THE EFFECT OF DIETARY SUPPLEMENTATION OF FISH OIL ON EXPERIMENTAL MYOCARDIAL INFARCTION

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Abstract

The effect of altering the abundance of precursors and inhibitors of prostaglandin formation by dietary supplements of fish oil was investigated in dogs with experimentally induced myocardial infarction. Prior to induction, 10 male mongrel dogs were fed standard dog chow supplemented with 25% of the total calories as menhaden oil for 36 to 45 days. The fatty acid composition of the lipids in plasma and platelets changed to reflect the increased intake of polyunsaturated fatty acids of the n-3 type. Thrombosis and subsequent infarction was induced by electrical stimulation of the left circumflex coronary artery of ambulatory dogs that were monitored by telemetry. Upon stimulation of control animals, the frequency of ectopic beats rose from less than 10% at the beginning to about 80% after 19 hours. In contrast, the oil-fed dogs maintained a more normal ECG pattern, showing less than 30% ectopic beats after 19 hours. In these animals, the size of infarction (measured by formazan formation) was 3% of the left ventricle compared to 25% in the control animals. The results suggest that dietary supplementation with fish oil may be beneficial in reducing myocardial damage associated with coronary artery thrombosis.

INTRODUCTION

Platelet aggregation and the formation of microthrombi are important pathophysiological mechanisms for the development of myocardial ischemia and infarction (1-2). The aggregation is dependent upon the biosynthesis of prostaglandin derivatives from arachidonic acid (3) and upon interactions of platelets with endothelial cells (4).

Several pharmacological strategies have been suggested to block *in vivo* platelet aggregation and thus help to reduce the incidence of myocardial ischemia and infarction. These include administering prostacyclin, specific thromboxane A_2 synthase inhibitors or cyclooxygenase inhibitors such as aspirin and ibuprofen. All of these strategies, while worthy of further pursuit, have various limitations including drug toxicity and transient inhibitory levels. An alternate approach has recently been suggested on the basis of reports of lipid composition in Eskimos in Greenland (5-7). In these reports, a high ratio of 20:5 n-3 to 20:4 n-6 fatty acid in platelet and plasma lipids was related to a low incidence of myocardial infarction. The n-3 fatty acids, so abundant in marine food, are effective inhibitors of the oxygenation of arachidonate by cyclooxygenase (8). The inhibitory function of the n-3 fatty acids could reduce thromboxane formation and thereby platelet aggregability, vasospasm and the incidence of coronary disease (2).

Recent studies from our laboratory on the consequences of myocardial infarction showed beneficial effects of dietary fish oil supplementation in limiting the infarct size in an experimental mode of cerebral artery ligation (9). The present study was undertaken to determine the effect of fish oil supplementation on infarct size in a model of coronary artery thrombosis in the dog.

METHODS

Animals and Diet

Ten male mongrel dogs weighing between 12 and 22 kg were fed a standard diet of dog chow (Friskies Dinner, Carnation, Los Angeles, CA) supplemented with 25% of their calories as menhaden fish oil (Zapata Haynie Corp., Reedville, Virginia) for 36 to 45 days before coronary artery thrombosis. The diets of all the animals were isocalorically adjusted to maintain their initial body weight. Water was provided *ad libitum*. All dogs were alert and active. They readily consumed the chow supplmented with fish oil. The amount of polyunsaturated fatty acids in the dog chow, menhaden oil and in the supplemented diet are shown in Table 1. Prior to starting the diet, and weekly thereafter, venous blood samples were obtained for determination of plasma and platelet composition of lipid as well as the percent of platelet aggregation *in vitro*. The 10% (w/w) supplementation with fish oil provided total lipids in the diet containing 14% of the fatty acid of the n-3 type and decreased the mol % of linoleate (18:2 (n-6)) to one half that found in the dog chow. Seventeen control animals were maintained on normal chow for 1 to 7 days and subjected to the same surgical and experimental protocols as the experimental animals.

