Regular Article
Insights into the inhibition of platelet activation by omega-3 polyunsaturated fatty acids: Beyond aspirin and clopidogrel

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Objective: We sought to examine the effects of escalating doses of omega-3 polyunsaturated fatty acid (PUFA) supplements on platelet function using light transmission aggregometry (LTA) and electrophoretic quasi-elastic light scattering technology (EQELS).

Background: PUFA may inhibit platelet function through fatty acid substitution in the platelet membrane by changing the surface charge density and causing decreased production of thromboxane A2. EQELS can measure platelet surface charge density and determine whether the platelet is in resting or activated state.

Methods: A total of 30 volunteers were divided in 3 groups of 10 as follows: Group A, no antiplatelet agent; Group B, daily aspirin only, and Group C, daily aspirin and clopidogrel. All patients received escalating doses of omega-3 PUFA from 1 to 8 g daily over 24 weeks. Platelet function was measured by template bleeding time, LTA, and EQELS at baseline and at 6, 12, 18 and 24 weeks.

Results: Mean bleeding time increased in a dose-dependent manner with escalating omega-3 PUFA doses. LTA confirmed expected antiplatelet effects of aspirin and clopidogrel, but did not detect any additional antiplatelet effects of omega-3 PUFA. EQELS showed a significant increase in the negative resting platelet charge compared to baseline and an attenuated response to arachidonic acid mediated platelet activation. No bleeding events were observed.

Conclusions: In this pilot study we were able to successfully measure platelet surface charge variation as a measure of omega-3 PUFA effect on platelets. Our results suggest that omega-3 PUFA increase the total amount of omega-3 PUFA from 1 to 8 g daily over 24 weeks. Platelet function was measured by template bleeding time,

Introduction

The cardiovascular benefit of diets rich in polyunsaturated fatty acids (PUFA) has been the focus of interest for several decades. Diets with high content of omega-3 fatty acids have been associated with a lower rate of cardiovascular events [1–3]. The lower incidence of myocardial infarction reported in individuals on these diets is thought to be, in part, derived from polyunsaturated fatty acids’ effect on platelet function. The exact mechanism of platelet inhibition is not entirely understood, but factors that affect signal transduction and thromboxane metabolism have been implicated [4]. Prolonged bleeding times have been observed in Eskimos who ingest diets high in polyunsaturated fatty acids and this observation ultimately led to the identification of polyunsaturated fatty acids as the cause of their mild platelet dysfunction [5,6]. Multiple studies have demonstrated that adding cis-polyunsaturated fatty acids to isolated platelets decreases activation possibly through an effect on platelet surface charge; this may be due to increased cAMP levels, decreased cytosolic calcium concentrations, or via the promotion of lipid raft formation or alterations in membrane fluidity [7–14]. The augmentation of negative platelet surface charge induced by fatty acids is speculated to affect activation.

Under normal circumstances, human platelets circulate in a resting state and carry a negative surface charge. Upon activation, the physical character of the platelet membrane undergoes drastic modification that includes rearrangement of the cytoskeleton to

Abbreviations: PUFA, Polyunsaturated Fatty Acid; EQELS, Electrophoretic quasi-elastic light scattering; LTA, Light transmission aggregometry; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; INR, International normalized ratio; PRP, Platelet rich plasma; PPP, Platelet poor plasma; ADP, Adenosine diphosphate; AA, Arachidonic acid.
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develop pseudopods, activation of flipase and scramblase, movement of phosphatidylserine and phosphatidylethanolamine from the inner to the outer membrane surface [15,16], activation of numerous surface molecules such as integrin α₃β₁, an increase in cytosolic and surface calcium concentration, and ultimate development of a positive surface charge [9,10,17].

We conducted a pilot study to assess the effects of escalating doses of an FDA-approved formulation containing a combination of DHA and EPA (Lovaza®, Glaxo Smith Kline, Research Triangle Park, NC) on platelet function and bleeding risk using multiple methods to assess platelet function including LTA and bleeding time. In addition, we evaluated EQELS, a novel platelet assay that can detect platelet surface charge density and determine whether the platelet is in a resting or activated state.

