Fish oil supplementation reduces markers of oxidative stress but not muscle soreness after eccentric exercise

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Abstract

Due to the potential anti-inflammatory properties of fish-derived long chain n-3 fatty acids, it has been suggested that athletes should regularly consume fish oils, although evidence in support of this recommendation is not clear. While fish oils can positively modulate immune function, it remains possible that, due to their high number of double bonds, there may be concurrent increases in lipid peroxidation. The current study aims to investigate the effect of fish oil supplementation on exercise-induced markers of oxidative stress and muscle damage. Twenty males underwent a six week double blind randomised placebo-controlled supplementation trial involving two groups (fish oil or placebo). After supplementation, participants undertook 200 repetitions of eccentric knee contractions. Blood samples were taken pre-supplementation, post-supplementation, immediately, 24, 48 and 72h post-exercise and muscle soreness/maximal voluntary contraction (MVC) assessed. There were no differences in creatine kinase, protein carbonyls, endogenous DNA damage, muscle soreness or MVC between groups. Plasma thiobarbituric acid reactive substances (TBARS) were lower ($P<0.05$) at 48 and 72h post exercise and H_2O_2 stimulated DNA damage was lower ($P<0.05$) immediately post-exercise in the fish oil, compared with the control group. The current study demonstrates that fish oil supplementation reduces selected markers of oxidative stress after a single bout of eccentric exercise.

Introduction

Increased consumption of fish-derived n-3 long chain polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as originally described in Inuit populations (Bang *et al*, 1980), seems to be beneficial in the management of many inflammatory conditions (Calder, 2006a) including heart disease (Lemaitre *et al*, 2003). Recommendations for intake have been issued for different populations including athletes, who are advised to consume 1-2 grams per day of n-3 PUFAs at a ratio of 2:1 for EPA:DHA (Simopoulos, 2007), although the evidence for this recommendation is not clear.

It is possible that due to the high number of double bonds present in these fatty acids, any increase in n-3 PUFA consumption could potentially result in a concomitant increase in lipid peroxidation and the generation of a state of oxidative stress. Oxidative stress can be defined as an imbalance in the concentrations of reactive oxygen species (ROS) and antioxidants in favour of oxidant species (Aruoma, 1998), and prolonged oxidative stress is linked to various disease states such as Parkinson's disease (Jenner, 2003) and cardiovascular disease (Madamanchi *et al*, 2005). However, a further positive role as signalling molecules may exist for ROS (Barbieri and Sestili, 2012), which could potentially be important for those participating in exercise. Indeed, a bout of intense exercise can lead to the development of oxidative stress via several potential mechanisms (Nedeljkovic and Gokce, 2005). Approximately 0.15% of oxygen metabolised during mitochondrial oxidative phosphorylation is believed to form free radicals (St-Pierre *et al*, 2002) and other potential sources of contraction-induced free radical production include nitric oxide synthase, xanthine oxidase and NAD(P)H oxidase (Powers and Jackson, 2008). The precise physiological role of exercise-induced free radicals are yet to be determined but may be involved in many physiological processes associated with adaptations to exercise, such as angiogenesis, mitochondrial biogenesis and skeletal muscle hypertrophy (Gomes *et al*, 2012).

Resting studies have shown either an increase (Allard *et al*, 1997, Mazière *et al*, 1999, Wander and Du, 2000) or a decrease (Mori *et al*, 1999, Nälsén *et al*, 2006) in markers of lipid peroxidation and enhanced protection against cellular DNA damage (Kikugawa *et al*, 2003) after fish oil supplementation. Furthermore, higher plasma F_2-isoprostane concentrations were found in volunteers, consuming fish oil supplements compared with a placebo group, after three days of 3h intense cycling per day (McAnulty *et al*, 2010). However, the effect of fish oil consumption on the oxidative responses after a single bout of eccentric exercise, where ROS may mediate force recovery and adaptations (Close *et al*, 2004), remains to be established.