TABLE 1. MOLE % FATTY ACID COMPOSITION OF CONTROL DIET, FISH OIL SUPPLEMENT AND 25% CAL% FISH OIL DIET

FATTY ACID	DOG CHOW	MENHADEN OIL	DIET
14:0	1.9	9.6	6.1
16:0	21.3	20.0	20.5
18:0	8.6	3.4	5.5
18:1 (n-9)	33.9	11.0	20.4
18:2 (n-6)	29.2	1.3	12.7
20:4 (n-6)	0.2	2.0	1.3
20:5 (n-3)	0.1	14.1	8.3
22:5 (n-6)	0.1	0	<0.1
22:5 (n-3)	0	1.1	0.6
22:6 (n-3)	0	8.8	5.1

Fatty acids are designated by chain length:number of double bonds, with the number in parenthesis representing the carbon atoms between the terminal bond and the methyl group.

Operative Procedure

Coronary artery thrombosis was induced using techniques described in detail elsewhere (10). Briefly, a 28 gauge teflon-coated silver wire was inserted through the wall of the left circumflex coronary artery under sodium pentobarbital (30 mg/kg i.v.) anestheia, the dogs were intubated and ventilated via a Harvard respirator. The silver electrode was placed within the vessel lumen and in contact with the intimal lining. An electromagnetic flow transducer (Carolina Medical Electronics, Inc.) was placed around the left circumflex coronary artery (LCX) proximal to the point of entry of the electrode. Lead II electrocardiographic (ECG) data were obtained from subcutaneous electrodes. The animals were allowed to recover from surgery.

Induction of Coronary Artery Thrombosis

Twenty-four hours post-surgery the animals were placed in a quiet laboratory and electrocardiographically monitored. Anodal current from a 9 volt battery was delivered to the intimal surface of the LCX via the indwelling electrode. By controlling a 250,000 ohm potentiometer placed in a series with the battery, 50 microamperes of current were delivered to the vessel for 24 hours. The ECG data were received via telemetry and recorded on a tape recorder programmed for 28 seconds of tracing every 15 minutes. A permanent record was obtained by replaying the tape into a Grass Polygraph.

Myocardial Infarct Size

After 24 hours of electrical stimulation, the current was terminated. The animal was anesthetized, intubated, and placed on the respirator. The original thoracotomy incision was then reopened to expose the heart. A 20% patent blue violet solution (1 ml per 5 kilograms body weight) was injected into the left atrial appendage. The visual absence of dye in an area of myocardium was an indication of defective circulation in that area. After removal of the heart, the LCX in the region of the electrode was dissected free of the surrounding tissue and opened lengthwise. The thrombus was scraped free from the intimal wall and its wet weight was determined. The heart was then sectioned transversely from apex to base into roughly 1.5 cm thick slices and areas of non-perfusion noted. The area of infarcted myocardium was determined by incubation of the slices in 2,3,5,-triphenyltetrazolium chloride. This salt formed a red formazen derivative in tissue areas with normal dehydrogenase activity and failed to form the intracellular pigment in areas of impaired cellular function. The non-stained, infarcted regions were excised and weighed to determine the percent of infarcted tissue.

Lipid Analyses

Plasma and platelets were acidified with 0.2 ml formic acid and homogenized with 6 ml of chloroform:methanol (2:1). Arachidic acid (20:0) was added as an internal standard for calculation of recovery and quantitation of non-esterified fatty acids. The homogenate was heated at 70°C for 10 minutes, and two milliliters of water was added. The lower phase was removed, evaporated to dryness under nitrogen, and the residues dissolved in 50 µl of chloroform: methanol (2:1). The

total lipids were then fractionated into lipid classes by thin-layer chromatography (TLC) using silica gel H plates. The solvent for the development of neutral lipids was petroleum ether, anhydrous diethyl ether, and acetic acid (60:40:1). Neutral lipid and phospholipid standards were chromatographed in parallel with the samples. The TLC regions corresponding to these standards were located by iodine vapor and then scraped from the plate. Pentadecanoic acid was added and methanolysis performed at 70°C for 45 minutes with 8% H_2SO_4 in anhydrous methanol without previous extraction of the lipids from the silica gel.

