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Medium-chain triglycerides and monounsaturated fatty acids potentiate the beneficial effects of fish oil on selected cardiovascular risk factors in rats

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Abstract

Fish oil (FO) rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is known to reduce the risk of cardiovascular diseases (CVDs). Little information is known regarding the influence of lipid composition in the background diet on the modulatory effect of FO supplementation on CVDs. The present study was designed to investigate the influence of various background dietary lipids and FO on selected cardiovascular risk factors in rats. Adult Wistar rats were fed semisynthetic diet with FO at 1.0% or 2.0% along with other lipids, namely, medium-chain triacylglycerols (MCTs), monounsaturated fatty acids (MUFAs), n-6 polyunsaturated fatty acids (PUFAs) and n-3 PUFAs, for 5 weeks. Some of the potent CVD risk factors were estimated in the rats. FO at 1.0% and 2.0% has significantly reduced serum lipid peroxides, total cholesterol, triglycerides (TAGs), tumor necrosis factor-α, interleukin-6 and C-reactive protein; liver and adipose TAG and cholesterol levels in MCT, MUFA and n-6 PUFA diet groups. Notably, these alterations were comparatively higher in 1.0% FO-substituted MCT and MUFA diet groups. Interestingly, feeding of FO along with n-3 PUFAs did not show additive effect in attenuation of these factors. Serum liver EPA and DHA levels were remarkably elevated in rats fed FO-enriched MCT or MUFA diets. Our results suggest that MCTs or MUFAs in the background diet might promote the beneficial effects of FO on CVDs.

Keywords: Fish oil; Cardiovascular disease; n-3 PUFA; Fatty acids; Cytokines; Arachidonic acid

1. Introduction

Long-chain n-3 polyunsaturated fatty acids (LC-PUFAs) including eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) are found in fatty fish and in fish oils (FOs). Existing data suggest that dietary intake of LC-PUFAs reduces the potential cardiovascular risk factors including plasma cholesterol, triacylglycerols (TAGs), inflammatory cytokines, chemoattractants, cell adhesion molecules, eicosanoids and blood pressure and decreases the mortality in people suffering from cardiovascular diseases (CVDs) [1,2].

The important members of the n-6 and n-3 fatty acid families are linoleic (18:2n-6, LNA) and α-linolenic (18:3n-3, ALA) acids, respectively. Mammalian cells cannot synthesize LNA and ALA, but they can metabolize them to corresponding LC-PUFAs, namely, arachidonic acid (ARA), EPA and DHA, by desaturation and elongation process. The competition between n-3 LC-PUFAs and ARA reduces the availability of ARA as a substrate for eicosanoid synthesis and inhibits ARA metabolism [3].Therefore, a high dietary intake of LNA has been proposed to increase the required dose of EPA and DHA to exert their beneficial effects in various ailments including CVDs, bipolar disorders and postpartum depressions [4]. Moreover, LNA may also increase inflammation and endothelial activation [5,6]. Hence, the reduction of LNA in the diet during FO intervention may reduce the competition between ARA and EPA and promotes the accretion of EPA and DHA in the membranes.

Olive oil, a Mediterranean oil rich in oleic acid (18:1 n-9), is reported to be atheroprotective [7]. Dietary intake of olive oil reduces the plasma very low density lipoprotein (VLDL) and triglyceride levels in healthy subjects [8]. The supplementation of saturated fatty acids along with oleic acid lowers concentrations of both total and low-density lipoprotein cholesterol (LDL-C) [9]. It has been shown that monounsaturated fatty acid (MUFA) has favorable effects on insulin sensitivity in healthy subjects [10]. Moreover, the MUFAs and antioxidants of olive oil may protect the circulating lipoproteins against detrimental effects of lipid peroxides [11]. Therefore, it is reasonable to assume that consumption of olive oil and FO together may diminish the generation of lipid peroxides besides the hypolipidemic effects.

Medium-chain triglycerides (MCTs) with 8–12 carbons are found mainly in coconut oil. The MCTs are digested and transported directly to the liver through the portal venous system. On the contrary, the LC-PUFAs are incorporated into chylomicrons for transport through the lymphatic

Abbreviations: MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; LNA, linoleic acid; ALA, α-Linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LC-PUFAs, long-chain polyunsaturated fatty acids; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-4, interleukin-4; IL-4, interleukin-4; IL-10, interleukin-10; TAGs, triacylglycerols; ELISA, enzyme-linked immunosorbent assay.

