Blood Coagulation and Fibrinolysis of Rats Fed Fish Oil: Reduced Coagulation Factors Especially Involved in Intrinsic Pathway and Increased Activity of Plasminogen Activator Inhibitor

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Received March 4, 2003; Accepted June 23, 2003

Differences in the coagulation and fibrinolytic system of rats fed a fish oil based diet (fish oil diet) and fed a soybean oil based diet (control diet) were determined. Concentrations of plasma lipids were depressed in rats fed the fish oil diet. Prothrombin time (PT) and activated partial thromboplastin time (APTT) of rats fed the fish oil diet were longer than for the rats fed the control diet. Fish oil intake lowered the activities of most of the blood coagulation factors, and strongly depressed the factors involved in the intrinsic pathway. Fish oil also affected the fibrinolysis of rats. Plasminogen activator inhibitor (PAI) activity was elevated in rats fed the fish oil diet. In this study, both blood coagulation and fibrinolysis were down-regulated by feeding the fish oil diet.

Key words: fish oil; coagulation; fibrinolysis; intrinsic pathway; plasminogen activator inhibitor

Thrombotic diseases, stroke, and ischemic heart disease have killed or caused serious disability to many people in industrialized countries. The prevention of clot formation has become a crucial issue in medical and health care for elderly people. Some factors are known to influence blood coagulation, constituents of foods like vitamin-K and vitamin-E, hormonal agents like estrogen, or immune functional compounds like tumor necrosis factor a. Some correlations between fibrinolytic or coagulation parameters and daily habits such as alcohol drinking, smoking, and nutrient intake have been observed. Epidemiological studies have suggested that people who eat fish, have a heavy dependence on fish-eating have less risk of heart disease, hyperlipidemia, and atherosclerosis than people with a heavy dependence on meat-eating. Eskimos have a prolonged bleeding time and low risk of hyperlipidemia and coronary heart disease, and this has been suggested as a consequence of the large amount of fish intake, especially fish oil.

The effects of fish oil or n-3 polyunsaturated fatty acids on blood coagulation and fibrinolysis have been examined. However, the mechanism by which they have effects on hypocoagulant activity remains unclear. Adhesion of platelets is the trigger of blood coagulation, and the coagulation cascade is activated on the platelets. There are two main pathways of the blood coagulation cascade: intrinsic pathway and extrinsic pathway. These two pathways converge on a common pathway and end up with fibrin formation. The intrinsic pathway is started with the contact system and includes coagulation factors XII (FXII), FXI, FIX, and FXIII. The extrinsic pathway is started with a tissue factor, and includes FVII. In association with fish or fish oil intake, the activities of coagulation factors II, V, and VII are relatively well documented, but the activities of coagulation factors involved in the intrinsic pathway have been less well investigated. The blood coagulation is complex and involves many factors that interact. Some coagulation factors have blood coagulation activating ability and also converse activity. Thus, it is difficult to determine whether blood coagulation as a whole is increased or not with the limited information on the various factors involved in the blood coagulation and fibrinolytic system. So we did an experiment to analyze most of the factors involved in the coagulation cascade and fibrinolysis to enable a comprehensive assessment of blood coagulation in rats fed experimental diets. In this report, we investigated the effects of fish oil on the blood coagulation and fibrinolytic system
comparing rats fed the fish oil diet to rats fed the control diet.

**Materials and Methods**

Male five-week-old Sprague-Dawley rats obtained from the Charles River (Kanagawa, Japan) were kept in an air-conditioned room (temperature: 20–22°C; humidity: 55–65%; lighting: 0700–1900), and they were fed on a commercial nonpurified diet (Type NMF; Oriental Yeast Co., Tokyo, Japan) for 1 week. After acclimation to the housing conditions, the rats were divided into two dietary groups: control group and fish oil group. The control diet was prepared according to the recommendations of the American Institute of Nutrition (AIN-93G) and the fish oil diet was made with fish oil substituted for soybean oil in the composition of the control diet (7% of total volume). All the other ingredients in the two types of diets were of equal amounts. Fish oil was obtained from Nippon Chemical Feed Co., (Hokkaido, Japan) and the fatty acid composition was as follows: 16:0, 10.3; 16:1, 8.1; 18:0, 2.6; 18:1, 11.4; 18:2, 1.3; 18:3(n-3), 1.0; 18:4(n-3), 5.1; 20:4, 1.8; 20:5(n-3), 34.2; 22:6(n-3), 17.3. All the other diet ingredients were products of the Oriental Yeast Co. The fatty acid composition of soybean oil was as follows: 16:0, 10.3; 16:1, 8.1; 18:0, 2.6; 18:1, 11.4; 18:2, 1.3; 18:3(n-3), 7.9; 20:0, 0.3; 20:1, 0.1; 22:0, 0.4.

