Environmental and dietary effects on the composition and metabolism of lipids in fish (Practical effects of research)

a Ph.D. Thesis

By
Hossam Mohamed kotkat
B.Sc. Vet. Med.
M.Sc. Vet. Med. Biochemistry

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Jozsef Attila University, Faculty of Science
Szeged, Hungary

Supervisor Prof.Dr. Nemcsok Janos D.Sc.

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Scientific background and project objectives

For years we have told that cholesterol is the major killer and we should avoid foods high in cholesterol, such as eggs. This has been extremely damaging to the egg industry. Many National heart foundations have recognized their serious error and are now allowing 4 eggs to be consumed each week per person. Today, we know that dietary cholesterol is not an important risk factor in heart disease for 98% of the population. There are many other factors that are more important, such as the amount and nature of the fat in the diet.

Fish oils are unique fats in the human diet because they are a rich source of n-3 fatty acids or omega-3 FAs (n minus three). This family of fatty acids is characterized by the presence of a double bond, three carbon atoms away from the terminal methyl group. The major n-3 Fatty acids found in fish-oil are eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). Dietary intake of these long-chain highly polyunsaturated fatty acids reduces the synthesis of prostaglandins, thromboxanes, prostacyclins and leukotrienes from arachidonic acid. Since all n-3 fatty acids from linolenic (18:3) to DHA can be elongated and desaturated.

Phospholipids are an important component of the lipid bilayer of cell membrane and contain high levels of polyunsaturated fatty acids, particularly EPA and DHA. For any naturally occurring phospholipid, a large number of molecular species many exist. Each molecular species is defined by the chemical nature of the polar head groups, the type of linkage to glycerol and the aliphatic chains at both sn-1 (α-) and sn-2 (β-) positions. Different molecular species would be expected to have different metabolic and physical properties, and the distribution of molecular species composition of membrane phospholipids was found to be closely associated with cell membrane fluidity, function and the activity of membrane - bound enzymes.

The aims of the present study

1- To compare the n-3 fatty acid compositions of the poikilothermic and homothermic animals.

2- To explore the useness of fish oil as a source of eicosapentaenoic (20:5 n-3) acid, to assess the safety of long-term fish oil consumption for the prevention and treatment of human disease.

3- To examine the dietary incorporation of n-3 fatty acids into egg yolk to produce an omega-3 fortified egg.

4- To obtain an insight into the mechanism of action of an insecticide (deltamethrin), a herbicide (atrazine) and a petroleum derivative (phenol) on the omega-3 fatty acids of the fish erythrocyte plasma membrane.

Materials and Methods

Experimental animals:-

1- Collection of fish:

In Egypt, there are three main regions for the collection marine fish; all are located along the coastal line of the Mediterranean sea, around or near lake mouths or river mouths. These regions are Mex, Rosetta and Damietta. Freshwater fish were collected from their natural habitats and nearest fish farms. The three pelagic sardine spp. were caught by purse-serine. The water temperature varied according to the season.

2. Dietary supplementation of fish oil to:-

2.1. Freshwater fish

A group of young tilapia (Oreochromeous niloticus), mean weight 5 g, caught in autumn, were fed a semisynthetic fat-free diet for three months and were then divided into four groups in twelve circular tanks each measuring 38.5 cm in diameter and 41 cm in high. One group was kept on a fat-free diet, while the others were received fatty acid-supplemented diets (Cat-fish feed, marine oil or active EPA-30) for the next two months.

2.2. Rats:

Sixty health (3-months old) young male albino rats weighing 110-160 g were housed in separated cages (22X36X15 cm) throughout the experimental period (two months) and were allowed food and water ad libitum. The rats were randomly assigned into three groups.

One group was fed a semi-purified diets containing 5% pig-fat. The second group received a modified diet in which 50% of the pig-fat was replaced by active EPA-30, while third group fed on the lab-pellets acted as control animals.

The same experiments were repeated using (18-month old) Old male albino rats, weighing 250-300 g.

2.3. Laying hens:

A total of 120 pullets from fayioumy (17 weeks old) were housed in a conventional three-tiers laying battery (3 birds/cage of 40X40X44 cm) throughout the experimental period (two months). They were divided into three groups (three replicates of 10 birds for each group) and fed on semipurified diets for at least two months. For the control group, the fat source was soybean oil which contained certain amounts of both linoleic and linolenic acids. For the supplemented animals, the fat sources were fish oil (active EPA-30), which contained high levels of

linolenic (18:3 n-3), eicosapentaenoic (20:5 n-3) and docosahexaenoic (22:6 n-3) acids. The first supplemented group was switched to a fish oil replacing 50% of the soybean oil, and the second supplemented group to a fish oil replacing 100% of the soybean oil.