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system [12]. MCTs quickly enter the hepatocyte mitochondria and undergo rapid β-oxidation to ketones [13,14], whereas most of the PUFAs remain as triglycerides in the hepatocyte. Therefore, MCTs have been used therapeutically in the treatment of fat malabsorption, cystic fibrosis and epilepsy because of the unique structure, absorption and metabolic characteristics [15]. In this context, we propose that combined feeding of FO and MCTs protects the dietary EPA and DHA against β-oxidation and elevates the hepatic EPA and DHA levels. Further, the present study aimed to investigate the influence of various background dietary lipids along with FO on selected cardiovascular risk factors in male adult rats.

2. Materials and methods

2.1. Materials

Fish oil (sea cod oil, Sanofi Pharmaceuticals, India), virgin olive oil (Delmonte brand, Spain), coconut oil (cooking grade) and sunflower oil (Sunpure brand, India) were purchased from suppliers in Mysore. Garden cress (*Lepidium sativum* L.) seeds, which are rich in ALA, were purchased from a local market in Mysore, India. The seeds were identified and authenticated at the Department of Horticultural Sciences, University of Agriculture Sciences, Bangalore, India. Seeds were air dried, flaked in a roller flaker (Kvarnmaskiner, Malmo, Germany) and stored in plastic containers. Flaked Garden cress (GC) seeds were pressed with a hydraulic press (B Sen Barry & Co., New Delhi, India) at a pressure of 10 tons for 10 min at room temperature. The oil obtained from the seeds was collected in a clean vessel and flushed with nitrogen, and the weight of the oil was recorded. The oil was stored in polyethylene terephthalate bottles at −20°C until further analysis [16]. All the chemicals used in the present study (except where specifically mentioned) were procured from Sigma Chemical Company, India.

2.2. Methods

2.2.1. Determination of fatty acid composition of experimental oils

Fatty acid compositions of oils were analyzed by gas chromatography (GC-14B, Shimadzu Corporation, Japan). Individual oils were saponified with 0.5 M KOH and methylated with 40% boron trifluoride in methanol [17].The methyl esters of fatty acids were separated on a fused silica capillary column (BP 21: 30-m length, 0.30-mm internal diameter,0.50-mm film thickness). GC was equipped with a flame ionization detector and Clarity Lite 420 integrator. The operating conditions were as follows: column temperature 220°C, injection temperature 230°C and detector temperature 240°C. Nitrogen was used as the carrier gas. Individual fatty acids were identified by comparing with the retention times of reference fatty acids (Supelco Chemical Co., St. Louis, MO, USA) quantified by Clarity Lite integrator (Data Apex, Czech Republic).

2.2.2. Estimation of antioxidants in experimental oils

Tocopherols in the oils were estimated using high-performance liquid chromatography (HPLC) method described previously [18]. In brief, tocopherols were separated using Shimadzu LC-10A (Shimadzu Corporation, Tokyo Japan) HPLC fitted with C18 column (25 cm×4 mm length, 5 μm, Supelco, USA) and fluorescence detector was used with excitation and emission wavelengths of 292 nm and 326 nm respectively. The mobile phase consists of acetonitrile/methanol/isopropanol/water (48:45:5:2) in isocratic condition at a flow rate of 1.0 ml/min. Individual tocopherol peaks (alpha, beta, gamma and delta) were identified and quantified with respective standard tocopherols. The results are expressed as total tocopherol content mg/kg of oil. Total phenolics were quantified using a colorimetric method described previously [19]. Briefly, phenolic compounds were isolated from 10 g of oil and dissolved in hexane with a water/methanol mixture (60:40, v/v). The Folin–Ciocalteau reagent (SRL Chemicals, Mumbai, India) was added to a suitable aliquot of the extracts, and the absorption of the solution was measured at 725 nm. Total phenolics were quantified using gallic acid as a standard.

2.2.3. Animals and diets

The protocol adopted for this study was approved by the Institute Animal Ethics Committee (IAEC-191/11), Central Food Technological Research Institute (CFTRI), Mysore, India. Seven-week-old male Wistar rats (n=78) weighing 95–100 g were procured from the Animal House Facility, CFTRI, Mysore, India. The rats were housed in individual cages; were acclimatized to constant conditions of humidity (70%–80%), temperature (22°C–25°C) and light (12-h light and dark cycle); and had unrestricted access to food and water. The rats were randomly assigned into 12 groups (n=6) based on the type of lipid source provided including MCTs, MCTs+F1, MCTs+F2, MUFAs, MUFAs+F1, MUFAs+F2, n-6 PUFAs, n-6 PUFAs+F1, n-6 PUFAs+F2, n-3 PUFAs, n-3 PUFAs+F1 and n-3 PUFAs+F2. Rats were fed isocaloric AIN-93 diet containing 50% starch, 10% sucrose, 20% casein, 5% cellulose, 10% oil as fat, 3.5% mineral mix, 1.0% vitamin mix, 0.2% choline chloride and 0.3% L-cysteine. FO was substituted in the diets at 1.0% and 2.0% in different native oil groups (Table 1). The rats were fed respective diets *ad libitum* for 5 weeks; food intake and body weight gain were monitored every week during the course of the experiment. The rats were terminated (overnight fasted) under mild ether anesthesia, and the blood was collected by direct heart puncture. Sera were separated and stored at −20°C for further analysis. The major organs such as liver, kidney, brain, lungs and heart were collected, weighed and stored at −80°C. The adipose tissue from the three major fat depot areas (retroperitoneal, epididymal and inguinal) was collected, weighed and stored at −80°C until the analysis.