The care and treatment of the experimental animals conformed to the National Research Institute of Fisheries Science guidelines for the ethical treatment of laboratory animals.

After free feeding of the experimental diets for 2 weeks, rats were lightly anesthetized with diethyl ether and blood samples were withdrawn from the abdominal aorta into plastic syringes containing 3.8% sodium citrate solution (1/9 volume of the blood) and collected into plastic tubes. Plasma samples were obtained by centrifugation (for 30 minutes at 2000 g) and kept frozen until the day of measurements. Blood samples were collected in alternate shifts for each experimental group to exclude the time relevance of various factors.

Plasma lipids were measured with commercially available kits using the enzymatic method (Wako, Japan). Plasmin-α2-plasmin inhibitor complexes (PIC) and D-dimer were measured by latex agglutination (International Reagents Corporation, Japan).

PT, APTT, fibrinogen, and the activities of the coagulation factors (II, V, VII, X, VIII, IX, XI, and XII) were measured using CA-50 and clinical diagnostic agents (Sysmex, Japan). The CA-50 is a measuring device for coagulation, which can detect the change of turbidity by the dispersion of light when fibrinogen is converted to fibrin. PT was measured with thromboplastin (from rabbit brain) and the time of fibrin formation was detected. APTT was measured with cephalin (phosphatidylethanolamine) from rabbit brain. Fibrinogen was measured in the presence of excess thrombin. The fibrinogen concentration of each sample was calculated from the time taken for coagulation compared to the reference standard. The activities of the coagulation factors were measured by using coagulation-factor-deficient plasma. For the factors included both in the extrinsic pathway and in the common pathway (II, V, VII, and X), PT reagent (thromboplastin) and the appropriate factor-deficient plasma were used. For the factors included in the intrinsic pathway (VIII, IX, XI, and XII), APTT reagent (actin: cephalin from rabbit brain) and appropriate factor-deficient plasma were used. And the activity of each sample was expressed as the percentage of the pooled plasma of the control group.

FXIII activity was measured by the method previously described by Anwar et al. with some modifications. The following reagents used in this assay, fibrinogen, thrombin, streptavidin-alkalinephosphatase (SAAP), and p-nitrophenyl phosphate (pNPP) were purchased from Sigma and 5-(Biotinamido) pentylamine from Pierce Biotechnology Inc., IL. A reference standard curve was obtained by plotting serial dilution of the pooled control plasma and the activity of FXIII expressed as a percentage of the control.

Antithrombin III (AT III) and α2-plasmin inhibitor were measured by using commercially available kits (Chromogenix, Italy). AT III activity was assessed using S-2238 (H-D-phenylalanyl-l-pipecolyl-l-arginyl-p-nitroanilide·2HCl) which is the substrate of thrombin and produces optically detectable p-nitroaniline. AT III activity was measured from remaining thrombin activity compared to a reference standard in the presence of an excess of thrombin. α2-Plasmin inhibitor activity was measured using a substrate of plasmin (S-2251; H-d-valyl-l-leucyl-l-lysyl-p-nitroanilide·2HCl). When an excess amount of plasmin and S-2251 are added to plasma, plasmin forms an inactive complex with α2-plasmin inhibitor (PIC) and the remaining plasmin reacts with the substrate S-2251 and the amount of released p-nitroaniline indicates the activity of remaining plasmin. Adding certain amounts of excess plasmin to the plasma samples, α2-plasmin inhibitor activity is measured from the remaining plasmin activity. The absorbance was read at 405 nm and the α2-plasmin inhibitor activity was calculated by comparison with the standard serum.

