Design criteria: tests used to assess platelet function\textsuperscript{1,2}

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\textbf{ABSTRACT} \hspace{1em} A brief overview of platelet function tests used in the past to assess relations between dietary fatty acids and thrombogenicity reveals problems that need to be recognized and addressed before planning future studies. Implementation of new strategies that integrate new technologies with measures of two or more markers of platelet activity may be more successful in predicting the thrombotic potential of dietary fatty acids. \textit{Am J Clin Nutr} 1997;65(suppl):1665S–8S.

\textbf{KEY WORDS} \hspace{1em} Platelets, aggregation, thrombosis, fatty acids, diagnostic test, bleeding time, platelet aggregation, optical method

\textbf{INTRODUCTION} \hspace{1em} Investigators can select from an array of functional tests for platelet activity when designing studies to assess the thrombogenicity of dietary fatty acids. Unfortunately, use of these tests in experiments with both human subjects and in animal models has failed to provide definitive answers about the important question of thrombosis and fatty acids. Careful assessment of the tests, using both current information on platelet physiology and advances in technology, is needed to produce the desired result—relevant, reliable data.

\textbf{HUMAN STUDIES} \hspace{1em} In 1962 the optical method of testing the ability of platelets to aggregate was introduced (1, 2). Since then, this has been the test most often used by investigators to monitor platelet status in both epidemiologic studies and clinical trials designed to study the influences of dietary fatty acids on thrombogenicity (3–14). Meade et al (3), describing platelet aggregability in participants of the Northwick Park Heart Study, acknowledged that the optical method was selected to characterize the platelet responses in their population because of the established precedent for its use as a marker of hyperactivity.

The application of this technique is based on the premise that increased in vitro platelet aggregation indicates an increased tendency for thrombogenesis in vivo. However, the responsiveness of platelets measured in vitro depends on many factors that produce paradoxical and artificial results, making interpretations difficult. These conflicting results have contributed greatly to the confusion and controversy that exists about which dietary fatty acids promote and which inhibit a thrombotic tendency in healthy humans (15).

Studies of platelet reactivity in patients with coronary and thrombotic cerebrovascular diseases have been more successful in demonstrating the concept of in vivo and in vitro hyperresponsiveness of these cells. Application of a noninvasive technique that uses gas chromatography–mass spectroscopy to analyze urine for platelet arachidonic acid (20:4) metabolites has been instrumental in assessing in vivo activation. Knapp et al (16) used this method to show that excretion of thromboxane metabolites, indicative of in vivo platelet activation, was increased in patients with atherosclerosis. They also showed that supplementation with \(n\)-3 polyunsaturated fatty acids (PUFAs) decreased the excretion of urinary thromboxane metabolites both in patients and healthy control subjects.

Other tests, such as assays for \(\beta\)-thromboglobulin and platelet factor 4, the platelet-count ratio method, and measurement of bleeding times, have also shown that platelets are activated in subjects with cardiovascular and cerebrovascular diseases (17–20). Study designs for prospective and intervention trials have rarely used combinations of noninvasive and invasive tests to assess the effects of dietary fatty acids, but when they have, the results have been inconclusive (4, 8, 10).

An additional complicating factor in studies with healthy subjects is that in vivo platelet activation should be low and difficult to assess in these subjects even though diets are well controlled. Thromboxane metabolite excretion remained unchanged when subjects consumed diets in which linoleic acid (18:2) or palmitic acid (16:0) and stearic acid (18:0) intakes were varied to assess the effects of these individual fatty acids on thrombotic potential (21–23).

\textbf{Bleeding times} \hspace{1em} Bleeding times have been used to assess in vivo platelet function, especially in clinical trials designed to investigate the antithrombotic potential of \(n\)-3 PUFAs compared with \(n\)-6 PUFAs (4, 10, 13). Increases in bleeding time were observed in some but not all studies using this measurement as a biomarker.

Platelet counts have been used as an indicator of effects of diet on homeostatic responses controlling platelet turnover. Ingestion of \(n\)-3 fatty acids resulted in decreased platelet counts in some but not all intervention trials. This observed variability may be a consequence of study design and amount and type of supplement used. However, the decreases may be transient, for example, platelet counts for participants in trials

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lasting 9 mo and 7 y, which used n−3 supplements, were not lower than pre-study values (9, 24).

Platelet aggregation

Platelet aggregation in platelet-rich plasma was determined in both prospective and intervention trials in attempts to study relations among dietary fatty acids and risk for thrombosis (4, 10, 13). Collagen, ADP, thrombin, and epinephrine are the agonists used most frequently by investigators to test the in vitro responses of platelets. Because of inherent problems associated with this method and with study designs, it has not been possible to correlate increases and decreases in platelet responsiveness to specific dietary fatty acids. However, data from these investigations tend to support the hypotheses that saturated fatty acids (SFAs) are thrombogenic and that PUFAs, especially those from the n−3 family, are antithrombogenic (5, 8).