Therefore the aim of the current study is to investigate the effects of six weeks supplementation with fish oil on muscle damage, muscle soreness and markers of oxidative stress following a bout of eccentric exercise. Monitoring oxidative stress is not straightforward and so to give a detailed overview of the effects of fish oil on oxidative stress we have chosen to measure 3 markers of oxidative stress. The markers chosen provide information on lipid peroxidation (thiobarbituric acid reactive substances - TBARS), protein oxidation (protein carbonyls) and oxidative damage to lymphocytes (DNA damage).

Materials and methods

Subjects

Twenty males (age 23 ± 2.3 years, height 180 ± 6.5cm, body mass 79 ± 11.4kg) volunteered to take part in the study. All participants were recreationally active but none were specifically trained. The study was approved by the University of Aberdeen College of Life Sciences and Medicine Ethics Review Board and participants were made aware of the aims, risks and discomfort associated with the study, before providing written informed consent. Participants were free to withdraw at any time.

Supplementation

Participants were randomly assigned to either a placebo (n=10) or fish oil (n=10) group. Capsules were closely matched for both colour and shape and both participants and investigators were blind to the supplementation group. After the baseline visit, participants in the placebo group consumed 3g of olive oil (0.4g palmitic acid, 0.08g stearic acid, 2.1g oleic acid and 0.37g linoleic acid) daily while those in the fish oil group consumed 3g of fish oil (in triglyceride form: 1.3g EPA, 0.3g DHA and 45I.U. d-α tocopherol) daily for a six week period. Fish oil and placebo capsules were provided by Nordic Naturals. Throughout the supplementation period fish consumption was recorded using a weekly checklist and weekly consumption of EPA and DHA estimated based on published food tables (McCance & Widdowson, 2002).

Intervention

Fasting blood samples were collected, and muscle soreness and maximal voluntary contraction (MVC) measured, at baseline and after 6 weeks of supplementation prior to and immediately following an eccentric exercise protocol. Three additional fasted blood samples

were collected, and muscle soreness and MVC determined at 24, 48 and 72 hours after the exercise bout.

During each visit, participants were asked to assess perceived muscle soreness by means of a ten-point visual analogue scale (VAS). Participants were then seated on a dynamometer (Biodex Medical, NY, USA) and performed an MVC for 10s of the knee from a fixed angle of 90°. This was repeated twice with 120s rest in between each contraction. Participants were given 120s of rest before beginning the eccentric exercise protocol which consisted of twenty sets of ten eccentric contractions at 0.52 rads/s between 90° and 120°. Each set was separated by 120s on the dynamometer. Immediately after the eccentric exercise an additional blood sample was taken, muscle soreness assessed and MVC measured.

During the 24h period prior to each visit, participants were instructed to refrain from the consumption of alcohol and strenuous exercise.

Blood Sampling and Preparation

Blood samples were drawn from an antecubital vein using a butterfly needle (21G). Blood was collected in vacutainers coated with K^+EDTA (BD, Oxford, UK), for separation of plasma. Plasma was isolated by centrifugation (800 g, 10 minutes, 4°C) and stored at -80°C until analysis. Peripheral blood mononuclear cells (PBMCs) were then isolated, for the measurement of lymphocyte DNA damage, as previously described (Thies *et al*, 2001a) and frozen to -80°C at a cell density of 3×10^6 in FCS containing 10% dimethyl sulphoxide (DMSO) at approximately -1°C/min (Jenkinson *et al*, 1999).

Creatine Kinase

All measurements were performed using a commercially available kit (Randox, N Ireland). Samples were analysed spectrophotometrically (Spectronic Genesis 10 Bio, Thermo Scientific, USA) at 340 nm at baseline and 1, 2 and 3 minutes of incubation. Creatine kinase activity was determined by substituting the change in absorbance between baseline and 3 minute timepoint (ΔA 340 nm/min) into the following formula:

Creatine kinase activity (u/l) = 4127 x ΔA 340 nm/min

Lymphocyte DNA Damage

Cellular DNA damage was assessed using alkaline single-cell gel electrophoresis (Jenkinson *et al*, 1999). DNA strand breakage was assessed following treatment with and without 200 μM H_2O_2. DNA damage was quantified by visual scoring validated using computerised image analysis and expressed in arbitrary units (Duthie *et al*, 1997).