**Methods**

**Patients and Study Design**

A total of 30 volunteers who were healthy (Group A) or had a stable cardiovascular condition necessitating treatment with aspirin (Group B) or dual antiplatelet therapy with aspirin plus clopidogrel (Group C) were asked to participate in the study. Relevant exclusion criteria were known chronic liver disease or liver transaminase levels greater than the upper limit of normal, renal insufficiency with a calculated creatinine clearance <60 mL/min or serum creatinine >2 mg/dL, recent bleeding episodes, thrombocytosis (>600,000 /mm³) or thrombocytopenia (<100,000 /mm³), history of anemia, or baseline hemoglobin <11.0 g/dL, bleeding diathesis, stroke in the past 12 months, recent gastrointestinal or genitourinary bleeding within 3 months, INR >1.3, and previous treatment with an intravenous platelet inhibitor (epti- or abciximab) within the past 30 days. Patients taking over-the-counter nutritional supplements of fish or flaxseed oil were allowed to participate after a 2-week wash-out period. The study was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill and registered with ClinicalTrials.gov under the identifier NCT00515541. All patients signed an informed consent before participation. The study was conducted at the Clinical and Translational Research Center of the University of North Carolina at Chapel Hill in four sequential groups of 10 patients. Subjects in group A were not on antiplatelet therapies, subjects in group B were on aspirin alone at a dose ≤325 mg daily and subjects in group C were on aspirin at a dose ≤325 mg and clopidogrel 75 mg daily. After baseline assessments, all patients received escalating doses of Lovaza® of 1 g, 2 g, 4 g and 8 g daily in consecutive periods of 6-weeks. Thus, the study duration was 24 weeks for each patient. In addition, each patient received a clinical evaluation at the end of every escalation period, which consisted of a bleeding questionnaire, physical examination, and laboratory studies, including a complete blood count, liver enzymes, bleeding time, and partial thromboplastin time. Study treatment for the subsequent 6-week period was dispensed at each visit.

**Study Assessments**

Samples of 40 mL of blood for platelet function assessments were drawn (without use of a tourniquet) at baseline, at each study visit before dose escalation, and at the end of the study. All samples were collected into a plastic syringe containing 0.11 M sodium citrate (1:9, citrate:blood, vol:vol) at room temperature, and processed within 3 hours.

**Study Endpoints**

The primary endpoints of the study were the effect of a change in platelet membrane surface charge density and its effect on platelet activation as assessed by EQELS; change in platelet aggregation, as assessed by LTA; and change in bleeding time.

**PRP Preparation**

Blood was centrifuged using a BioData PDQ centrifuge at 2220 g for 30 sec at room temperature to remove the red and white cells. The top layer containing the PRP was carefully removed and transferred into labeled polypyrrolyene tubes and capped. Platelet count was measured on a Coulter counter (Beckman Coulter, Act 10, Hialeah, FL) and the samples were allowed to rest for at least 30 minutes prior to further analysis.

**PPP Preparation**

In order to adjust the platelet counts of the PRP in the light transmission platelet aggregation studies, platelet poor plasma (PPP) was obtained by centrifugation as above at 8880 g for 120 sec at room temperature. Following centrifugation, the PPP was carefully transferred into labeled polypyrrolyene tubes and the tubes were capped.

**LTA**

The PAP-8E LTA (BioData, Corp., Horsham, PA) measurements were made in duplicate. Agonists included AA, ADP, collagen, and epinephrine (BioData, Corp., Horsham, PA). The instrument was blanked (100% baseline) using a siliconized tube containing 0.225 mL patient PPP. A siliconized tube containing 0.225 mL of patient PRP with a standard magnetic stir bar was then placed in a mixing channel for 2 minutes at 37 °C. After 2 minutes, the tube was removed and placed into the test-blanked channel. Testing was initiated by addition of 0.025 mL agonist and evaluated over 10 minutes. A final percentage aggregation less than 60% compared to baseline was considered to show inhibition.