The composition of the fatty acid methyl esters from the triglycerides, non-esterified fatty acids, and phospholipids was measured by gas chromatography using a Varian Model 3700 gas chromatograph with a flame ionization detector. A 6' glass column of 2 mm I.D. packed with 10% diethylene glycol succinate (DEGS) coated on porapak type S, 80/100 mesh was used. The flow rate was 28.6 ml of hydrogen/minute at an inlet pressure of 30 psi. The inlet heater was kept at 261°C and the detector cell at 241°C. All the esters were chromatographed at 185°C column temperature. Each individual methyl ester was identified by its derived equivalent chain length calculated relative to the 15:0 internal standard.

Platelet Aggregation

In vitro assessment of platelet aggregation was performed according to the spectrophometric method of Born (11) utilizing a Bio-Data aggregometer. Citrated venous blood (3.8%) was obtained. Centrifugation of blood at 310 x g for 3 minutes yielded platelet-rich plasma (PRP) and at 2,200 x g for 10 minutes to obtain platelet-poor plasma (PPP). Platelets in PRP were adjusted to 200,000/µl with autologous PPP. The baseline for 0% transmission was set with PRP and that for 100% with PPP. The change in light transmission after the addition of aggregating agents was recorded as a percentage value. The following aggregating agents were tested less than 2 hours from the time of blood sampling: 3 and 6 µg/ml collagen (Ethicon collagen Dispersion-TD 150); 2,11,50 and 110 Mu ADP (Sigma, St. Louis, MO) and 0.66,2,5,7, and 10 mM Arachidonate (Sigma, St. Louis, MO).

RESULTS

This experimental model for the induction of coronary artery thrombosis produced sudden death in 5 of 17 control animals (29%) and 3 of 10 oil-supplemented dogs (30%). Constant monitoring of the ECG by telemetry indicated that the ventricular fibrillation occurred from 45 minutes to 18 hours after initiating the electrical stimulation. The similar incidence of fibrillation in the two groups suggest that the diet did not influence the tendency for sudden death in these animals and no further observations will be reported on this subset. In the 19 dogs which survived 24 hours of 50 microamperes of electrical stimulation, the ECG recording showed some depressed ST segments within 6 to 8 hours and some episodes of ventricular tachycardia. In control animals, the frequency of ectopic beats rose from less than 10% at the beginning of the experiment to about 80% after 19 to 24 hours of

stimulation. In contrast, the oil-fed dogs maintained a more normal ECG pattern, showing less than 30% ectopic beats after 19 hours.

The oil fed dogs exhibited smaller areas of impaired circulation as indicated by the distribution of injected patent blue violet. This was confirmed by small corresponding areas of impaired myocardium that were detected by tetrazolium reduction. The mean infarct size in the 7 oil-supplemented dogs was 11% of the left ventricle as compared to 23% for the 12 control dogs ($p=0.08$; Table 2). Two dogs in the fish oil supplemented group had infarcts of 30 and 33%, more than four standard errors of the mean from the mean value (11%), and were clearly outliers of the group. When these values are set aside, the mean infarcts size for the 5 remaining oil-supplemented animals is 3±1%. The control group included one animal with a 3% infarct size which was five standard errors below the mean (23%) of this group. Excluding this value from the results from the control group gave an average infarct size of 25±3% significantly greater than that for the experimental animals ($p<0.001$).