Thiobarbituric acid reactive substances (TBARS)

All measurements were performed using a commercially available kit (Cayman Chemicals, MI, USA). 100 μl sodium dodecyl sulphate (SDS) solution was added to 100 μl plasma sample or standard solution in 5 ml tubes and vortexed. 4 ml colour reagent was added to each tube. Tubes were placed in water bath at 100°C for 1h. Immediately after boiling, tubes were incubated on ice for 10 minutes to stop reaction. Tubes were centrifuged at 1600 g and 4°C for 10 minutes. 150 μl of each sample were added to wells of a 96-well plate. Samples were analysed within 30 minutes using a microplate reader at 530 nm.

Protein Carbonyls

All measurements were performed using a commercially available kit (Cayman Chemicals, MI, USA). 200 µl plasma was aliquoted into two microtubes – sample and control. 800 µl 2,4-dinitrophenylhydrazine was added to the sample tube and 800 µl 2.5M hydrochloric acid was added to the control tube. Tubes were incubated at room temperature, protected from light for 1h. 1 ml of 20% trichloroacetic acid (TCA) was added, tubes incubated on ice for 5 minutes and centrifuged at 10000 g at 4°C for 10 minutes. The supernatant was discarded, protein pellet reconstituted with 1 ml of 10% TCA and tubes were centrifuged at 10000 g at 4°C for 10 minutes. The supernatant was discarded and the pellets washed (10000 g at 4°C for 10 minutes) 3 times with 1 ml of ethanol:ethyl acetate (1:1 ratio). Supernatant was discarded and the pellet resuspended using 500 µl guanidine hydrochloride before centrifugation at 10000 g at 4°C for 10 minutes. 220 µl of the supernatant was added to a 96-well plate and the absorbance measured at 370 nm. Protein carbonyl content (nmol/ml) was then calculated relative to the total protein content of the sample, which was calculated from a BSA standard curve (0.25-2.0 mg/ml) dissolved in guanidine hydrochloride read at 280 nm.

EPA/DHA Analysis

Plasma total lipids were extracted as previously described (Bligh and Dyer, 1959). Briefly, lipids were extracted under N_2 in a mixture of chloroform/methanol containing 0.05% butylated hydroxytoluene. Sodium chloride was then added, samples centrifuged at 400 g at 20°C for 5 minutes and the bottom phase collected and dried under N_2. Chloroform/methanol containing 0.05% butylated hydroxytoluene was then added and phospholipids separated by thin layer chromatography, extracted using isohexane and fatty acid methyl esters analysed by gas chromatography as previously described (Thies *et al*, 2001b).

Statistical Analysis

Data analysis was carried out using Prism version 5 software. Normality of data was tested using the Shapiro-Wilk test, with all data being found to be normally distributed. Baseline characteristics data were compared between groups using independent t-tests. All other data were analysed using a two way (group and time) repeated measures ANOVA with bonferroni corrected post hoc t-tests, where appropriate. When a time effect was noted a one way ANOVA was carried out for each group, with bonferroni correct post hoc t-tests comparing each time point with baseline where appropriate. Statistical significance was accepted at P <0.05. Data are presented as mean $\pm$ SD.

Results

Subject Baseline Characteristics

Subject baseline characteristics and weekly EPA/DHA intake were similar between the groups (Table 1).

Effects of 6 weeks supplementation on resting measurements

There were no differences in either group for MVC, soreness, creatine kinase, TBARS, protein carbonyls or lymphocyte DNA damage after the supplementation period (Table 2). Plasma concentrations of EPA were higher ($P<0.05$) in the fish oil group, compared with the control group, after 6 weeks of supplementation (Figure 1). However, plasma DHA concentration remained unchanged ($P>0.05$) after supplementation with fish oil (Figure 1).