**Bleeding Time**

Forearm bleeding times were determined using a standard Surgicutt device (ITC, Edison, NJ). A manual blood pressure cuff was placed 2 inches above the antecubital fossa and inflated to 40 mmHg. Using the Surgicutt device, a small incision was made and a stopwatch started. The incision edge was blotted at 30 second intervals with standard filter paper until bleeding stopped. Time to hemostasis was noted.

**EQELS**

Measurements were made using a modified device (EQELS, Invitrox, Inc., RTP, NC) to specifications of constant current, high electric field and a scattering angle of 30 degrees. EQELS provides a sensitive assessment of subtle changes in the cell surface that occurs with activation, ligand binding or apoptosis. These changes are a result of different distributions of charged groups that define a surface charge finger print for the current state of activation of the cell. Resting state platelets have a negative surface charge; fully activated platelets have a positive surface charge (typically >0.5 mobility units, micron-cm/volt-second). By monitoring membrane changes, the degree of platelet activation can be determined. Using our buffer conditions, a fully activated human platelet has an electrophoretic mobility greater than +0.5 mobility units at 10 minutes after the addition of an agonist. An electrophoretic mobility greater than −0.1 mobility units, but less than +0.5 mobility units indicates intermediate activation. EQELS buffer consisted of 5 mM NaCl, 2 mM HEPES (pH 7.4), 280 mM Sucrose.[18,19] Each sample was comprised of 200uL PRP added to a final volume of 2.8 mL EQELS buffer with or without platelet agonist. Resting platelets (no agonist) and two
different concentrations of each agonist were measured at 37 °C for each sample. Agonists tested included AA and ADP.

Statistical Analysis

Categorical variables are presented as frequencies with the respective percentages. Continuous measures are presented as medians with 25th and 75th percentiles. For observations recorded before and after treatment in same individuals, the Wilcoxon signed-rank test was used to compare continuous variables and the McNemar’s test for categorical variables. For observations recorded in different individuals, Wilcoxon rank-sum test was used to compare continuous variables and the Chi square test (or Fisher’s exact test if model assumptions were not met) for categorical variables. As this is a hypothesis-generating pilot study (as opposed to a strict hypothesis-testing study), an unadjusted, nominal p level of less than 0.05 was considered statistically significant. To investigate interactions of trends in outcome variables over time by treatment group, nonparametric Kruskal-Wallis tests were performed. Statistical analysis was performed in Stata version 11 (www.stata.org).

Results

Study Patients

A total of 30 subjects were enrolled in the study between 9/24/2007 and 10/13/2008. The median age of group A subjects was 43 years old, group B was 51 years old and group C 54 years. There were 3 females in group A, 4 in group B and 5 in group C. There were 2 African American subjects in groups B and C and none in group A. Diabetes was present in 3 subjects in group C. All patients in group C, and none in groups A and B, had known coronary artery disease. No subjects withdrew from group A (no antplatelet therapy). Two subjects withdrew from group B (aspirin alone) when they reached an omega-3 PUFA dose of 8 g daily. One subject withdrew because of nausea and one withdrew because of a finger injury. No subjects withdrew from group C.

Bleeding Time Assessment

There was a trend towards increased bleeding times among patients taking omega-3 PUFA compared to baseline measurements among groups A-C (Table 1). Among group A participants, the difference did not reach statistical significance (150 sec at baseline vs. 240 sec at week 12, p = 0.14). The effect of omega-3 PUFA on bleeding time was more apparent among group B participants, (240 sec at baseline vs. 390 sec at week 12, p = 0.01). Omega-3 PUFA had very little effect on bleeding time among group C patients who already had significantly prolonged values due to the combined antplatelet effects of aspirin and clopidogrel. Among patients in groups A-C, there was a significant increase in mean bleeding time at 6 and 12 weeks compared to baseline (Table 2), however this effect did not persist at higher omega-3 PUFA doses. In fact, there was a slight decrease in bleeding time at weeks 18 and week 24 compared to week 12. This trend was most apparent for group C, suggesting no additional effect of omega-3 PUFA on bleeding time was not beyond 2 g daily.