3- Fish and environmental pollution:

The carp (Cyprinous carpio L) which were used in our experiments weighing (150-200 g) were purchased from the Alexandria Governorate fish farm and acclimatized to laboratory conditions for two weeks in large tanks at about 18°C with filtered tap-water and fed daily the commercial pellets. They were brought into the test aquaria (100 L) two weeks before the experiment started. Each aquarium contained nine fish. The fish were not fed for two days before or during the experimental period.

The pollutant used in these experiments were of the purest chemical grades and commercial available. The durations of treatment were 48 and 96 hr for all pollutiontants.

3.1. The pesticide deltamethrin or decamethrin, DM; type II pyrethroid;(s) α-cyano-3-phenoxybenzyl-(1R)-cis (2,2-dimethyl-3-2,2-dibromovinyl) cyclopropane carboxylic acid ($C_{22}H_{19}Br_2NO_3$) was used at the concentrations of 0, 0.5, 1and 2 µg/L.

3.2. The herbicide atrazine (triazine derivatives) was used at concentration of 0, 10, 100 and 1000 µg/L.

3.3. Phenol (oil refineries): was used at concentrations of 0, 5, 10 and 20 ppm/L (µg/L)

Methods:

Collection and preparation of samples:

The animals were killed by vertebral rupture and their organs muscle or livers were rapidly removed, and cleaned from accessory connective and adipose tissues and washed with tris-sucrose solution to remove blood. The net weights of the tissues were measured. In some cases, blood sample was collected and erythrocyte plasma membrane was isolated according to (Soresen, 1990).

Lipid analysis:

Extraction of total lipids: These were extracted according to Folch et al (1957). The tissue was homogenized with a 2:1/v/v. chloroform-methanol mixture to a final value 20 times the weight of the wet tissue sample.

Separations of polar lipids (PLs): The phospholipid pools were purified by silicic acid column chromatography, using chloroform to elute neutral lipids and methanol for phospholipid (Leray et al., 1987).

Separation of phospholipid subfractions: These were subfractionated by thin layer chromatography according to Fine and Sprecher (1982) on precoated G-60 silica gel TLC plates F254, Merck, Darmstdt, Germany). The spots were detected under UV at 254 nm and removed for quantitative determination of phosphorus according to Rouser et al. (1970).

Determination of fatty acid composition: The fatty acid composition of total lipids or polar lipid subfractions were transesterfied in the presence of dry methanol containing 5% HCl at 80°C under CO_2 or N_2 within 2-3 hrs. The methyl esters were separated by using a Hewlet packard 5890 II equipped with a capillary column coated with SP2330 of 0.25 m thickness (0.25 mm I.D. X 30 mi CPS-Li Quadrex, New Haven, CT, USA).

Separation of molecular species quantitation by high performance liquid chromatography (HPLC): The phosphotidylethanolamine (PE) was hydrolysed with phospholipase from Bacillus cereus (Sigma-chemicals, St. Louis, MO) by a modification of the method of Takamura et al. (1986). Dinitrobenzol derivatives prepared and separated by HPLC (water associated model 440) on a Nucleosyl C-18 column (5 μm, 4 mm i.d X 250 mm), using acetonitrile - propanol (80:20 v/v) of HPLC grade (Carlo, Erba, Milano, Italy) as mobil phase with a flow rate of 1.0 ml/min pressure 54 kg/cm^2, temp. 250°C.

Other biochemical measurements:

Plasma total lipids: These were determined calorimetrically by using the sulphophosvanillin reaction as described by Frings et al.(1972).

Plasma triacylglycerol: This was determined with the triacylglycerol kits of Sclavo, by the methods of Young et al. (1975).

Plasma total cholesterol: This was measured by using the cholesterol kit of Sclavo according to Watson, (1960).

Plasma phospholipids: These were measured by the method of Connerty et al. (1961).

High density lipoprotein (HDL): This was estimated by using the kits of Sclavo, according to Lopes et al. (1977).

Very low density lipoprotein (VLDL): This can be calculated in mg/dl

$$= \frac{\text{plasma triacylglycerol}}{5}$$

Low density lipoprotein (LDL): This can be calculated in mg/dl

= total cholesterol (VLDL + HDL).

Statistical analysis:

The data obtained were subjected to statistical analysis system (SAS, 1994 microcomputer version).

Results and Discussion

1- Our project started by examining omega-3 polyunsaturated fatty acids for the phospholipid composition in important animals such as the poikilothermic freshwater fish and marine fish and the homothermic laying hens and lab animals. In these species, important differences were discovered in the quantitative compositions of omega-3 PUFAs.