TABLE 2 THE EFFECTS OF DIETARY FISH OIL UPON INFARCT SIZE*

NUMBER OF ANIMALS	CONTROL DIET	SUPPLEMENTED DIET
Total Studied	17	10
Sudden Death	5	3
Survivors (24 hrs)	12(23±4%)	7(11±5%)**
Outliers	1(3%)	2(30%,33%)
Final Subset	11(25±4%)	5(3±1%)***

*Values in parenthesis are the percent of the left ventricle that was infarcted with mean ±S.E.M. where indicated.
**p=0.08
***p 0.001

The average wet weight of the thrombus in the oil-fed dogs was 20±6 mg, similar to the value of 23±2 mg for control dogs. Furthermore, repeated *in vitro* measurements of platelet response to the aggregants were unaffected by diet, remaining unchanged throughout the feeding period. The mean hematocrit (42±0.2% SEM) for the dogs receiving the fish oil supplement was comparable to that reported elsewhere for control animals (42±2%) (12). Thus, the general tests of hemodynamic properties did not appear to be appreciably affected by the diet.

Prior to dietary supplementation with fish oil, the plasma phospholipids contained 13% arachidonate and less than 1% of each of the n-3 type of fatty acids (20:5,22:5 and 22.6) (Table 3). Linoleic acid concentration was the highest in the triglycerides (together with that for 20:4) and it showed the greatest variance among the individual dogs prior to the 5 week dietary oil supplementation. This variance may reflect responses to dietary supplies of these essential acids. Supplementation with 10% (w/w) menhaden oil for 5 weeks decreased the content of oleic (18:1) and linoleic (18:2) acid in plasma triglycerides to approach the content in the diet. On the other hand, the content of oleic and linoleic acid in plasma phospholipids dropped below that of the diet, reflecting the competitive entry of dietary polyunsaturated fatty acids into the 2-position of the plasma

TABLE 3 DOG PLASMA LIPIDS BEFORE AND AFTER FISH OIL SUPPLEMENTATION

FATTY ACID	PHOSPHOLIPIDS		TRIGLYCERIDES		FREE FATTY ACIDS	
	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
	n=9		n=5		n=5	
14:0	0.8±0.3	0.5±0.1	1±0.2	2±0.3	2±0.5	3±0.5
16:0	18 ±0.3	18 ±0.6	13±0.5	13±0.9	28±3	24±2
18:0	25 ±1	30 ±1	4±1	3±0.3	15±1	8±0.4
18:1 (n-9)	13 ±1	10 ±0.5	27±0.2	19±0.4	27±0.3	28±3
18:2 (n-6)	21 ±2	9 ±1	45±3	30±0.8	18±2	17±2
20:4 (n-6)	13 ±2	9 ±1	5±1	8±1	3±0.6	3±0.4
20:5 (n-3)	0.7±0.2	11 ±1	0.9±0.2	16±0.4	3±1	6±1
22:5 (n-6)	0.2±0.1	0.2±0.1	trace	trace	1±1	0.1±0
22:5 (n-3)	0.8±0.1	2 ±0.4	0.1±0.1	1±0.3	0.4±0.1	0.2±0.1
22:6 (n-3)	0.6±0.1	6 ±1	0.2±0.1	3±0.5	0.6±0.3	2±0.1
TOTAL NMOLES/ML	1518±235	1400±181	636±65	670±79	135±11	147±16

Fatty acids are designated by chain length:number of double bonds with the number in parenthesis representing the carbon atoms between the terminal bond and the methyl grou
Values expressed as mole % are mean ± SEM.

phospholipids. Also, arachidonate (20:4) decreased in the phospholipids as other dietary polyunsaturated fatty acids were incorporated. In contrast, 20:4 increased slightly in the triglycerides from 5% to 8% even though the diet contained only 1%. This suggests that the triglycerides secure extra 20:4 from tissue reserves. In fact, the triglycerides contained higher amounts of 18:2,20:4 and 20:5 than appeared in the diet, apparently reflecting some selective incorporation of these polyunsaturated fatty acids. The compositional pattern of the free fatty acid fraction closely reflected the daily dietary intake (Table 1) with increases from 3 to 6% for 20:5 and from 0.6 to 2% for 22:6 although no significant change occurred for 18:2.