PAI activity was measured for its inhibitory activity against tissue-type plasminogen activator (t-PA) in the presence of t-PA and substrate S-2288 (H-D-Ile-Pro-Arg-p-nitroanilide·2HCl (Chromogenix), which releases p-nitroaniline cleaved by t-PA).
Table 1. Plasma Concentration of Lipids (mmol/l)

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<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fish Oil</th>
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<tbody>
<tr>
<td>Triacylglycerol</td>
<td>1.72 ± 0.13</td>
<td>0.49 ± 0.05***</td>
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<tr>
<td>Cholesterol</td>
<td>1.24 ± 0.06</td>
<td>0.56 ± 0.03***</td>
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<tr>
<td>Phospholipid</td>
<td>3.63 ± 0.12</td>
<td>1.73 ± 0.04***</td>
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Values are the mean ± SEM (n = 7). ***p < 0.001: Significance of difference compared with the control group.

Table 2. Coagulation Time and Plasma Fibrinogen Concentration

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<tr>
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<th>Control</th>
<th>Fish Oil</th>
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<tbody>
<tr>
<td>PT (s)</td>
<td>16.3 ± 0.06</td>
<td>17.0 ± 0.14***</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>21.8 ± 0.38</td>
<td>23.4 ± 0.30**</td>
</tr>
<tr>
<td>Fibrinogen (mg/ml)</td>
<td>2.48 ± 0.018</td>
<td>2.43 ± 0.028</td>
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Values are the mean ± SEM (n = 7). *p < 0.01, **p < 0.001: Significance of difference compared with the control group.

Thromboxane B₂ (TXB₂) and 6-keto-prostaglandin F₁₀ (6kPGF₁₀) were measured using commercially available ELISA kits (Neogen Corp.).

Results

During the experimental period, no significant difference was observed in the growth and food intake of rats between the two dietary groups. The final body weight was 285.2 ± 2.6 g in the control group and 291.3 ± 3.7 g in the fish oil group. And the average of their food intake throughout the experimental period was 24.6 ± 0.12 g and 24.3 ± 0.17 g per day, respectively. All statistical analyses were done with student’s t-test between the two dietary groups.

Lipid concentration
Plasma concentrations of triacylglycerol and phospholipid and cholesterol were significantly lower in the fish oil group than the control group (Table 1).

Blood coagulation
PT, APTT, and fibrinogen concentration were measured (Table 2). PT and APTT of the fish oil group were significantly longer than that of the control group. There was no significant difference in the plasma concentration of fibrinogen between the two dietary groups. The activities of the blood coagulation factors were measured. The activities of all the measured factors in rats fed fish oil were lower than that of the control group rats (Fig. 1).

Fibrinolytic parameters and AT III
PIC, D-dimer, PAI, α₂-plasmin inhibitor, and AT III were measured (Table 3). No significant difference was observed between the two diet groups in measured values of PIC and D-dimer. The activities of PAI and plasmin inhibitor were elevated significantly in the fish oil fed group.

Thromboxane B₂ and 6kPGF₁₀
We measured the plasma level of TXB₂ and the
6kPGF_{1\alpha} level to estimate the levels of thromboxane A_{2} (TXA_{2}) and prostaglandin I_{2} (PGI_{2}), respectively. Concentrations of TXB_{2} and 6kPGF_{1\alpha} were both significantly decreased in rats fed the fish oil diet (Table 4).

**Discussion**

There is a growing interest in the functionality of n-3 polyunsaturated fatty acids to modulate health and disease. There have been reports of modulatory effects and prevention of diseases such as coronary heart disease, stroke, autoimmune disorders, Crohn's disease, rheumatoid arthritis, and some kinds of cancer. The effects of n-3 polyunsaturated fatty acids on lipid metabolism are well documented and the mechanisms of reducing serum lipids have been reported. As in these reports, the plasma concentrations of lipids were found to be strikingly suppressed in rats fed the fish oil diet.

Both PT (which indicates the extrinsic pathway coagulation activity) and APTT (which indicates the intrinsic pathway coagulation activity) were elongated significantly by fish oil feeding. This means that fish oil has a hypocoagulant ability and the activity of some coagulation factors might be depressed. Then, we measured the activity of each coagulation factor. The coagulation factor activities were generally lower in rats fed the fish oil diet and it was notable in factors involved in the intrinsic pathway. As most of the coagulation factors are produced in the liver, we tested to see if the fish oil caused damage to the liver. However, the fibrinogen concentration of the two dietary groups were found to have the same level, and no elevation was observed in the levels of γ-glutamyl transpeptidase (γ-GTP), glutamate oxaloacetate transaminase (GOT), and glutamate pyruvate transaminase (GPT), used as markers of liver damage (data not shown). So, it is considered that the fish oil rich diet influenced the rat blood coagulation without causing damage to the liver.