MVC and muscle soreness

MVC significantly decreased immediately after exercise in both groups ($P<0.05$) compared with pre-exercise values. However, there were no differences between groups, nor was any interaction effect observed ($P>0.05$; Figure 2). Muscle soreness increased 48h after exercise in both groups ($P<0.05$ compared with pre-exercise values), with no group or interaction effects observed ($P>0.05$).

Creatine Kinase

Creatine kinase activity significantly increased in both groups 24h and 48h after exercise ($P<0.05$ for both groups) compared with pre-exercise values, with no group or interaction effects observed ($P>0.05$; Figure 2).

TBARS

With plasma TBARS concentration the ANOVA showed a trend for a time effect ($P=0.06$) with no effect of group observed ($P>0.05$). An interaction effect ($P<0.05$) was observed, with post-hoc tests showing that plasma TBARS concentration was lower in the fish oil group compared with the control group at 48h and 72h ($P<0.05$) post-exercise (Figure 3).

Protein Carbonyls

No group or interaction effects were noted from the ANOVA ($P>0.05$) when comparing protein carbonyls. A time effect ($P<0.05$) was noted for plasma protein carbonyls with post-hoc tests showing an increase ($P<0.05$) in the fish oil group at 24h and 72h post-exercise compared with pre-exercise concentrations. There were no differences within the control group (Figure 3).

Lymphocyte DNA Damage

There were no differences in endogenous lymphocyte DNA damage between groups, nor was an interaction effect noted ($P>0.05$; Figure 4). An effect of time was noted from the ANOVA ($P<0.05$) and post-hoc tests revealed that endogenous lymphocyte DNA damage was significantly higher ($P<0.05$) immediately post-exercise compared with pre-exercise, and significantly lower ($P<0.05$) than pre-exercise levels 72h post exercise, in the control group (Figure 4). No differences with time were observed within the fish oil group ($P>0.05$).

The ANOVA revealed no time or interaction effects ($P>0.05$) with H_2O_2-induced lymphocyte DNA damage, but a group effect ($P<0.05$) was noted. Post-hoc tests revealed that H_2O_2-induced lymphocyte DNA damage was significantly higher in the control group ($P<0.05$) immediately post-exercise compared with the fish oil group.

Discussion

Our results show that six weeks supplementation with 3.0g/day of fish oil, rich in EPA/DHA, significantly decreased plasma TBARS 48 and 72h after a single bout of eccentric exercise. In addition, H_2O_2-induced lymphocyte DNA damage was lower in the fish oil group compared with the control group, immediately after the eccentric exercise bout, suggesting that n-3 PUFA enrichment may reduce exercise-induced oxidative stress and provide protection against oxidative damage. It is, perhaps, worth noting at this point that the eccentric exercise protocol employed in the current study induced relatively mild muscle damage and the response observed may differ if a more severe damaging protocol was used. One limitation to this study is that, whilst we monitored weekly fish intake and asked participants to fast before each visit, a full analysis of nutritional intake was not made. It is possible, therefore, that large differences in nutritional intake may influence the findings of the study, although with the monitoring of fish intake and an overnight fast we believe a major effect of this is unlikely.

Previous studies investigating the effect of fish oil supplementation on markers of oxidative stress and DNA damage are somewhat ambiguous. Plasma TBARS concentration increased in basal/resting conditions after 5 weeks supplementation with EPA and DHA (2.5g EPA and 1.8g DHA per day) in females, while protein carbonyls concentration remained unchanged (Wander and Du, 2000). In a similar study, plasma TBARS concentrations increased by 23% after six weeks' fish oil supplementation (2.0g EPA and 1.4g DHA per day) with no effect on F2-isoprostanes concentrations (Higdon *et al*, 2000). Similar findings were also reported by Allard *et al* (1997). However Mori *et al* (1999) found that increased fish consumption for 8 weeks (3.6g n-3 PUFA per day) reduced urinary F2-isoprostanes in sedentary patients with type 2 diabetes, with similar findings reported by Nalsen *et al* (2006). When exercise is superimposed on top of fish oil supplementation (2.2g EPA and 2.2g DHA per day for 6

