHA	week 0	17·50	5·32	24·66	10·25	15·64	6·38	14·09	3·57
		week 12	16·56	3·03	18·26	5·44	14·77	5·88	15·83	6·19
		change	−0·94	3·60	−6·41	7·73	−0·88	7·18	1·74	4·55
		n	7		6		7		6	
IL-2†	PHA	week 0	0·80	0·40	0·71	0·28	1·88	0·78	0·63	0·33
		week 12	0·45	0·25	0·24	0·08	0·51	0·16	0·18	0·05
		change	−0·35	0·36	−0·45	0·23	−1·29	0·80	−0·58	0·35
		n	7		5‡		6‡		5‡	
IL-1β†	LPS	week 0	0·37	0·19	0·44	0·20	0·65	0·35	0·46	0·20
		week 12	1·30	0·30	1·16	0·35	1·00	0·15	1·56	0·33
		change	0·67	0·34	0·73	0·28	0·36	0·37	1·10	0·34
		n	6‡		6		7		6	
IL-6†	LPS	week 0	3·00	0·85	5·80	2·34	4·97	2·28	4·55	2·12
		week 12	8·22	1·60	7·25	0·65	7·54	1·81	11·33	1·63
		change	5·22	2·22	1·45	1·83	2·57	2·74	6·78	2·44
		n	7		6		7		6	
TNFα†	LPS	week 0	0·42	0·16	0·32	0·08	0·56	0·16	0·60	0·23
		week 12	2·08	0·37	1·53	0·52	1·82	0·39	2·15	0·60
		change	1·67	0·45	1·21	0·50	1·26	0·35	1·54	0·61
		n	7		6		7		6	

LT, lymphotoxin; Con A, concanavalin A; PHA, phytohaemagglutinin; IFNγ, interferon γ; LPS, lipopolysaccharide.

* Data were analysed with a 3-factor repeated measures ANOVA with interaction for oil, exercise and time, with $P<0·05$ considered significant. No 3-or 2-factor interactions were detected.

† Significant main effect of time, $P<0·05$.

‡ Subject excluded due to insufficient specimen.

reduction in TNFα IL-1β and IL-6 following 12 weeks of supplementation with 2·4 g/d of EPA-rich fish oil; however they did not evaluate the effects of DHA.

Varying doses of fatty acids may also account for inconsistencies between reports of anti-inflammatory effects of *n*-3 PUFA. Trebble[58] found a U-shaped dose–response relationship between EPA intake and cytokine production, wherein maximum inhibition occurred with 1 g EPA-rich oil/d. However, a dose–response curve for DHA is yet to be established. In studies using subjects characterised by elevated cytokine production[59–61], decreases in IL-1β, TNFα, IL-2 and IFNγ production by stimulated MNL have been elicited by comparable or lower doses of *n*-3 PUFA than used in the present study. The supplementation in this trial may have been of insufficient dose to produce changes in cytokine production, as although exhibiting some risk factors for CVD, participants in this trial were generally healthy and did not present with raised serum cytokines at baseline. With high doses of *n*-3 PUFA, several studies have shown a reduction in both cytokine production and neutrophil function[16,18,53,61].

Regular moderate exercise (2·5 h/week) has been shown to decrease MNL production of TNFα and IFNγ in subjects with risk factors for CVD[26] and to reduce the pro-inflammatory cytokines, C-reactive protein, IL-1, IL-6 and IFNγ in patients with coronary artery disease[25]. Much research has focused on the independent effect of exercise in populations with elevated inflammatory markers, and data from randomised controlled trials are conflicting[62]. Variations in protocol may partially explain the differences in cytokine production as investigators have used a range of MNL stimulants including lipopolysaccharide[6,58] and phytohaemagglutinin[26]. Furthermore, the small sample size and large inter-individual subject variation may have limited our ability to detect effects of intervention (fish oil or exercise) compared to controls.

While several studies have investigated the effect of an acute exercise bout on neutrophil respiratory burst, chemotaxis and adherence, these reports are inconsistent and likely to be confounded by factors such as training status, exercise intensity and population differences[31,32]. Fewer studies have investigated the effect of regular exercise training on

neutrophil function, although there is some evidence to suggest that it may reduce neutrophil respiratory burst activity[31]. While we observed no effect of regular aerobic training on superoxide production, we did observe a protective effect of exercise on bactericidal activity. As for other measures of neutrophil function, there is limited data on the effect of aerobic exercise training on this parameter. Lewicki[63] reported that bactericidal activity of trained sportsmen was significantly reduced compared to untrained individuals. Conversely, Benoni[64] observed an increase in bactericidal activity during the sports season of elite basketball players, which returned to baseline levels at the end of the season. Although significant, the change in bactericidal activity observed in this trial was marginal and unlikely to be of clinical significance.