Several reports have examined the effects of fish oil on blood coagulation, Nieuwenhuys *et al.* and Leray *et al.* suggested that the hypocoagulant effects of fish oil were caused by reduction of the vitamin-K-dependent coagulation factor. In this study, we found that most of the coagulation factors were depressed and it was very significant in the intrinsic pathway. It has been reported that high levels of factor VIII and factor IX, which are included in intrinsic pathway, increase the risk of thrombosis. Thus, fish oil may have beneficial effects for the control of this type of disease by controlling plasma levels of these factors.

Fibrinogen, soluble fibrin monomer (converted from fibrinogen by thrombin), and insoluble fibrin polymer (cross-linked by FXIII) are all decomposed by plasmin. D-dimer is the degradation product of fibrin polymer. The D-dimer level indicates the amount of produced fibrin polymer and its decomposition by plasmin. And PIC increases with excessive amounts of plasmin. Although no significant difference was seen in the PIC and D-dimer concentrations, significant elevation of PAI activity was found in the fish oil group compared to the control group. The elevated PAI activity suggests the lowered activity of fibrinolysis. We speculate that the activity of α2-plasmin inhibitor also increased for the reduction of the activity of plasmin.

Byberg *et al.* reported that dietary intake of unsaturated fatty acids is positively associated with PAI-1, the main physiological inhibitor of plasminogen activator, in human. It has also been reported that PAI-1 activity is correlated with the amount of VLDL and adipose tissue. In this study, the adipose tissue weight of the rats fed the fish oil diet was significantly lower than the rats fed the control diet (data not shown), VLDL was also speculated to be lower in the rats fed the fish oil diet from the significant reduction of triacylglycerol in plasma and PAI activity was elevated. These results are contradictory but it is considered that n-3 polyunsaturated fatty acids directly increased PAI-1 in this study, as unsaturated fatty acids reported to increase PAI-1 in vitro.

The effects of n-3 polyunsaturated fatty acids upon cyclooxygenase activity and biosynthesis of eicosanoids have been well documented. In our study, the level of eicosanoids also changed dramatically. TXA_{2} has platelet aggregation activity, and PGI_{2} can inhibit platelet aggregation. TXA_{2} and PGI_{2} are both unstable and readily metabolized into TXB_{2} and 6kPGF_{1\alpha}, respectively. Hence, low levels of TXA_{2} and PGI_{2} were observed in the plasma of the rats fed the fish oil diet. Plasma TXB_{2} level of the fish-oil-diet-fed rats was 45% lower than that of the control-diet-fed rats and the 6kPGF_{1\alpha} level of the fish-oil-diet-fed rats was 58% lower than that of the rats fed the control diet. The decrease of the 6kPGF_{1\alpha} level was larger than the TXB_{2} level, and the ratio of 6kPGF_{1\alpha}/TXB_{2} was larger in the rats fed the fish oil diet. So platelet aggregation activity of the rats fed the fish oil diet is speculated to be lower than the control-diet-fed rats.

In this study, the following effects of fish oil were confirmed; lipid-lowering activity, reduction of plasma coagulation factor level, reduction of fibrinolysis speculated from increased PAI activity. So that lipids are involved in blood coagulation, reduced blood coagulation factor activities might be correlated with the reduction of the plasma lipid level. Our research has expanded the possibility that fish oil has beneficial effects, in the point of finding reduction of the intrinsic coagulation factors.

In conclusion, fish oil rich in n-3 polyunsaturated fatty acids can act as a modulator of blood coagulation and fibrinolysis. So as rats and humans have a...
similar mechanism of blood coagulation and fibrinolysis, this is also expected to be applicable to human. Thus, habitually taking food like fish, shellfish, or seaweed, rich in n-3 polyunsaturated fatty acids, is expected to be effective to prevent thrombus diseases.

**Acknowledgments**

This work was in part supported by the Ministry of Agriculture, Forestry, and Fisheries, Japan and by the cooperative system for supporting priority research (Japan Science and Technology Corporation).

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