Dose Response of Dietary Fish Oil Supplemenations on Platelet Adhesion

Xiaolin Li and Manfred Steiner

A dose–response study of dietary fish oil supplementation on platelet adhesion was performed in three groups of five normal individuals each. Fish oil equivalent to 3, 6, or 9 g eicosapentaenoic acid (EPA)/day was administered for 3 weeks, and platelet adhesion was evaluated under high and low shear rate conditions in a laminar flow chamber before, during, and after termination of fish oil administration. Platelet adhesion to collagen I and fibrinogen, the two test surfaces in this study, was greatly reduced in response to fish oil. The inhibitory effect was similar whether platelet adhesion was evaluated at high or low shear rates. Maximal inhibitory activity was noted at 6 g EPA/day. A delayed onset and prolonged washout period characterized the response. The washout period of the fish oil effect was inversely related to the level of dietary supplementation. Measurement of total fatty acid distribution in platelets showed a dose-related increase in n-3 polyunsaturated fatty acids. From these studies, it is concluded that fish oil is an effective inhibitor of platelet adhesion, which reaches its maximum effect at approximately 6 g EPA/day. (Arteriosclerosis and Thrombosis 1991;11:39–46)

A diet rich in fish oil containing n-3 polyunsaturated fatty acids has been thought to be the reason for the low incidence of thromboembolic disease in Eskimos.1-4 Fish oil supplementation of the diet has been shown in a number of groups of normal individuals to cause a slight inhibition of platelet aggregability, 5-16 which could be a contributing factor in the fish oil–induced reduction of thromboembolic phenomena. Previous studies by us17 have shown that fish oil can induce a highly significant inhibition of platelet adhesiveness when tested in a laminar flow chamber. In these studies, fish oil was administered at a single concentration and compared with an equal concentration of a vegetable oil–based dietary supplement.

In the present study, we have determined the dose–response relation between fish oil intake and inhibition of platelet adhesiveness. A progressive increase of fish oil equivalent to 3, 6, and 9 g of 5,8,11,14,17-eicosapentaenoic acid (EPA) was evaluated for its effect on platelet adhesion as measured under high and low shear rate conditions. Analyses of platelet fatty acids revealed compliance with the supplementation schedule. Our studies show that there is no further benefit to be gained by raising the supplementation dose of fish oil above 6 g EPA/day.

Methods

Experimental Design

Fifteen normal, healthy volunteers (11 women, four men), all nonsmokers aged 24–52 years, were studied. The participants in the study were advised to abstain from all medications for the entire period of the investigation. They were also urged to continue their normal, regular diet and to refrain from alcoholic beverages for the duration of the study. Fish oil was administered for a 21-day period in the form of 1-g capsules for a total equivalent to 3, 6, or 9 g EPA/day. Analysis of the fish oil capsules showed an EPA content of 13.6% and a 4,7,10,13,16,19-docosahexaenoic acid (DHA) content of 8.1% of the total lipids present. The total concentration of n-3 fatty acids was 308.3 mg/g, whereas that of n-6 fatty acids was 28.4 mg/g fish oil. Each 1-g capsule of fish oil contained 0.9 mg α-tocopherol and 1 mg γ-tocopherol. This project was reviewed and approved by the Institutional Review Board for Human Studies. Informed consent was obtained from all volunteers participating in this study.

Preparation of Platelets

Blood was obtained from the antecubital vein and collected into 1:10 volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described.18 To prepare plasma-free suspensions of platelets, platelets were isolated from PRP,18 and after two washes with 0.05 M phosphate buffer containing 0.14 M NaCl,
pH was adjusted to 6.5 with acid–citrate-dextrose, USP (ACD), and the platelets were then suspended in 3 mM N-morpholinoethanesulfonic acid containing 0.147 mM NaCl, 3 mM KCl, and 4.8 mM d-glucose (MES buffer), pH 6.65. Platelet suspensions were adjusted to approximately 300–350,000 cells/μl.

