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Original Article

Fish oil increases atherosclerosis and hepatic steatosis, although decreases serum cholesterol in Wistar rat

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Abstract

BACKGROUND: It is known that fish oil consumption decreases incidence of cardiovascular disease. However, some studies showed that it increases atherosclerosis as it does not get completely metabolized by the liver. The aim of the present study was to investigate the effects of fish oil on aortic atherosclerosis, hepatic steatosis and serum lipids in rats.

METHODS: Twenty pregnant Wistar rats were fed with a fish oil-containing diet or standard diet (containing soy bean oil) during pregnancy and lactation and the pups were weaned onto the same diet. Fasting blood samples, hepatic and aortic specimens were taken from pups on day 70 postnatal. Data were analyzed with SPSS software, using t-test, Mann-Whitney test and Spearman correlation coefficient. Values of $p < 0.05$ were considered significant.

RESULTS: Medians for fatty streak in aorta of fish oil fed and soy bean oil fed pups were 1.00 and 0.00, respectively, and P value was 0.042. Also, medians for ductular cell hyperplasia of liver in fish oil fed and soy bean oil fed pups were 1.00 and 0.00, respectively, and P value was 0.014. Total cholesterol in pups fed with fish oil was 52.20 mg/dl and in pups fed with soy bean oil was 83.90 mg/dl ($p < 0.00$) and for low density lipoprotein cholesterol (LDL-C) values were 8.79 mg/dl and 13.16 mg/dl, respectively ($p = 0.031$).

CONCLUSIONS: According to the results of the present study, a diet which provided 15.9% of energy from fish oil as the only source of dietary fat, induced aortic atherosclerosis as well as hepatic steatosis in Wistar rat, although it decreased total cholesterol and LDL-C.

KEYWORDS: Fish Oil, Omega-3, Liver, Aorta.

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It is well known that fish oil consumption decreases incidence of cardiovascular disease and enhances blood lipid profile.[1-3] Lower prevalence of cardiovascular diseases in communities with higher consumption of dietary fish is attributed to preventive effect of omega-3 fatty acids from cardiovascular diseases .

However, some studies showed that fish oil increases atherosclerosis due to increase in fatty streaks in arteries.[4,5] Development of atherosclerotic plaque is a well known mechanism in cardiovascular disease pathogenesis and accumulation of macrophages in the arterial intima or so called fatty streak is a critical event in atherosclerotic plaque development.[6]

As the liver is the principal site of fatty acid metabolism, some researchers have postulated that fish oil adverse effects on atherosclerosis may be due to its very long chain of polyunsaturated fatty acids which doesn't get completely metabolized by the liver and therefore, develops hepatic steatosis and leads

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to atherosclerosis as well.[7] The pathogenesis of hepatic steatosis is complex but often explained by liver inflammation and fibrosis, mainly oxidative stress.[8] Little is known about simultaneous effect of fish oil on hepatic steatosis and aortic atherosclerosis and data regarding simultaneous effect of fish oil on aortic atherosclerosis and hepatic steatosis is variable.

Ritskes-Hoitinga et al. fed four groups of rabbits with diets containing 0%, 1%, 10% and 20% of energy from fish oil. Animals fed 10% and 20% fish oil diets developed more severe hepatic steatosis and aortic atherosclerosis. Fasting total cholesterol, low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were higher in the above mentioned animals.[7] Verschuren et al[9] and Brenner et al[10] showed that fish oil induces hepatic fatty micro-vesicle and aortic atherosclerosis in rabbits due to fatty acid peroxidation. Larter et al. fed mice a diet enriched with fish oil and observed that feeding a fish oil-enriched diet suppressed hepatic de novo lipogenesis, but failed to prevent development of steatohepatitis and they concluded that very high levels of hepatic lipoperoxides could be responsible for lipotoxic hepatocellular injury and inflammatory recruitment.[11]