Problems that contribute to interpretation of results from intervention trials are related to type and amount of test fatty acid fed, background diet, control diet and group, age, sex, length of study, and test sample collection and preparation (25). A complex network linking receptors and intracellular signaling processes controls platelet responses. The functional status of this network can easily be affected by both in vivo and in vitro perturbations. These perturbations can be caused by injuries to vessel walls or by procedures such as phlebotomy and preparation of samples for in vitro testing.

Platelets are easily activated by many post-sampling manipulations, such as exposure to 4°C. Cold temperatures stimulate quiescent disc-like platelets to change shape to more spherical, activated forms (26). Depending on time of exposure, such treatment can produce a refractoriness to subsequent stimuli agents. Testing of such previously activated platelets can lead to paradoxical conclusions about interactions of dietary fatty acids with platelet function. For example, in a study in which subjects were fed diets high in milk fat or in sunflower or rapeseed oils, the investigators noted, contrary to expectations, that platelet aggregation was decreased after consumption of the SFA-rich diet compared with the PUFA-rich diets (12). In this study, however, samples of platelet-rich plasma were chilled before being tested for aggregability. A greater cold-induced activation may have produced a more refractory state in the platelets collected after ingestion of the SFA diet compared with platelets collected after ingestion of the two PUFA-rich diets.

Impedance method

To permit the study of platelet function under more physiologic conditions, Cardinal and Flower (27) introduced a new technique for measuring platelet aggregation in whole blood. This method determines the impedance changes that occur when platelets adhere to an electrode. Although the advantages and disadvantages of this whole-blood method have been characterized (28), it has seldom been used by investigators to assess platelet function. In an intervention trial, the impedance method was used to show a modest effect of n−3 PUFAs on in vitro platelet function (29). A decrease in velocity of collagen-induced aggregation in whole blood was observed for samples from subjects who consumed mackerel paste for 6 wk compared with the control subjects who consumed meat paste for 6 wk. Increased impedance aggregation was correlated with risk of ischemic heart disease in a cohort of men participating in the Caerphilly Prospective Study (30).

When threshold doses of ADP required to produce an impedance increase were determined, a noticeably but not significantly lower threshold dose was observed between subjects with a past history of myocardial infarction and subjects free of cardiovascular events (31). A lower ADP threshold dose, a measure of increased platelet sensitivity, was correlated with higher platelet counts and a higher mean platelet volume. This comparison was limited, however, by the low incidence of myocardial infarction in the population whose platelets had been tested for dose responses to ADP with the impedance method (8 of 242 subjects).

Information from 7-d diet records from another cohort of the Caerphilly Prospective Study was used to calculate the thrombogenicity index of the diet and to relate the scores with ability to predict risk for ischemic heart disease (32). [The thrombogenicity index of a diet was defined by Ulbright and Southgate (33) as the ratio derived by combining intake of the SFAs 14:0, 16:0, and 18:0 and dividing by weighted factors for monounsaturated (MUFA) and PUFA intakes (0.5 MUFA + 0.5 n−6 PUFA + 3.0 n−3 PUFA + n−6 PUFA).] Participants with higher scores tended to have increased risk for subsequent development of ischemic heart disease. The investigators concluded that the proposed indexes offered no advantage over total SFA intake or ratios of PUFAs to SFAs in predicting ischemic heart disease.

Platelet-count method

Another test that uses whole-blood samples is the platelet-count ratio method (18). This test is based on the comparison of counts of platelets collected into anticoagulant containing EDTA with and without formalin. The latter reagent fixes any aggregates that are present in the blood sample. If aggregates are present and fixed, a decrease is seen in single platelet counts compared with the unfixed sample. In one study with 12 healthy young men fed a butter-rich diet followed by a diet high in n−6 PUFA, the platelet-count ratio changed significantly after the PUFA-rich diet, indicating less aggregate formation in whole blood and supporting a divergence in thrombotic potential between SFAs and PUFAs (34).

Animal Studies

The same dissonance exists between in vivo and in vitro measurements of platelet function in animal studies designed to test the thrombogenicity of various fatty acids (14). Hornstra and Lussenburg (35) determined the in vivo arterial thrombotic tendency in rats fed diets containing various combinations of SFAs and PUFAs by noting the obstruction time in aortic loops. Their data support the theory that diets high in SFAs enhance thrombosis compared with diets high in either n−6 or n−3 PUFAs (36). The nature of the fats and oils used to prepare the diets did not allow these investigators to distinguish the thrombotic tendency of individual fatty acids. This same problem prevails in human studies as well, because natural food components contain a mixture of fatty acids.

In subsequent studies, Hornstra (8) noted the lack of correlation between the in vivo experimental thrombotic model and in vitro platelet aggregation by the optical method. That is, platelets from rats fed diets high in SFAs showed less aggre-
gability in vitro than did platelets from rats fed diets high in either n-6 or n-3 fatty acids.