LTA

In general, LTA results confirmed the expected antplatelet effects of aspirin and clopidogrel (Table 3). As expected, patients in group A had the highest levels of platelet aggregation. Platelet function inhibition using AA agonist suggested aspirin effects in groups B and C; aggregation in response to ADP was lowest in Group C due to aspirin and clopidogrel treatment. The results of a trend test assessing changes in aggregation within each group with escalating doses of omega-3 PUFA were not statistically significant.

Table 1

<table>
<thead>
<tr>
<th>Week</th>
<th>All patients</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Baseline)</td>
<td>255 (180, 390)</td>
<td>150 (120, 180)</td>
<td>240 (210, 330)</td>
<td>570 (440, 720)</td>
</tr>
<tr>
<td>6 (1 gram daily)</td>
<td>330 (240, 480)</td>
<td>240 (180, 300)</td>
<td>345 (240, 450)</td>
<td>720 (450, 720)</td>
</tr>
<tr>
<td>12 (2 grams daily)</td>
<td>330 (240, 480)</td>
<td>240 (180, 300)</td>
<td>390 (240, 450)</td>
<td>960 (450, 720)</td>
</tr>
<tr>
<td>18 (4 grams daily)</td>
<td>300 (240, 450)</td>
<td>255 (180, 270)</td>
<td>330 (330, 450)</td>
<td>600 (540, 720)</td>
</tr>
<tr>
<td>24 (8 grams daily)</td>
<td>300 (240, 450)</td>
<td>255 (180, 270)</td>
<td>300 (300, 420)</td>
<td>600 (360, 780)</td>
</tr>
</tbody>
</table>

All values are medians and 25th, 75th percentiles and expressed in seconds. *Kruskal-Wallis tests indicate a significant change in bleeding response rates across weeks (p < 0.05 compared to baseline).

Table 2

<table>
<thead>
<tr>
<th>Week</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>All patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Baseline)</td>
<td>79.5 (36, 100)</td>
<td>0 (0, 42)</td>
<td>5.5 (2.10)</td>
<td>6 (0, 81)</td>
</tr>
<tr>
<td>6 (1 gram daily)</td>
<td>79.5 (73, 100)</td>
<td>3 (0, 81)</td>
<td>6.5 (2.15)</td>
<td>13 (2, 74)</td>
</tr>
<tr>
<td>12 (2 grams daily)</td>
<td>87.5 (35, 93)</td>
<td>2.5 (0, 7)</td>
<td>6 (3.10)</td>
<td>7.5 (1, 86)</td>
</tr>
<tr>
<td>18 (4 grams daily)</td>
<td>82 (47, 82)</td>
<td>0 (0, 2)</td>
<td>4 (2.8)</td>
<td>6 (2.47)</td>
</tr>
<tr>
<td>24 (8 grams daily)</td>
<td>83 (80, 85)</td>
<td>3 (0, 85)</td>
<td>5 (1.14)</td>
<td>15.5 (1.5, 83.5)</td>
</tr>
</tbody>
</table>

All values are medians and 25th, 75th percentiles. Trend tests assessing for changes in aggregation by week in all patients and within each group were all not statistically significant.

greater than +0.5 mobility units indicating full activation (data not shown).

**Effect of omega-3 PUFA on ADP platelet activation**

As expected, no platelet inhibition was observed (Table 3) when low-dose ADP was used as agonist in subjects not on clopidogrel therapy (Groups A and B). In group C, there appeared to be a synergistic effect between clopidogrel and Omega-3 PUFA at 8 grams daily when low-dose ADP was used as the agonist. This was suggested by a decreased EQELS value in week 24 compared to baseline. Therefore, our results suggest that platelet inhibition is enhanced at higher doses of omega-3 PUFA in subjects taking clopidogrel.

**Discussion**

Our study results showed that treatment with omega-3 PUFA is associated with an increase in the negative platelet surface charge as assessed with the novel EQELS method. The increased negative charge of the platelet surface diminishes the response of platelets to known agonists. However, doses greater than 1 g/day appeared to have a similar effect in inhibiting platelet aggregation.