However, Saraswathi et al. used diets enriched with 60 g/kg fish oil in mice and they found that fish oil decreased aortic atherosclerosis and hepatic steatosis as well as fasting total cholesterol and triglyceride.[12] Bringhenti et al. fed weaning mice with a diet enriched with fish oil and observed that these animals reduced hepatic steatosis.[13] Zampolli et al. fed mice diets with 1% fish oil; these mice had lower levels of LDL-cholesterol and triglycerides and had less atherosclerosis in the aorta at the end of study.[14] In a study performed by Casós et al. mice were fed diets containing 5% menhaden fish oil, a decrease in the surface area of atherosclerotic lesions at the aorta were observed in fish oil fed mice.[15]

The aim of the present study was to investigate simultaneous effects of a diet containing fish oil as the only source of dietary fat, on aortic atherosclerosis and hepatic steatosis in Wistar rats. Due to possible effect of serum lipid profile on aortic atherosclerosis and hepatic steatosis,[16] we examined fasting serum lipid profile as well as aortic and hepatic samples of fish oil fed rats. As the period of prenatal and postnatal development is accepted as the most important time for effect of dietary fatty acids,[17] the present study was performed from fetal period till puberty.

Methods

An experimental study was designed in that 20 virgin (70-day old) female Wistar rats weighting 164.11 ± 18.60 g and 10 male Wistar rats were purchased from Neuroscience Research Center, Shahid Beheshti University of Medical Science. After two weeks of acclimation period, they were kept for breeding in the female to male ratio of 2 to 1. Female rats were checked every morning for mating plug to establish day 0 of pregnancy. Female rats in which mating plugs had been observed, were placed individually in cages from day 0 of pregnancy and randomly allocated to two dietary groups (n=10 in each group). Dams in each group were fed one of the fish oil containing or standard diets during pregnancy and lactation and the pups were also weaned onto the same diet.

The purified diets were made weekly in Faculty of Nutrition Science and Food Technology, Shahid Beheshti University of Medical Science and stored at -20°C. The diets were similar in composition except for the types of fats used: the standard diet was formulated based on AIN93-G recommended by American Institute of Nutrition[18] for rodent feeding studies which contained 70 g/kg soybean oil as the only source of fat. Fish oil diet was the same as AIN93-G except for the type of fat used which was 70 g/kg Menhaden fish oil. The dietary ingredients are given in Table 1. As the Table indicates, 15.9% of energy of the diets was provided by fish oil or soy bean oil. Fish oil and soy bean oil used for diets were analyzed by "Oily Seeds Culture and Development Company", Tehran, Iran, and the fatty acid composition of the oils is presented in Table 2.

Animals allowed free access to food and tap water. The food was given every 48 hours and daily food intake was determined by weighting the food remaining in the cages.

A standard 12-hour photoperiod (07-19 h) and appropriate ventilation were maintained, temperature was kept at 20-25°C and humidity was kept close to 50%.

Pups birth weight and their weight and height on day 70 postnatal were measured. The height of pups couldn't be measured at birth. The birth weights were calculated as sum of the neonates' weights divided by their number.

On day 70 postnatal (day of puberty) one female offspring from each mother was randomly selected, its weight and height was measured and then fasted overnight for 12 hours. The next morning, 5 cc blood from carotid artery was taken, after a brief exposure to CO_2.

Blood samples were centrifuged at 2500 rpm for 10 minutes and freezed in -20°C. Fasting total cholesterol and triglyceride (photometry), LDL-C and HDL-C (direct enzymatic), were checked with special kits for rats (Mercodia Company, Uppsala, Sweden).

The animal was euthanized by cervical dislocation after blood sampling. One centimeter from thoracic aorta was cut immediately after euthanasia and fixed in formalin 10%. Three vertical and three horizontal sections were made from each sample and colored with Hematoxylin & Eosin. The sections were studied by an expert pathologist who wasn't aware from the type of diets. Each sample was evaluated for presence of following lesions: fatty streak, fibrous plaque, calcification in media and intimal thickening with foam cells and cholesterol crystals.[19] Each type of lesion was scored on a semi-quantitative three point scale (0: absence of aortic lesion, 1+: aortic lesion in less than 50% of the sample, 2+: aortic lesion in more than 50% of the sample).