The fatty acid composition of platelet lipids before and after fish oil supplementation is illustrated in Table 4. The content of 18:1 and 18:2 decreased while the amount of 20:5, 22:5 and 22:6 increased. No significant change was observed in 20:4. One difference noted between the control and experimental groups may become important in further investigations. The two dogs designated as outliers in the oil-fed group had a lower degree of overall incorporation of the n-3 fatty acids (18.6 mole %) in platelet lipids compared to a mean of 26 1 mole % in the rest of the oil-fed animals (individual results not shown). Future studies may show differences among different animals in the esterification of the n-3 type of fatty acids which would cause less of the inhibitory analogs to be released during stimulation of the platelets.

DISCUSSION

We found less severe myocardial damage in animals with elevated n-3 fatty acids.

The mechanism whereby the increased abundance of n-3 fatty acids might exert a beneficial effect may depend on the ability of these competitive inhibitors to impair arachidonate conversion to the dienoic prostaglandins and thromboxanes (8). Dyerberg and Bang (13) were the first to suggest that "20:5 protects against thrombocyte aggregation, either by competitively inhibiting TXA_2 synthesis, or by generating prostaglandins which inhibit thrombocyte aggregation". Recent work by Culp et. al. (14) have confirmed that eicosapentaenoic acid (20:5) is a reversible competitive inhibitor of arachidonate oxidation under physiologic conditions supporting the hypothesis of Dyerberg and Bang. In the present study, the ratio of 20:5 to 20:4 in the platelet lipids of the menhaden oil-supplemented dogs was changed from 0.06 to 1.8 (Table 4) to resemble that found in Eskimos in Greenland (0.94;5). The ratio found in Danes on a conventional diet was 0.02 (5) which compared with the value obtained in dogs in the present study during the control period. The levels of the n-3 fatty acids incorporated into the platelets of the oil-fed animals may be causally related to the lower infarct sizes produced.

Although the experimental design in these studies emphasized thrombogenesis in the left circumflex artery, we observed no correlation between the size of the thrombus and the area of myocardial damage. The *in vitro* assays of platelet function appeared to be insensitive to the factors that produced decreased infarct sizes. Decreased platelet function may have occurred *in vivo* in this study, and the use of lower concentrations of collagen might have allowed

TABLE 4 FATTY ACIDS IN THE TOTAL PLATELETS OF DOGS BEFORE AND AFTER MENHADEN OIL SUPPLEMENTATION

FATTY ACID	BEFORE	AFTER
14:0	0.6±0.1	1.1±0.5
16:0	15.8±0.3	10 ±1
18:0	17.5±0.5	19.7±0.6
18:1 (n-9)	19 ±1	12.2±0.6
18:2 (n-6)	32 ±2	15.8±0.7
20:4 (n-6)	10 ±2	9 ±0.7
20:5 (n-3)	0.6±0.2	10 ±1
22:5 (n-6)	0.1±0.1	0.1±0
22:5 (n-3)	0.6±0.2	2.6±0.3
22:6 (n-3)	0.6±0.1	5.8±0.8

Fatty acids are designated by chain length:number of double bonds with the number in parentheses representing the carbon atoms between the terminal bond and the methyl group. Values expressed as mole % are mean ± SEM for six dogs.

in vitro detection of subtle changes in platelets as recently reported for human subjects fed a mackerel diet (15).

On the other hand, it seems important to emphasize that our previous results with cats indicated a major role of the collateral microcirculation in controlling tissue infarct size. Following complete closure of the middle cerebral artery, cats on a diet of normal cat chow had 19% of the cerebral tissue damaged. In contrast, cats supplemented with fish oil had much less brain damage (7% of brain volume) and showed less neurological defects. Since the tissue maintained normal function in the animals receiving supplements of n-3 fatty acids, the flow of oxygen and nutrients through the microvasculature appears to have been maintained even when the main artery was totally occluded.

The size of the infarction may be regulated more by the maintainance of microvascular flow than by the flow through the main vessels. There are three possible ways in which the n-3 acids may have maintained the tissue integrity in the studies with cats and dogs.