In our experiment, we observed that the inhibition of platelet activation by omega-3 PUFA may impact platelet function. The effects of AA on platelet surface charge in our experiment are provocative. AA is a fatty acid and also incorporates into the platelet outer membrane. Thus, as the concentration of AA increases, the platelet surface charge density increases until a critical concentration is reached causing platelet activation. When this occurs, the platelet surface charge density becomes positive possibly due to calcium release, membrane rearrangement, activation of other surface molecules and other events. EQELS is able to reliably detect these subtle changes in the electrokinetics in the platelet surface charge. As seen in our study, increasing omega-3 PUFA dose increased the negativity of the baseline platelet surface charge. Regardles of its molecular form, the membrane content of DHA has been implicated in the modulation of molecules involved in signal transduction. Examples include G-coupled signaling pathway, protein kinase C, phospholipase C, and lecithin:cholesterol acyltransferase [24–27]. The change in surface charge has been linked to regulation of some membrane ion channels [28].

Our results showed an increase in the negative surface charge density of platelets in response to escalating doses of omega-3 PUFA. At doses of AA that usually activate platelets, the platelet surface charge remained negative indicating a resting state. These data suggest that the increase in the negativity of the platelet surface charge creates an additional barrier that must be overcome before platelets can become fully activated. The exact dose that provides this antiplatelet effect could not be determined from this pilot study, however doses greater than 1 g/day appeared to have a similar effect on platelet function, suggesting that relatively low doses of omega-3 PUFA may impact platelet function.

Table 3

Median electrophoretic motilities of resting platelets with escalating doses of Omega-3 PUFA in mobility units ([micron-cm]/[volt-sec]) by Group and Omega-3 PUFA dose.

<table>
<thead>
<tr>
<th>Week</th>
<th>All patients</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Baseline)</td>
<td>-0.72 (−1.26, 0.14)</td>
<td>-0.24 (−0.60, 0.69)</td>
<td>-0.32 (−0.93, 0.14)</td>
<td>-1.23 (−1.49, −0.85)</td>
</tr>
<tr>
<td>6 (1 gram daily)</td>
<td>-1.33 (−1.45, −1.15)*</td>
<td>-1.11 (−1.27, −0.92)*</td>
<td>-1.38 (−1.44, −1.26)*</td>
<td>-1.42 (−1.54, −1.24)</td>
</tr>
<tr>
<td>12 (2 grams daily)</td>
<td>-1.47 (−1.63, −1.36)*</td>
<td>-1.51 (−1.61, −1.38)*</td>
<td>-1.55 (−1.63, −1.48)*</td>
<td>-1.41 (−1.42, −1.30)</td>
</tr>
<tr>
<td>18 (4 grams daily)</td>
<td>-1.46 (−1.55, −1.26)*</td>
<td>-1.50 (−1.57, −1.41)*</td>
<td>-1.51 (−1.57, −1.39)*</td>
<td>-1.23 (−1.41, −1.13)</td>
</tr>
<tr>
<td>24 (8 grams daily)</td>
<td>-1.44 (−1.56, −1.25)*</td>
<td>-1.54 (−1.56, −1.43)*</td>
<td>-1.36 (−1.45, −1.22)*</td>
<td>-1.40 (−1.54, −1.26)</td>
</tr>
</tbody>
</table>

All values are medians and 25th, 75th percentiles. *p<0.05 compared to baseline (week 0).

charge becomes positive indicating full activation. Therefore, it is possible that the use of lower concentrations of an agonist can uncover subtle inhibition to platelet activation when coupled to a sensitive monitoring device such as EQELS.