Adhesion Measurements

Low shear rate adhesion. Platelet adhesion was measured in a laminar flow chamber as previously described.19 Two adhesive surfaces were examined in this study, human fibrinogen and collagen I. Time-resolved measurements were made over a 13-minute period at 30-second intervals in a 71,000-μm² area at a shear rate ranging between 20 and 25 sec⁻¹. Shear rates were calculated according to the methods described by Batchelor20 for Hele-Shaw cells. Computer-aided analysis of the data was performed on the series of developed photomicrographs that resulted from each experiment. We evaluated the following parameters for each discrete time period: 1) occupied sites, that is, the total number of sites occupied by platelets at the present time; 2) fresh adhesion sites, that is, the number of currently occupied sites that were not occupied during the immediately preceding time period; 3) new sites, that is, the number of currently occupied sites that had never been occupied until the present time; and 4) cumulative sites, giving a running total of the fresh adhesion sites. From these data we determined the adhesion rate, represented by the slope of the linear regression line of a plot relating cumulative sites versus time, and a reuse-of-sites index, which represents the slope of the least-squares linear regression line for a plot of the natural logarithm of the total number of sites occupied by platelets versus the different number of times such occupations occurred.

It should be stressed that this method of measuring platelet adhesion generally does not produce platelet thrombi. At times, a few of the platelets can be seen to adhere to each other, but even in such areas, the platelets remain in monolayers. The activating stimulus is clearly the contact of the platelet with the adhesive surface. The products released by the activated platelets are rapidly diluted by the large volume of the perfusate.

High shear rate adhesion. The system for the measurement of platelet adhesion under high shear rate conditions also uses a laminar flow chamber whose upper deck is coated with human fibrinogen but differs from the above in that whole blood is perfused at high shear rates of at least 760 sec⁻¹.

The platelets from 6 ml PRP (approximately 2.1×10⁸ platelets) were separated after acidification of the PRP with 15% ACD. After two washes with MES buffer, they were suspended in 1 ml of the same buffer and incubated with 40 μCi [¹¹¹In]oxine for 20 minutes at room temperature. The platelets were then washed twice and resuspended in 6 ml PPP from the same donor. After this [¹¹¹In]PRP was reconstituted with 4 ml packed red blood cells, the mixture was temperature equilibrated to 37°C and placed in a temperature-controlled syringe pump. The same Hele-Shaw laminar flow chamber used for low shear rate measurements of platelet adhesion was used with the reconstituted blood for 5 minutes at a speed of 16.5 ml/min. The effluent of the flow chamber was collected in a reservoir, which was used to resupply the syringe pump. The entire setup, except for the flow chamber, was temperature controlled (37°C). The run was terminated by flushing the flow chamber with 10 ml lactated Ringer's solution. The upper deck of the flow chamber containing the adhesive surface was then rinsed with a 1% sodium dodecyl sulfate solution. The entire volume of this solution was collected and its radioactivity measured. Completeness of removal of adherent platelets was ascertained by phase microscopy. Shear rates were calculated as described above.