The entire liver was also dissected out immediately after euthanasia. Liver was weighted and one piece of 1 in 2 cm from median lobe was removed and fixed in 10% buffered formalin for histological examination. Three sections were taken from each sample and stained with Hematoxylin and Eosin. Samples were examined blindly by an expert pathologist who wasn't aware from type of diets. Each sample was evaluated for presence of following lesions: lipidosis (presence of micro or macro-vesicles containing fat), ductular cell hyperplasia, hepatocellular hypertrophy and hepatocellular inflammation.[7] Each type of lesion was scored on a semi-quantitative four point scale (0: absence of liver pathology, 1+: mild, 2+: moderate, 3+: intensive).

The data were analyzed by SPSS software version 11.5 (IBM Corporation, New York, USA). The results for mean daily ingested food, weights and heights and also blood chemistry were compared between dietary groups using t-test. The results for hepatic and aortic lesions were compared between dietary groups with Mann-Whitney test. Spearman correlation coefficient was used to investigate correlation between aortic and hepatic lesions. Values of $p < 0.05$ were considered statistically significant.

The present research is extracted from a Ph.D. dissertation of National Nutrition and Food Technology Research Institute, Shahid Beheshti University by the Research Project Number of P25/47/1048 and it is approved by ethical committee of National Nutrition and Food Technology Research Institute by the number of P 47/1703.

Results

Average daily food intakes in fish oil fed and standard diet fed animals were 16.3 ± 2.5 g and 15.9 ± 1.9 g, respectively, and the difference wasn't statistically significant.

Mean birth weight in pups fed with fish oil and standard diet were 6.07 ± 0.21 g and 5.93 ± 0.19 g, respectively. Mean weight and height on day 70 postnatal in pups fed with fish oil and standard diet were also 167.40 ± 21.75 g, and 1702 ± 77 mm and 175.44 ± 3.68 g, and 1917 ± 19 mm, respectively. Mean liver weight on day 70 postnatal in pups fed with fish oil and standard diet were

6.25 ± 0.71 g and 6.46 ± 0.56 g, respectively. None of the above mentioned values were significantly different between dietary groups.

Results of analysis of fish oil and soy bean oil used for diets are presented in Table 2. As the Table indicates, omea-3 to omega-6 ratio in Menhaden fish oil and soy bean oil was 6 to 10 and 1.1 to 10, respectively. Soy bean oil contained alfa-linolenic acid (18:3 n-3) as the omega-3 fatty acid source and fish oil contained eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3).

Results of pathologic examination of aortic samples are shown in Table 3. As the Table indicates fatty streak in fish oil fed pups were significantly more than that in the other group. Table 4 shows pathological liver changes in pups. As the Table indicates, ductular cell hyperplasia in pups fed with fish oil was significantly more than that in animals fed with standard diet. There was a positive relationship between fatty streak in aorta and ductular hyperplasia in liver ($r = 0.470$ and $p= 0.037$).

Fasting blood lipids in pups are presented in Table 5. As the Table indicates, total cholesterol, LDL-C and HDL-C in pups fed with fish oil diet were significantly lower than those in pups fed with standard diet. Total cholesterol in fish oil fed pups was decreased by 37% comparing to pups fed with standard diet. LDL-C and HDL-C were also decreased by 33% and 45%, respectively. Triglyceride level was not significantly different between the two groups.

Discussion

Our results showed that animals fed with fish oil as the only source of dietary fat, developed fatty streak in aorta as well as ductular cell hyperplasia in liver more than the animals fed with standard diet. Our findings also indicated that aortic and hepatic lesions were positively correlated. The food ingested by animals, as well as their weight and height were not significantly different between dietary groups, so it seems the difference between groups may be due to the type of fats used in diets. Increased aortic and hepatic lesions may be due to presence of very long chain polyunsaturated fatty acids in fish oil which increases fatty acid oxidation. Studies showed that omega-3 polyunsaturated fatty acids from fish oil origin are very long chain with couples of double bonds; so they accumulate in hepatocytes and increase lipid peroxidation. Lipid peroxidation products inhibit glucose-6-phosphate dehydrogenase which in turn decreases NADPH production and reduced form of glutathione. Hepatic oxidative stress and hepatic steatosis positively correlate with dosage of fish oil used.[20,21]