weeks) there was no effect of fish oil on plasma TBARS after 1h of treadmill exercise (Bloomer *et al*, 2009), with similar findings in rats (Venkatraman et al, 1998). On the other hand, McAnulty *et al*. (2010) showed that post-exercise plasma concentrations of F2-isoprostanes were higher in participants supplemented with fish oil (2g EPA and 0.4g DHA per day for 6 weeks) alone, compared to those supplemented with fish oil plus antioxidants, antioxidants alone or placebo, following three days of 3h cycling sessions. However the current study found that fish oil supplementation decreased post-exercise plasma TBARS and H_2O_2-induced DNA damage. This is the first study, to our knowledge, to investigate the effects of fish oil supplementation on post-exercise H_2O_2-induced DNA damage.

These differences may be due to the aforementioned differences in supplementation dose/duration. Furthermore the current study employed eccentric exercise and may highlight a differential oxidative response to fish oils with varying modes of exercise. Indeed it is likely that the mechanisms through which reactive oxygen species are generated will depend upon the mode of exercise employed (Vollaard *et al*, 2005) and so any modulation of these mechanisms (e.g. via fish oil-derived n-3 PUFAs) will be dependent upon the exercise mode employed. However, as the precise mechanisms responsible for exercise-induced free radical production, under these various exercise modes, remains to be elucidated, uncovering the mechanisms underlying the findings of the current studies are problematic. Regardless, these potential mechanisms remain worthy of further discussion.

Previous work has suggested that EPA/DHA may act as a free radical scavenger (Barbosa *et al*, 2003) which may partly explain the fall in production of TBARS and DNA damage in fish oil supplemented participants in the current study. A further possible mechanism for these findings may involve the modulation of the production of prostaglandin E2 (PGE-2), a metabolite of arachidonic acid metabolism. The consumption of fish oil increases the cell membrane incorporation of EPA at the expense of arachidonic acid, resulting in a decrease in

the production of PGE-2 and a concomitant increase in production of PGE-3, a less "potent" inflammatory eicosanoid (Calder, 2006b). In relation to the current findings, a link has previously been shown between the synthesis of PGE-2 and lipid peroxidation. It has been found that the cyclooxygenase enzymes (COX1 and COX2), which are responsible for the generation of PGE-2 can also generate free radicals (Pepicelli *et al*, 2005), although it must be pointed out that others dispute this COX activity-associated increase in lipid peroxidation (Manabe *et al*, 2004). It may therefore be the case that this decrease in cyclooxygenase mediated arachidonic acid metabolism, after fish oil consumption, results in not only a decrease in PGE-2 production but also a decrease in the generation of free radicals, although further work is required to test this hypothesis. The need for further work is highlighted by our findings in protein carbonyls, with an increase over time only observed in the fish oil group, with no change in the control group. Although no group or interaction effects was observed these findings may indicate a dichotomous effect of fish oil on markers of oxidative stress, which, as indicated, highlights the need for further work in this area.

The production of PGE-2 has also been linked to the sensation of pain, with PGE-2 causing pain via excitation of nociceptors and by stimulating the release of neuropeptides known to be related to pain (Kawabata, 2011). In addition to changes in oxidative stress, we also investigated whether fish oil consumption could modulate post-exercise muscle soreness. We found that fish oil had no effect on self-reported muscle soreness, or force recovery, which is in contrast with previous work reporting a significant decrease in perceived pain during the recovery period following eccentric stepping exercise after fish oil supplementation (Tartibian *et al*, 2009). However, our finding agrees with those of Lenn *et al*. (2002) who found no effect of fish oil supplementation on levels of muscle soreness following eccentric arm contractions (Lenn *et al*, 2002). We also found no effect of fish oil supplementation on post-exercise plasma creatine kinase levels, suggesting that levels of muscle damage were

unchanged by fish oil consumption. Similar findings were also reported by Toft *et al.* (2000), although in that study participants undertook endurance rather than eccentric exercise.