Lipid Analyses

Washed suspensions of platelets were extracted with chloroform/methanol (1:2, vol/vol) containing 50 μg/ml butylated hydroxytoluene (BHT). To 1 volume cell extract were added 1:10 volume 0.2 M EDTA, 1 drop 88% formic acid, and 3.7 volumes chloroform/methanol. An additional 1.25 volumes 2 M KCl and 1.25 volumes chloroform with BHT were added. The lipid extract was recovered, and the remainder was reextracted with 1.5 volumes chloroform containing BHT. The combined extracts were concentrated under a stream of N₂ and redissolved in a small amount of chloroform/methanol. To this lipid extract was added 5 μg heptadecanoic acid as an internal standard. Fatty acids were then transesterified with boron trifluoride at 100°C for 90 minutes. The fatty acid methyl esters were extracted twice with petroleum ether. The extracts were combined, evaporated, and dissolveld in a small amount of methylene chloride containing BHT (5 mg/ml). Gas chromatography was performed in a Model 8500 Perkin-Elmer gas chromatograph (Norwalk, Conn.) using a capillary column SP-2230 (Supelco, Bellefonte, Pa.). The injector had a 1:10 split ratio. Bleed calibration of a blank run was automatically subtracted from the experimental run. After an initial isothermal period of 5 minutes at 85°C, the temperature was raised to 185°C at a heating rate of 20°C/min. This temperature was maintained for 20 minutes and then raised to 200°C again at 20°C/min, at which temperature it remained for an additional 10 minutes. Using this temperature program, we were able to elute all the fatty acid methyl esters within a 42-minute period. In case gas chromatographic analyses could not be performed immediately, the extracted fatty acid methyl esters were stored at −80°C. Most of the fatty acid methyl esters could be identified by comparing their retention times with those of authentic standards. However, certain fatty acid methyl esters were also identified by mass spectroscopic analyses, which were performed on a Finnegan mass spectrometer (San Jose, Calif.).
Li and Steiner  Fish Oil and Platelet Adhesiveness  41

FIGURE 1. Line plots of time-resolved evaluation (minutes) of platelet adhesion to fibrinogen (cumulative sites) at three different levels of fish oil supplementation (3, 6, and 9 g eicosapentaenoic acid [EPA]/day). Adhesion (shear rate, 25 sec\(^{-1}\)) was measured before (D0), during (D7 and D21), and after (D28 and D35) fish oil supplementation. Each point represents the mean of five normal individuals. For baseline and day 21, data±SEM are shown.

Statistical Evaluations

Each individual participating in this study served as his/her own control. Baseline values obtained in the week preceding the administration of fish oil represent controls, which are compared with the samples obtained in the course of the fish oil administration, as well as during a 2-week period after the termination of the fish oil regimen. Paired differences were evaluated by \(t\) tests using a statistical software program (Crunch Software Corp., Oakland, Calif.).

Materials

Human fibrinogen was purchased from Kabi Diagnostica, Uppsala, Sweden. Rat tail collagen type I was obtained from Serva Biochemicals, New York, N.Y. The purity of these two coating materials was tested as described before.\(^{14}\) There was no recognizable contamination of fibrinogen with fibronectin or laminin. Von Willebrand factor did not appear to be a contaminant of our protein. Boron trifluoride in 14% methanol was obtained from Analabs, Norwalk, Conn. The fish oil capsules were obtained from the National Oceanic and Atmospheric Administration, Charleston Laboratory, Charleston, S.C.

Results

Evaluation of Adhesion at Low Shear Rates

Platelet adhesion to fibrinogen showed a remarkable decrease in response to fish oil administration (Figure 1). The first sampling period, which was conducted on day 4 of the fish oil regimen, showed no statistical significance when compared with baseline values. Day 7, however, provided the first indication that fish oil induced a reduction of platelet adhesiveness. At an intake level of 3 g EPA/day, the inhibition of platelet adhesion was, on average, 38% at day 7 of the fish oil-supplementation period. At day 21 of the study, the nadir of adhesion was reached. At that time, the inhibition was 81.7%. After discontinuation of the fish oil supplementation, adhesion began to return toward normal, but even 2 weeks after cessation of the dietary supplementation, the cumulative adhesion was depressed compared with normal (38.4%). At 6 g EPA/day, the maximal inhibition of platelet adhesion was only slightly greater than at 3 g EPA/day (85.5%). It was interesting to note that the return toward normal adhesion values during the washout period progressed considerably faster after the 6 g EPA/day supplementation than after the 3 g EPA/day regimen. This observation received further confirmation by analyzing the data of the effect of 9 g EPA/day. The reduction of platelet adhesion was in the same range as that obtained with 3 and 6 g EPA/day. Nadirs of adhesion were obtained on day 21 of the dietary supplementation. A return toward normal values after cessation of the fish oil regimen was even faster after 9 g EPA/day than after 6 g EPA/day.