A second mechanism of platelet inhibition by omega-3 PUFA derives from their metabolic end products. As part of the platelet activation process, phospholipase A2 clips the fatty acid at the sn2 position of the inner membrane phospholipids. In the case of the platelet, the fatty acid is then metabolized through COX-1 eventually to a thromboxane [31–34]. In most Western diets, AA is the fatty acid usually metabolized to thromboxane A2, a potent platelet activator and vasoconstrictor [35]. In contrast, omega-3 PUFA are metabolized to thromboxane A3, a poor platelet agonist and vasoconstrictor [31–34]. When omega-3 PUFA are ingested in sufficient doses, AA may be replaced by an omega-3 PUFA. Omega n3 fatty acids, and specifically DHA, are reported to have a preference for incorporation into the sn2 position of phosphatidylethanolamine, a component of the inner leaflet of resting cell membranes [36]. Thus, production of thromboxane A3 from omega-3 PUFA underlies the second potential platelet inhibition effect of omega-3 PUFA.

LTA is not a sensitive method to detect subtle changes in platelet function. We could not detect the effect of omega-3 PUFA in group A using LTA. It is plausible that the agonist concentration could be optimized at lower levels to better demonstrate an inhibitory effect of omega-3 PUFA. LTA did show additional (and expected) platelet inhibition with aspirin when WA was used as agonist. A decrease in maximal platelet aggregation was also observed in group C subjects (aspirin and clopidogrel) when an ADP agonist was utilized (Table 2). Despite mild platelet inhibition, no bleeding or bruising was reported in our study. Goodnight et al. noticed a similar effect in a trial testing a diet rich in salmon and salmon oil in normal subjects [3]. Watson et al. observed no increase in bleeding risk with the addition of omega-3 PUFA to patients being treated with both aspirin and clopidogrel [37]. In addition to mild and transient platelet count reduction, Watson et al. also showed a mild reduction in platelet activation in response to ADP. In our study, LTA in response to ADP agonist did not suggest increased platelet inhibition with increasing omega-3 PUFA doses. However, we used a higher concentration of ADP than Goodnight, which could explain the discrepancy [3]. We did show a possible synergistic effect of clopidogrel and omega-3 PUFA when EQELS was used to monitor platelet activation.

Lev et al. showed that omega-3 PUFA could overcome aspirin resistance [38]. In our study, we showed that omega-3 PUFA, in the presence of clopidogrel, decreased the positive charge of platelets activated with low-dose ADP. In fact, at 8 grams daily of omega-3 PUFA the platelet surface charge remained negative despite the addition of low-dose ADP. However, because of the small sample size and the pilot nature of our study, further studies are needed to clarify these effects and to determine whether the EQELS measures observed at high doses of omega-3 PUFA were suggestive of a dose-response relationship, and whether the antiplatelet effects of omega-3 PUFA are synergistic with that of thienopyridines.

The main limitation of this study is low statistical power due to small sample size. However, the primary objective was to assess trends of the effect of omega-3 PUFA on platelet function in a small pilot study. Additionally, even though we performed careful drug accountability at each visit, we cannot exclude the possibility that some subjects were not fully compliant. Although the techniques involved in LTA and EQELS measurement have been established, the ideal dose of agonist used to assess response to aspirin and clopidogrel is not standardized. Greater sensitivity in platelet aggregation and EQELS studies may have been possible if lower agonist doses had been used. Because the results of platelet surface charge characteristics have not been correlated with clinical outcomes, any clinically significant platelet effect should not be inferred based on results presented here. Rather, this study generates the hypothesis that a reduction in resting platelet surface charge may attenuate the activation of platelets in clinical scenarios in which sustained and effective platelet inhibition are needed.

In conclusion, the effects of omega-3 PUFA on platelet membrane structure and surface charge and the differences in the efficacy of their metabolic products can account, at least in part, for the mild platelet inhibition seen in patients who ingest omega-3 PUFA. Reduction in platelet activation may provide a clinically beneficial response to patients at high risk for thrombosis, but additional clinical trials adequately powered to show statistical benefit are required. Although not powered to examine bleeding outcomes, there was no evidence of major or minor bleeding among patients taking omega-3 PUFA with antiplatelet agents or warfarin. Finally, EQELS was shown to be a sensitive means to monitor subtle changes in platelet function. Further studies are warranted to examine the clinical relevance of these measured changes.

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Conflict of interest statement

Dr. Gabriel is a cofounder of Invitrox, Inc.

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