Renaud and colleagues (37, 38) have used a combination of plasma clotting assays and aggregation in platelet-rich plasma to test the activity of platelets from rats fed different kinds of fat. Agonists used (ADP, thrombin, and epinephrine) produced equivocal outcomes on platelet responses, but these investigators interpreted their overall results as supporting the thombo-bogenicity of SFA's compared with PUFAs.

NEW APPROACHES TO ASSESS PLATELET FUNCTION

Measuring subtle effects of the individual fatty acids on platelet responsiveness in situations in which little in vivo activation is expected (ie, in subjects or animals free of vascular disease) has remained a challenging endeavor. The extensively used, classical optical method for assessing platelet function actually measures a late stage in a series of activation steps that culminate in aggregation. To see changes in light transmission, platelets must form large aggregates of seven or more platelets.

Approaches designed to look at early molecular and cellular events in the series of activation steps may be more appropriate for detecting subtle effects of dietary components on generation of signals controlling platelet responses (39). Flow cytometric analysis of platelets in whole blood offers such an opportunity (40, 41). Probing receptor status and exposure with monoclonal antibodies and studying platelet-leukocyte interaction are just two examples of the utility and potential of this technology.

Standardization of sampling procedures and validation of responses to suitable agonists and antagonists are necessary steps in devising methods that will detect the potential of platelet–signaling networks and track occurrence of prior events that establish this measured potential. Routine clinical variables carefully measured can also be useful indicators of platelet status.

Estimating the mean platelet volume through use of electronic whole-blood cell counters can supply information about the most immediate history of exposure to activating events (eg, venipuncture) because electronic volume reflects size and shape and not true volume (42). Thus, the apparent volume of nonactivated, disc-like platelets is smaller than the volume of activated, more spherical platelets. Two diets, one high in 16:0 and one high in 18:0, were fed to 10 healthy men in a crossover design. The diet high in 16:0 resulted in platelets with significantly larger apparent volumes than those for the diet high in 18:0. These results indicated that the two fatty acids did not have equal effects on platelet function and suggested that dietary 18:0 is less thrombogenic than 16:0. Data from two other endpoints, ADP-induced shape change and bleeding time, supported these conclusions.

Assessing the ability to respond to vascular stress by producing antithrombotic agents, in the presence or absence of vascular disease, may be another approach for investigating platelet–endothelial cell interactions after dietary intervention. Prostacyclin and nitric oxide production are increased by conditions that provoke vascular stress. Both compounds are powerful inhibitors of platelet aggregation and have been shown to be reduced by dietary n-3 fatty acids (43–46). Recently, methods have been developed to detect endogenous production of nitric oxide by measuring its stable metabolite, nitrate, in plasma and urine (47).

<table>
<thead>
<tr>
<th>Method</th>
<th>Comments</th>
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<tbody>
<tr>
<td>Aggregation optical method</td>
<td>Requires platelet-rich plasma or isolated platelets, artifacts easily introduced</td>
</tr>
<tr>
<td>Aortic loop model</td>
<td>In vivo model, invasive technique; poor correlation with aggregation</td>
</tr>
<tr>
<td>Platelet-count ratio</td>
<td>Rarely used; increased data to support physiologic relevance is needed</td>
</tr>
<tr>
<td>Assays for β-TG and PF-4</td>
<td>Highly variable results because of artifacts introduced during phlebotomy</td>
</tr>
<tr>
<td>Whole-blood impedance method</td>
<td>Seldom used, subject to same artifactual problems stated above</td>
</tr>
<tr>
<td>Urinary metabolites</td>
<td>Noninvasive, integrated value for TX-M production, analysis requires GC-MS; validated RIA and EIA can also be used</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Detection of surface activation markers with appropriate monoclonal antibodies; correlation with in vivo activation is needed</td>
</tr>
<tr>
<td>Shape change detection</td>
<td>Apparent volume can indicate degree of shape change; requires carefully standardized conditions; data to support relevance needed</td>
</tr>
<tr>
<td>Bleeding time</td>
<td>Highly variable results, operator dependent; measures interactions among blood cells and vessel wall</td>
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Additional background information can be found in the references listed in the text. Other papers in this supplement contain further information on tests used to evaluate the effects of dietary fatty acids on platelet function in humans and animals (13, 14). β-TG, β-thromboglobulin; PF-4, platelet factor 4; TX-M, thromboxane metabolite; GC-MS, gas chromatography–mass spectrometry; RIA and EIA, radio- and enzyme-linked immunoassay.

SUMMARY

This brief overview has outlined the hazards associated with many of the in vitro tests used to assess the effects of diet on platelet activity (Table 1). Results from these tests have been inconsistent and highly variable because of the activation of platelets that can occur during collection and preparation procedures.

As a necessary first step, phlebotomy procedures have to be standardized so that interpretable data can be produced. Until new techniques are devised to detect on-off status of the multiple signaling networks that control platelet responses, the best strategy is to measure two or more markers of platelet activity and evaluate correlative associations among the results. Such an approach should increase the likelihood of producing relevant, reliable data capable of predicting the effects of diet on the in vivo aggregatory and coagulant potential of platelets.

REFERENCES

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