Similar results were obtained when collagen I-coated surfaces were studied in the flow chamber (Figure 2). Cumulative adhesion values were generally lower than those observed when fibrinogen was the adhesive surface, and there was slightly more individual variability of baseline adhesion values. The remarkable decrease in adhesion in response to fish oil administration was again clearly evident. Low points were observed at the end of the supplementation period (3 weeks). The faster return toward normal during the washout period at higher EPA supplementation levels, that is, 6 and 9 g/day, was again apparent. Maximal inhibition of collagen-induced adhesion was slightly less than that obtained when fibrinogen was the adhesive surface. There were no substantive differences of the inhibition produced by 3, 6, or 9 g EPA supplementation/day.

Evaluation of the reuse of adhesion sites by platelets provided an additional aspect of the adhesion
FIGURE 2. Line plots showing platelet adhesion (shear rate, 25 sec\(^{-1}\)) to collagen I (cumulative sites) evaluated at three different levels of fish oil supplementation (3, 6, and 9 g eicosapentaenoic acid [EPA]/day). Time-dependent increase (minutes) of total number of adhesion sites is shown at various sampling times before, during, and after fish oil supplementation. Each point represents the mean of five individuals. Baseline and day-21 data are shown as mean±SEM.

process. Fish oil administration produced a remarkable decrease in the number of times adhesion sites on the fibrinogen-coated surface were used by newly arriving platelets (Figure 3). Low points for this parameter were also reached after 21 days of fish oil administration. After termination, a gradual return toward normal was apparent. It appeared to be faster after 6 and 9 g EPA/day than after 3 g EPA/day. The same trend was noted when collagen I was the adhesive surface (Figure 4).

Adhesion Measured at High Shear Rates

Evaluation of platelet adhesion under high shear rate conditions and in the presence of red blood cells reconstituted to a hematocrit of 40% also exhibited a fish oil-induced reduction (Table 1). For these experiments, only two sampling points were compared with baseline values. The greatest inhibitory activity of fish oil was demonstrated after 3 weeks of fish oil administration. A supplementation level of 6 g EPA/day provided a clearly superior inhibition than that obtained by 3 or 9 g EPA/day. The reduction of platelet adhesion was somewhat less than that observed when measured under low shear rate conditions; however, at each of the three different supplementation levels, statistically significant differences were noted between baseline and fish oil-supplemented periods. The acceleration of the washout period with increasing levels of fish oil supplementation was again noted, even more strikingly than under low shear rate conditions.

FIGURE 3. Regression lines from analysis of reuse of adhesion sites of platelets adhering to fibrinogen-coated surfaces (natural logarithm [Ln] of number of sites). Adhesion measurements (shear rate, 25 sec\(^{-1}\)) were performed at low shear rate. Data are shown at three levels of fish oil intake (3, 6, and 9 g eicosapentaenoic acid [EPA]/day) and at various times during the supplementation period (days [D] 0–35). Least-squares linear regression lines were fitted to data points, which represent mean of five individuals studied.
Fatty Acid Distribution in Platelets

The distribution of total fatty acids extracted and esterified from platelets before, during, and after the fish oil supplementation period are shown in Tables 2-4. The n-3 polyunsaturated fatty acids, that is, C20:5 and C22:6, showed a dose-dependent increase that reached its maximum on day 21 of the study. In addition, C20:4, C18:2, and C18:0 showed significant reductions over the same time intervals. There was no evidence of an inverse relation between the level of supplementation and the return toward baseline values of C22:6 and C20:5.