The physiological significance of the findings of the current study are that fish oil consumption may play a protective role, at least in part, against oxidation of lipids and DNA damage caused by eccentric exercise and the negative aspects associated with this process (Finaud *et al*, 2006). However, as mentioned, in recent years this classical role for free radicals and their negative consequences has been challenged, with the importance of free radicals in mediating positive adaptations to exercise being the subject of much debate. A recent study showed that supplementation with antioxidants (Vitamins E and C) reduced the insulin sensitising effects of exercise training and the upregulation of endogenous antioxidant defences (Ristow *et al*, 2009). This indicated that free radicals may be necessary for such, and other, exercise induced adaptations (Gomes *et al*, 2012). These findings have subsequently been challenged with other researchers finding no such negative, or positive, effects on exercise training adaptations with antioxidant supplementation (Higashida *et al*, 2011).

As mentioned previously it has been recommended that athletes should consume 1-2g fish oil per day (Simopoulos, 2007) and yet there remains a dearth of information regarding the interactions between fish oil and exercise. The current study has shown that fish oil supplementation can attenuate exercise induced lipid peroxidation after a bout of eccentric exercise, while others showed the opposite effects during 3 days of endurance training (McAnulty *et al*, 2010). Further studies have found that fish oil supplementation increases natural killer cell cytotoxic activity (Gray *et al*, 2012), reduces exercise induced bronchoconstriction (Mickleborough *et al*, 2006) and improves skeletal muscle blood flow in rats (Stebbins *et al*, 2010). It is not clear, however, what effect these changes have on exercise performance, recovery and adaptations to training and further studies are needed to investigate this.

In conclusion, the current study has shown that six weeks supplementation with fish oil, rich in EPA and DHA, can decrease production of TBARS and DNA damage after eccentric exercise.

Acknowledgements

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Table I – Baseline characteristics and performance measures of subjects in fish oil and control groups. Values are presented as mean ± SD.

Measure	Control group (n=10)	Fish oil group (n=10)	P value
Age (years)	23 ± 3.3	22 ± 1.3	0.590
Height (cm)	183.8 ± 7.5	178.1 ± 5.4	0.123
Weight (kg)	79.2 ± 12.1	80.0 ± 10.7	0.891
BMI (kg/m^2)	23.5 ± 3.0	25.3 ± 3.6	0.225

Table II – Pre- and post-supplementation measures of maximal voluntary contraction, muscle soreness, creatine kinase, protein carbonyls, TBARS and DNA damage. Values are presented as mean ± SD.

Measure	Control group (n=10) pre-supplementation	Control group (n=10) post-supplementation	P value	Fish oil group (n=10) pre-supplementation	Fish oil group (n=10) post-supplementation	P value
MVC (nM)	308.4 ± 66.8	313.0 ± 62.5	0.877	330.5 ± 104.2	349.5 ± 97.0	0.679
VAS (A.U.)	1.2 ± 0.2	1.1 ± 0.1	0.765	1.3 ± 0.7	1.2 ± 0.2	0.615
Creatine kinase (U/l)	133.9 ± 101.7	153.1 ± 122.5	0.706	84.1 ± 46.5	90.2 ± 57.3	0.796
Protein carbonyls (nmol/mg)	1.2 ± 0.7	0.9 ± 0.4	0.472	1.1 ± 1.2	0.7 ± 0.3	0.321
TBARS (μM)	1.0 ± 0.4	1.2 ± 0.3	0.244	1.2 ± 0.5	1.2 ± 0.6	0.805
Endogenous DNA damage (A.U.)	66.4 ± 34.8	75.9 ± 33.3	0.543	67.9 ± 34.4	54.4 ± 22.7	0.313
H_2O_2-induced DNA damage (A.U.)	229.7 ± 60.5	241.1 ± 53.0	0.661	204.6 ± 39.2	199.1 ± 66.7	0.824

FIGURE LEGENDS

Figure 1 – The effect of 6 weeks fish oil supplementation on plasma EPA and DHA concentration. * denotes a significant difference (P<0.05) compared with control group

Figure 2 – The effect of six weeks fish oil/placebo supplementation on MVC, muscle soreness and plasma creatine kinase pre- and post-exercise. * indicates a significant difference (P<0.05) from pre-exercise

Figure 3 – The effect of six weeks fish oil/placebo supplementation on plasma protein carbonyls and TBARS pre- and post-exercise. * indicates a significant difference from baseline. † indicates a significant difference (P<0.05) between groups.