1. The n-3 fatty acids may have helped to dilate the microvasculature allowing more effective flow after arterial occlusion.
2. n-3 fatty acids may have diminished any constriction of the microvasculature that may have occurred after occlusion.
3. n-3 fatty acids may have diminished formation of microthrombi that would tend to block the microvasculature.

Since the occurrence of microvascular constriction and microthrombi can depend upon cyclooxygenase products, it is reasonable to expect that the inhibitory action of the n-3 fatty acids upon cyclooxygenase did dimish those events.

Regardless of the mechanism involved, dietary supplementation with fish oil has resulted in reduced experimental infarct sizes in both dogs and cats. Although species differences in platelet aggregation and prostaglandin and thromboxane formation are of concern in translating the results of animal studies to man, the previous epidemiological studies in Eskimos and the results of our previous (9) and present study lend support to further trials in man.

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REFERENCES

1. Ross, R., and Glomset, J.A. The Pathogenesis of Atherosclerosis. New Engl. J. Med. 295: 369, 1976.

2. Lands, W.E.M., Pitt, B., and Culp, B.R. Recent Concepts on Platelet Function and Dietary Lipids in Coronary Thrombosis, Vasospasm and Angina. Herz 5: 34, 1980.

3. Smith, J.B., and Willis, A.L. Aspirin Selectively Inhibits Prostaglandin Production in Human Platelets. Nature 231, 235-238, 1971.

4. Moncada, S., and Vane, J.R. Role of Prostacyclin in Vascular Tissue. Fed. Proc. 38: 66, 1979.

5. Dyerberg, J., and Bang, H.O. Haemostatic Function and Platelet Polyunsaturated Fatty Acids in Eskimos. Lancett ii: 433, 1979.

6. Dyerberg, J., Bang, H.O., Moncada, S., and Vane, J.R. Eiconsapentaenoic Acid and Prevention of Thrombosis and Atherosclerosis? Lancet i: 117, 1978.

7. Dyerberg, J., Bang, H.O., and Hjørne, N. Fatty Acid Composition of Plasma Lipids in Greenland Eskimos. Amer. J. Clin. Nutr. 28: 958, 1975.

8. Lands, W.E.M., LeTellier, P.R., Rome, L.H., Vanderhoek, J.Y. Inhibition of Prostaglandin Synthesis. Advanc. Biosci. 9: 15, 1973.

9. Black, K.L., Culp, B., Madison, D., Randall, O.S., and Lands, W.E.M. The Protective Effects of Dietary Fish Oil on Focal Cerebral Infarction. Prostaglandins and Medicine. 5: 247, 1979.

10. Romson, J.L., Haack, D.W., and Lucchesi, B.R. Electrical Induction of Coronary Artery Thrombosis. In the Ambulatory Canine: A Model for in vivo Evaluation of Anti-Thrombotic Agents. Thrombos. Res. 17: 841, 1980.

11. Born, G.V.R. Aggregation of Blood Platelets by Adenosine Diphosphate and its Reversal. Nature 194: 927, 1962.

12. Julien, P., Gilles, D.A., Gailis, L., and Roy, P.E. Role of Cardiac Lymph and Interstitial Fluid in Lipid Metabolism of Canine Heart. Can. J. Pharmacol. 56: 1041, 1978.

13. Dyerberg, J., and Bang, H.O. Dietary Fat and Thrombosis. Lancet i: 152, 1978.

14. Culp, B.R., Titus, B.G., and Lands, W.E.M. Inhibition of Prostaglandin Biosynthesis by Eicosapentaenoic Acid. Prostaglandins and Medicine. 3: 269, 1979.

15. Siess, W., Scherer, B., Böhlig, B., Roth, P., Kurzmann, I., and Weber, P.C. Platelet-Membrane Fatty Acids, Platelet Aggregation and Thromboxane Formation during a Mackerel Diet. Lancet ii: 441, 1980.

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