It was established that more abundant amounts of omega-3 PUFAs are found mainly in the poikilothermic animals, and especially marine fish, as compared to the other species.

2- We next set out to determine the amounts and duration of omega-3 PUFAs supplementation required to explore the usefulness of fish oil as the main source of PUFAs (eicosapentaenoic, EPA, 20:5 n-3 and docosahexaenoic, DHA, 22:6 n-3). The result showed that:

2.1. In freshwater fish (Poikilothermic animals)

Fish fed on an omega-3 PUFAs - deficient diet displayed a drastic decrease of n-3 fatty acids and a compensatory increase of n-6 fatty acids in the PLs of their flesh. The feeding on a fish oil and active EPA-30 diet led to an increase in the proportion of 18:1/20:4, whereas 16:0/20:4 and 18:0/20:4 decreased. Thus, the total species containing Sn-1 (18:1) increased at the expense of Sn-1 (18:0) in the active EPA-30 fed animals.

2.2. In rats (homothermic animals)

The results showed that young rats fed on n-3 fatty acid- deficient diets exhibited a drastic decrease in the amount of PE and omega-3 fatty acids (EPA and DHA) and compensatory increase of PC, saturated fatty acids and n-6 polyunsaturated fatty acids. In contrast, the rats fed on active EPA-30 contained large amounts of PE and a complementary enrichment with n-3 FAs. At the same time, dietary supplemention of active EPA-30 did not effects on the phospholipid of livers of old rats.

Also in young rats, the molecular species containing long-chain PUFAs are in general altered by feeding on active EPA-30, while those that containe C_{16} or C_{18} acids are resistant to changes.

The PUFAs showed clear preferences for the β-position. These acids (20:5 n-3 and 22:6 n-3) are typical of fish oil, both types cannot be synthesized by animals, but are derived from the diet.

2.3. Introduction of active EPA-30 in laying hen's diet:

The addition of active EPA-30 to the laying hen's diet particularly lowers plasma triacylglycerol, total cholesterol and low density lipoproteins.

There are large increases in the three different omega-3 PUFAs (18:3 n-3, 20:5 n-3 and 22:6 n-3) in hen's liver and egg yolk, giving similar values after two months of feeding on these diets.

3. Omega-3 PUFAs and pollutants:

Pesticide deltamethrin, herbicide atrazine and phenol pollutants caused an oxidation enhancement in the carp tissues and influence the PLs composition of carp erythrocyte plasma membrane.

3.1. **Pesticide deltamethrin (DM):** High concentration of it eliminated phosphoglycerides and cardiolipin and led to increase levels of palmitic (16:0) and stearic (18:0) acids as well as of arachidonic acid. At the same time, the levels of omega-3 PUFAs are significantly ($P \leq 0.01$) decreased.

3.2. **Herbicide atrazine:** The amount of choline moiety (LPC + SM + PC) increased in parallel with atrazine concentrations, in contrast, PE was decreased. According to the quantitative changes in PLs fatty acids, the long-chain monounsaturated fatty acid (MUFAs) 20:1 and 22:1 disappeared at high concentrations of atrazine. The n-6 fatty acids linoleic (18:2 n-6) and arachidonic (20:4 n-6) constituted a major proportion of the total fatty acids. The n-3 fatty acids (20:5 n-3 and 22:6 n-3) were significantly ($P \leq 0.01$) decreased in polluted fish relative to the controls.

3.3 **Phenolic compound:** The high concentrations of phenol pollutant led to an increase of PC and eliminated the phosphatidic acid (PA). Arachidonic acid (20:4 n-6) was present in greatest amounts at both low and high concentrations. The n-3 fatty acids displayed a fairly varied picture after exposure to phenol pollutant. The long-term exposure to higher phenol concentration, leads to elimination of these acids and significantly ($P \leq 0.01$) decreased n-3/n-6 ratio.

Significance of the results:

1- Marine fish and fish oils are the main source of omega-3 PUFAs. The amounts and duration of supplementation with these acids had profound qualitative and quantitative effect on the PL molecular species of the freshwater fish and young rat tissues, and these changes have implications for possible functional changes.

2- The linkage of essential fatty acids to the β-position in general would play the role of a reservoir for PUFAs and protect them from oxidation.

3- It was also established that the inclusion of active EPA-30 in laying hen's diet enriched their eggs with omega-3 PUFAs as compared to the control eggs.

4- Increases in the concentration of water pollutants led to decreased amounts of omega-3 PUFAs and caused marked differences in the polar head group, which makes the membrane more rigid and less permeable.

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