Figure 4 – The effect of six weeks fish oil/placebo supplementation on endogenous and 200μM H_2O_2 induced lymphocyte DNA damage pre- and post-exercise. * indicates significant difference (P<0.05) from pre-exercise.
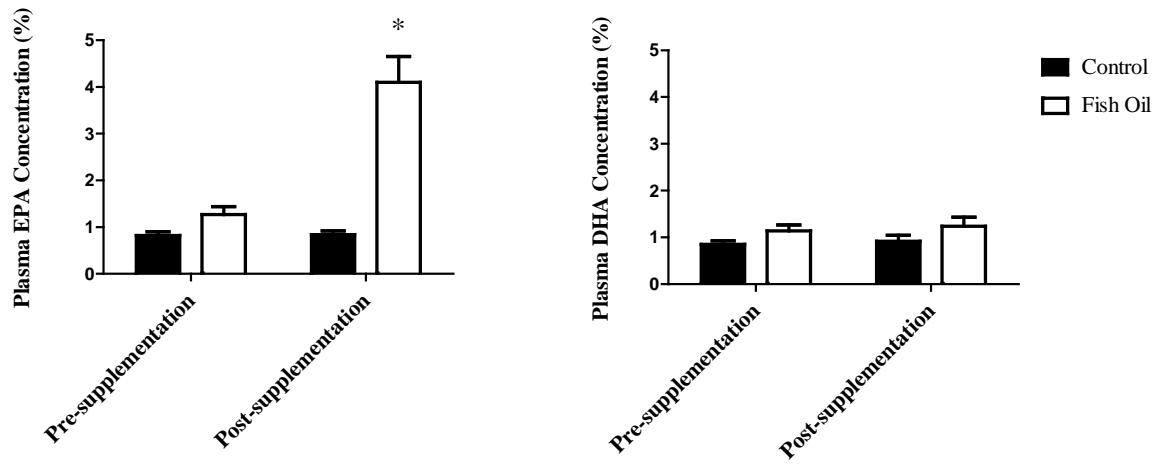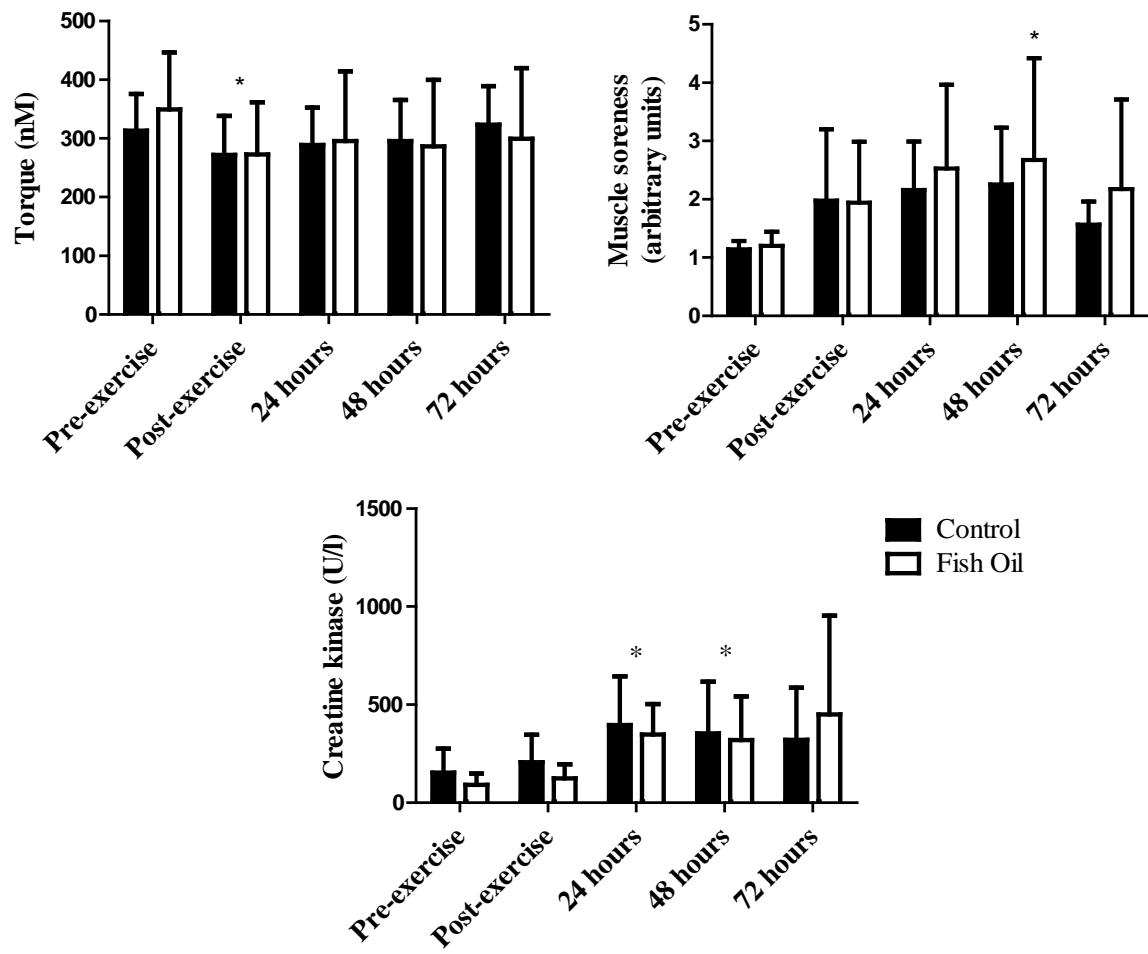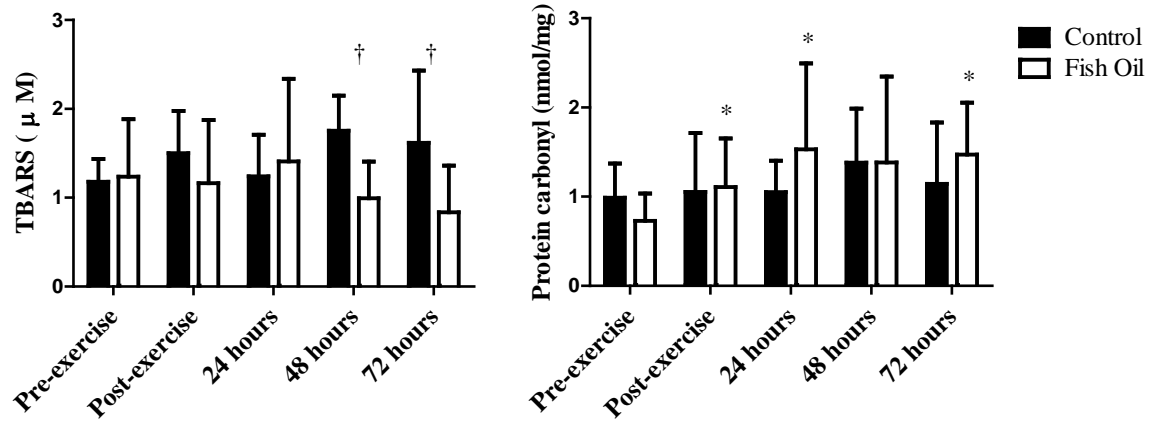
† indicates significant difference (P<0.05) between groups

Figure 1

Figure 2

Figure 3

Figure 4