Table 1. Composition of the fish oil containing diet and standard diet (AIN 93-G)

Component g/ kg diet	Fish oil diet	Standard diet
Casein (>85% protein)	200.000	200.000
L-Cystine	3.000	3.000
Sucrose	100.000	100.000
Cornstarch	529.486	529.486
Tertiary-Butylhydroquinone (TBHQ)	0.014	0.014
Cellulose	50.000	50.000
AIN-93G-Mineral Mix	35.000	35.000
AIN-93 Vitamin Mix	10.000	10.000
Choline chloride	2.500	2.500
Soybean oil	-	70.000
Menhaden fish oil	70.000	-
Hydrogenated cottonseed oil	-	-

Table 2. Fatty acid composition of fish oil and soybean oil

Fatty acid	Fish oil	Soybean oil
C4: 0	-	-
C6: 0	-	-
C8: 0	-	-
C10: 0	-	-
C12: 0	0.11	-
C14: 0	7.96	0.07
C14: 1	0.67	0.01
C15: 0	-	0.01
C15: 1	0.05	-
C16: 0	19.88	10.42
C16: 1	10.69	0.08
C17: 0	1.17	0.11
C17: 1	2.24	0.06
C17: 4	3.91	-
C18: 0	1.99	4.12
C18: 1t	0.32	0.07
C18: 1C	16.38	21.87
C18: 2t	0.34	0.04
C18: 2C	1.92	53.83
C18:3gamma	0.33	0.03
C20: 0	0.61	0.32
C18: 3 alpha	1.71	8.36
C20: 1	2.00	0.18
C18: 4w3	3.68	-
C20: 3w3	0.38	-
C20: 4w6	0.23	-
C22: 0	0.32	0.32
C22: 1	0.23	-
C20: 5	1.90	-
C24: 0	0.20	0.10
C22: 5	2.53	-
C22: 6	14.68	-

Fish oil and soy bean oil were analyzed by “Oily Seeds Culture and Development Company”, Tehran, Iran.

Table 3. Aortic atherosclerosis in pups fed with fish oil containing diet or standard diet †

variable	Fish oil diet n=10	Standard diet n=10	p
Fatty streak	1.00 (1.00-2.00)	0.00 (0.00-1.00)	0.042‡
Fibrous plaque	1.00 (0.75-1.00)	0.00 (0.00-1.00)	0.168
Calcification in media	0.00 (0.00-0.00)	0.00 (0.00-0.00)	1.00
Intimal thickening	1.00 (0.75-2.00)	0.00 (0.00-1.25)	0.147

† Values are median and interquartile range.
‡Significant difference with $p < 0.05$

Table 4. Hepatic steatosis in pups fed with fish oil containing diet or standard diet†

variable	Fish oil diet n=10	Standard diet n=10	p
Lipidosis	1.00 (1.00-2.00)	1.00 (1.00-2.00)	0.752
Ductular cell hyperplasia	1.00 (1.00-1.25)	0.00 (0.00-1.00)	0.014‡
Hepatocellular hypertrophy	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.317
Hepatocellular inflammation	0.00 (0.00-0.00)	0.00 (0.00-0.00)	1.00

† Values are median and interquatile range.
‡ Significant difference with $p < 0.05$

Table 5. Fasting blood lipids in pups fed with fish oil containing diet or standard diet†

Blood biochemistry	Fish oil diet n=10	Standard diet n=10	p
Total cholesterol (mg/dl)	52.20 ± 11.91	83.90 ± 16.11	0.000**
LDL-C (mg/dl)‡	8.79 ± 2.45	13.16 ± 5.39	0.031§
HDL-C (mg/dl)*	29.40 ± 6.57	53.80 ± 10.00	0.000**
Triglyceride (mg/dl)	62.57 ± 30.33	56.29 ± 9.05	0.226

† Values are mean ±SD
‡ Low density lipoprotein cholesterol
* High density lipoprotein cholesterol
** Significant difference with $p < 0.01$
§Significant difference with $p < 0.05$