Discussion

The beneficial effect of fish oil on platelet function has been thought to be due to the presence of n-3 polyunsaturated fatty acids. The high intake level of such fatty acids in certain population groups known to have a low incidence of thromboembolic disease has made a strong case for the causal association of n-3 polyunsaturated fatty acids and inhibition of events related to thrombosis.1-4 Aggregation has been extensively examined as a platelet function directly inhibited by n-3 polyunsaturated fatty acids. The inhibition, which is very modest at least in populations that consume a Western-type diet supplemented with fish or fish oil,5-9 has been thought to be related to a decrease in thromboxane A₂ and the generation of analogues (three series) of prostaglandins and thromboxanes that display varying functional alterations compared with their normal counterparts.12-14,23,24 We find it difficult to explain the difference between the modest efficacy of fish oil as a platelet antiaggregating agent and the potent reduction in thrombosis in Eskimos who habitually consume large amounts of foods rich in n-3 polyunsaturated fatty acids. To resolve this discrepancy, one could postulate that n-3 polyunsaturated fatty acids affect platelet adhesion more potently than aggregation.

We17 have shown in previous studies that fish oil supplementation of the diet strongly reduces platelet adhesiveness when tested in a laminar flow chamber. In those studies, platelet adhesion was measured at low shear rates, and only a single supplementation level of fish oil (6 g EPA/day) was

Table 1. Effect of Fish Oil Supplementation on Platelet Adhesion

<table>
<thead>
<tr>
<th>Supplementation</th>
<th>Day 0</th>
<th>Day 21</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 g EPA/day</td>
<td>100</td>
<td>55.6±8.9*</td>
<td>67.2±19.5†</td>
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<tr>
<td>6 g EPA/day</td>
<td>100</td>
<td>35.2±5.3†</td>
<td>77.8±11.4</td>
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<td>9 g EPA/day</td>
<td>100</td>
<td>50.6±8.1*</td>
<td>99.2±13</td>
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</table>

EPA, eicosapentaenoic acid.
Adhesion of 111In-labeled platelets reconstituted with red blood cells to fibrinogen-coated surfaces under high shear rate conditions. Data are presented at three different time points during the study, that is, before, during, and after fish oil supplementation at three different dosage levels. One hundred percent adhesion corresponds to 1.7×10⁹ (±0.14×10⁹) platelets/4.7×1.4-cm adhesive surface. For further details, refer to "Methods." Mean±SD are given for groups of five individuals each.

Paired t-tests: *p<0.005, †p<0.025, ‡p<0.05.
TABLE 2. Total Fatty Acid Distribution of Platelets Before and After Fish Oil Administration (3 g Eicosapentaenoic Acid/Day)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Baseline</th>
<th>Day 7</th>
<th>Day 21</th>
<th>Day 28</th>
<th>Day 35</th>
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<tr>
<td>C16:0</td>
<td>20.6±0.9</td>
<td>20.8±0.3</td>
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<td>21.8±1.7</td>
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<td>C16:1</td>
<td>1.4±0.4</td>
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<td>18.5±0.7</td>
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<td>18.5±0.9</td>
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<td>17.0±0.7</td>
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<td>17.2±0.3</td>
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<td>8.9±0.7</td>
<td>7.2±0.6</td>
<td>5.5±0.2</td>
<td>6.9±1.1</td>
<td>8.7±0.4</td>
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<td>C18:3</td>
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<td>1.9±0.2</td>
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<td>2.6±0.4</td>
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*Mean±SD of five individuals.  
\[ p<0.05, \| p<0.025, \| p<0.01, \| p<0.005, \| p<0.0001.  

studied. Although studies by Davies and colleagues have established that many of the thrombotic events that eventually result in myocardial infarction begin in fissures of the wall of coronary arteries, places where the flow is almost stagnant and shear rates are thus very low, most investigators are persuaded by the anatomic evidence that shows that arterial thromboses occur primarily in vessels with high shear rate flow conditions. Therefore, adhesions were made in this study under conditions of both high and low shear rates. In addition, the composition of the perfusate was varied, PRP for low shear rate measurements and whole blood for high shear rate measurements. Use of PRP allowed us to perform time-resolved measurements, which provided important clues to the effect of fish oil on platelet adhesiveness. Aided by computer-assisted fixation of the precise locations of each individual adhesion site, it is possible to obtain a "history" of such sites.