In this study, supplementation with DHA-rich tuna fish oil resulted in a significant reduction in superoxide production by stimulated neutrophils. This finding gives support to previous studies which have found decreases in superoxide production of 14–64 % following 4–6 weeks of supplementation with EPA-rich oil providing >4 g *n*-3 PUFA/d[16,19,65], which was substantially more than that provided in the present study. Although differences in the methods by which the reactive oxygen species are measured may explain some of the variation in the magnitude of change in superoxide production, it is more likely to be a function of the neutrophil stimulus used. Fisher[65] and Varming[19] used particles acting on neutrophil surfaces while we and others who found a smaller effect used the soluble stimulus PMA which acts on intracellular protein kinase C (PKC)[16]. Conversely, lower doses of *n*-3 PUFA (0·27–2·25 g/d) have not been shown to influence superoxide production by neutrophils[21].

The importance of modifying superoxide production is linked to its role in CVD, particularly in the pathogenesis of atherosclerosis. Reactive oxygen species, including superoxide, induce endothelial expression of cell adhesion molecules[66], increase microvessel permeability[67] and oxidised LDL[68]. Within the endothelium, excess reactive oxygen species can lead to a decrease in nitric oxide bioavailability[69], and this can impact negatively on vascular function. Moreover reactive oxygen species play a role in the activation of nuclear factor-κβ, a transcription factor that regulates the production and expression of numerous inflammatory cytokines and cell adhesion molecules[70]. The mechanism by which *n*-3 PUFA, particularly DHA, decreases superoxide production may be related to its incorporation into neutrophil membrane lipids. While we did not measure the fatty acid composition of neutrophil membranes, it is reasonable to assume that DHA incorporation into these cells would have mirrored the increase seen in erythrocyte membranes. Evidence for this relationship may be seen in the negative correlation between superoxide production and the increased incorporation of DHA in erythrocyte membranes.

While DHA as a NEFA has been shown to directly and strongly activate the neutrophil respiratory burst[71], increasing the *n*-3 PUFA concentration of neutrophil membrane phospholipids, such as phosphatidylcholine and phosphatidylinositol, may alter the ability to activate signaling molecules such as PKC, which are involved in oxidase stimulation. DHA, by replacing AA in the s*n*-2 position of the phospholipid, leads to the generation of DHA-containing diacylglycerol, possibly compromising PKC translocation and/or activation. The stimulus used in our studies, PMA, directly interacts with PKC to activate the NADPH oxidase. However as we and others have previously shown, PUFA act via phosphatidic acid which is involved in the generation of diacylglycerol, a process stimulated by PMA[72–74]. It is therefore not surprising that DHA supplementation suppresses T cell PKCθ lipid raft recruitment[75]. Indeed Madani[76] reported that DHA-containing diacylglycerol and AA-containing diacylglycerol activated different PKC isozymes differently *in vitro*. Finally intracellular AA is considered to promote the activation of small guanosine 5′-triphosphate binding protein Rac, a component of the neutrophil oxidase[77]. Replacing AA with DHA in the phospholipids may hinder this part of the oxidase activation process.

Our findings demonstrate that under these conditions, *n*-3 supplementation had no effect on *ex vivo* cytokine production by MNL and emphasises the controversies in this field. While we would have expected that this type of treatment should have resulted in reduced cytokine production[5–8,58,61,78,79], several studies have demonstrated either no effect[22,23,52,59,80–85] or even increased production of cytokines[86]. These discrepancies are difficult to explain and cannot be simply due to the dose of *n*-3. Other factors are likely to be the type of *n*-3 PUFA used, and the individual's health status, sex and age. Whether or not technical factors contribute to this difference needs to be given consideration. Our studies were conducted under NATA accredited conditions, ensuring a high level of standardisation and reproducibility in conducting cellular tests at different time intervals. Interestingly, contributing to these variations is the finding that polymorphism in the TNF gene and promoter can influence the effects of fish-oil supplement on the production of this cytokine[87]. Similarly, studies addressing *n*-3 supplementation and neutrophil functions have produced conflicting results, reporting either a decrease[3,15–17,19], no effect[13,21–23,52,84] or increase[86] in neutrophil function. Again, while more careful consideration of experimental conditions needs to be given, it is evident that doses, *n*-3 fatty acid type and the neutrophil stimulus used may give rise to these differences.

In summary, the present study found that supplementation with DHA-rich fish oil reduces superoxide production from stimulated neutrophils, while regular moderate exercise training protects bactericidal activity. Neither of these functions was enhanced by the combination of interventions. However it is unclear whether these changes are of biological significance. Other neutrophil functions (chemotaxis, adherence and cytokine production) were unaffected by intervention. Supplementation with about 2g *n*-3 PUFA/d therefore did not inhibit immune function in this study. Differences between this and other studies investigating the effect of *n*-3 PUFA on markers of inflammation may be due to variations in either the dose or fatty acid composition (i.e. DHA/EPA) of fish oil, clearly emphasising the need for a well-controlled dose–response study which differentiates between the effects of EPA from those of DHA. Nevertheless, given the independent beneficial effects of moderate exercise and fish-oil supplementation on CVD risk parameters, we support recommendations to combine these lifestyle interventions.

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