Some researchers have postulated that there is a relationship between fish oil adverse effects on atherosclerosis and hepatic steatosis, as it contains very long chain omega-3 polyunsaturated fatty acids which doesn't get completely metabolized by the liver.[22]

Our results are in agreement with those of Ritskes-Hoitinga et al[7], Verschuren et al,[9] and Brenner et al[10], however our findings do not support those of Saraswathi et al[12], Bringhenti et al[13], Zampolli et al,[14] and Casós et al.[15] One possible explanation for this discrepancy is that in our study animals faced higher amounts of dietary fish oil; Saraswathi et al. used 209 g/kg of mixed oils (including coconut oil, olive oil, corn oil and soy bean oil) plus 60 g/kg fish oil, while we used 70 g/kg fish oil which was the only dietary fat source. The dosage of fish oil used in Zampolli et al[14] and Casós et al[15] studies were 1% and 5%, respectively, which was lower than 15.9% used in the present study. In the study performed by Bringhenti et al[13] animals were fed with fish oil containing diet from weaning till puberty which is a shorter period comparing to ours.

We also investigated fasting serum lipid profile in pups. According to the results of the study, fasting triglyceride was not different between the two groups while total cholesterol, LDL-C and HDL-C in pups fed with fish oil diet were significantly lower than those in pups fed with standard diet. This may be due to the effect of omega-3 polyunsaturated fatty acids in fish oil which are known to lower serum cholesterol.[23] The results are in disagreement with those of Ritskes-Hoitinga et al[7] and Saraswathi et al[12] as in both studies, there was a positive relationship between aortic atherosclerosis and hepatic steatosis with serum cholesterol. The difference may be due to the fact that although in the present study total cholesterol, LDL-C and HDL-C decreased in fish oil

fed pups, the highest degree of decrease was observed in HDL-C (45% versus 33% and 37%) which is known as an anti-risk factor for atherosclerosis. Another possible explanation for this discrepancy is that Saraswathi et al[12] used LDL-receptor deficient mice and Ritskes-Hoitinga et al[7] used rabbits which may show different reaction to fish oil. Also, dosage and duration of fish oil feeding may affect the final outcome of different studies.

The interesting finding of the present study was that aortic atherosclerosis and hepatic steatosis increased in fish oil fed pups despite the decrease in serum cholesterol. We recommend further studies to investigate small dense LDL-C particles as well as apolipoproteins and indices of oxidative stress in serum simultaneously.

Strength of the present study lies in the use of fish oil from the beginning of fetal period till puberty which is a rather long duration comparing to most of the similar studies in which fish oil is used for shorter periods. One limitation of the present study was the animal model used; Wistar rat is a rather resistant animal model for development of hypercholesterolemia and atherosclerosis. To solve this problem, we used higher doses of fish oil that is to say the whole dietary fat was provided by fish oil and also the rats were fed fish oil for a long duration from the beginning of fetal period till puberty. However, the severity of aortic atherosclerosis and hepatic steatosis which was observed in our study was lower than that of Ritskes-Hoitinga et al. who used rabbit which is a more sensitive animal model for development of hypercholesterolemia and atherosclerosis.

Conclusions

According to the results of the present study, a diet which provided 15.9% of energy from fish oil as the only source of dietary fat, induced aortic atherosclerosis as well as hepatic steatosis in Wistar rat, although it decreased total cholesterol and LDL-C. The diet also decreased HDL-C which was more prominent than the decrease in total cholesterol and LDL-C. We recommend further studies to investigate effects of fish oil on small dense LDL-C particles as well as apolipoproteins and indices of oxidative stress in serum simultaneously.

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Conflict of Interests

Authors have no conflict of interests.

Authors' Contributions

MMS coordinated the study, analyzed the data and prepared the manuscript. FAT provided assistance in the design of the study, coordinated all the experiments and participated in manuscript preparation. ARA carried out the design, provided assistance for statistical analysis and participated in manuscript preparation. MS coordinated all the experiments and participated in manuscript preparation. All authors have read and approved the content of the manuscript.

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