TABLE 3. Total Fatty Acid Distribution of Platelets Before and After Fish Oil Administration (6 g Eicosapentaenoic Acid/Day)

<table>
<thead>
<tr>
<th>Fatty acid</th>
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<th>Day 28</th>
<th>Day 35</th>
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*Mean±SD of five individuals.  
\[ p<0.05, \| p<0.025, \| p<0.01, \| p<0.005, \| p<0.0001.  

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especially when platelet adhesion is evaluated under high shear rate conditions, but there are no platelet thrombi detected when measured at either of the two shear rate conditions. As previously noted, platelet adhesion shows considerable interindividual variability, but in the same individual when tested over long periods of time, it remains remarkably stable. The thrombi detected when measured at either of the two study days can be partially explained by this interindividual variability and by the paucity of male subjects (only one male volunteer in the group receiving 6 g EPA/day and none in the group receiving 9 g EPA/day) in these two study groups.

Our studies showed that fish oil supplementation is an effective means of reducing platelet adhesiveness. Whether measured at high or low shear rates, the dose-response effect of increasing levels of fish oil revealed a maximal reduction of platelet adhesiveness at an intake level of about 6 g EPA/day. It was interesting to note that higher dietary supplementation levels of fish oil, that is, 9 g EPA/day, provided no further inhibition of platelet adhesion above that obtained with 6 g EPA/day. In fact, total platelet adhesion was almost maximally inhibited at 3 g EPA/day. This suggests that a saturable mechanism is responsible for the fish oil-induced inhibition of platelet adhesion. From a practical standpoint, a fish oil supplementation providing 6 g EPA/day can easily be envisioned to lead to decreased platelet adhesiveness and to a reduction in the residues left behind by platelets that become dislodged from the adhesive surface.

We have previously demonstrated that the effect of fish oil is due to the presence of n-3 polyunsaturated fatty acids. A vegetable oil mixture administered in the same concentration as that of fish oil and of a similar distribution of saturated, monounsaturated, and polyunsaturated fatty acids did not inhibit platelet adhesiveness. Adhesion to fibrinogen and collagen I tested during the vegetable oil-supplementation period remained in the same range as the presupplementation (baseline) values. The mechanism by which n-3 polyunsaturated fatty acids are able to produce the altered pseudopodia forma-
tion has not yet been elucidated. We know, however, that fish oil–induced changes in eicosanoid products cannot be responsible for the reduced adhesiveness, as the platelet shape change occurs within fractions of a second after agonist stimulation, long before any thromboxanes are formed.

Fish oil–induced effects on platelet function are known to have a delayed appearance and a prolonged washout period. Our studies demonstrate this effect. It was, however, quite unexpected when we discovered that the washout period of the inhibitory effect of fish oil on platelet adhesiveness was inversely related to the level of fish oil supplementation. Not only cumulative adhesion but also the revisitation of previously vacated adhesion sites showed this effect.

Fish oil proved to be a dietary supplement that was well tolerated even at the high doses administered in this study. Except for the inconvenience of ingesting a large number of capsules, the side effects were primarily related to minor gastrointestinal discomfort at the highest dose level of fish oil corresponding to 9 g EPA/day. Although a systematic examination of bleeding times was not performed in this investigation, a few individuals with high fish oil supplementation were tested and found to have a 2–3-minute prolongation of their presupplementation bleeding times. However, in none of the volunteers was the bleeding time longer than 8 minutes.

We believe that our studies give convincing evidence of the antiadhesive properties of fish oil supplementation of the diet. We are especially encouraged by the fact that very effective inhibitions of platelet adhesion can be achieved with “reasonable” doses of fish oil. That adhesion is effectively reduced when measured both at high and low shear rates gives encouragement to the belief that fish oil could be an effective antithrombotic regimen when supplementing the Western-type diet of individuals.

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