2.2.4. Analysis of clinical markers in the serum

Serum glutamic–oxaloacetic transaminase (SGOT), serum glutamic–pyruvic transaminase (SGPT), ALP (alkaline phosphatase) and urea were analyzed using diagnostic kits (Agappe, Bangalore, India). The concentrations were determined according to the manufacturer's instructions [20].

2.2.5. Determination of lipid peroxides in the serum

Thiobarbituric acid reactive substances in serum were estimated as an indicator of lipid peroxides based on the method described previously [21]. Briefly, 0.05 ml of serum was added to a reaction mixture containing 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 0.8% thiobarbituric acid and 0.7 ml of distilled water. Samples were boiled for 1 h at 95°C and centrifuged at 3000×g for 10 min. The absorbance of the solution was measured spectrophotometrically at 532 nm. The lipid peroxides were quantified using tetramethoxy propane as a standard.

2.2.6. Analysis of serum lipid profile

Serum TAGs, LDL-C, high-density lipoprotein cholesterol (HDL-C) and total cholesterol levels were analyzed using diagnostic kits (Agappe, Bangalore, India). The concentrations were determined according to the manufacturer's instructions. The principle and methodology were described elsewhere briefly [20].

2.2.7. Determination of triglycerides and cholesterol in liver and adipose tissues

The liver and adipose tissues were homogenized in 0.74% potassium chloride solution; the total lipids were extracted using 2:1 chloroform and methanol according to the method of Folch *et al.* [22]. Aliquots of lipid extract (0.2 ml) were used to measure cholesterol using colorimetric method suggested by Searcy and Bergquist [23].The triglyceride content in the lipid extract was measured using the colorimetric method described by Fletcher [24]. The results are expressed as mg/g of tissue.

2.2.8. Analysis of cytokines in the serum

Serum tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-4 and IL-10 were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen Corporation, Frederick, MD, USA) according to manufacturer's instructions. The kits are based on solid-phase sandwich ELISA. Briefly, the monoclonal antibodies specific for rat TNF-α, IL-6, IL-4 and IL-10 coated onto the wells of the microtiter strips provided by the ELISA kit were used. One hundred microliters of the diluted serum (1:1) samples was loaded into the wells and incubated for 3 h at room temperature with gentle swirling. After washing thrice, 100 μl of biotinylated monoclonal antibodies specific to these cytokines was added to the wells and incubated for 1 h at room temperature. After removal of excess antibody, 100 μl of streptavidin–horseradish peroxidase solution was added and incubated at room temperature for 30 min. The wells were washed, and 100 μl of stabilized tetramethylbenzidine solution was added and incubated at room temperature in dark. The optical density of the samples after adding 100 μl of stop solution was measured at 450 nm. The calibration curves of these cytokines were plotted in the same manner using purified recombinant standards of these cytokines supplied in the kit. The concentration of each cytokine was determined by comparing to the respective calibration curve. The minimum detectable concentration of TNF-α, IL-6, IL-4 and IL-10 is <4 pg/ml, <5 pg/ml, <2 pg/ml and <5 pg/ml, respectively.

Table 1
Lipid composition of the experimental diets

Component (g/kg)	MCT	MCT+F1	MCT+F2	MUFA	MUFA+F1	MUFA+F2	n-6 PUFA	n-6+F1	n-6+F2	n-3 PUFA	n-3+F1	n-3+F2
Coconut oil	100	90	80	0	0	0	0	0	0	0	0	0
Olive oil	0	0	0	100	90	80	0	0	0	0	0	0
Sunflower oil	0	0	0	0	0	0	100	90	80	0	0	0
Garden cress oil	0	0	0	0	0	0	0	0	0	100	90	80
Fish oil	0	10	20	0	10	20	0	10	20	0	10	20

Coconut oil is a source of MCT, olive oil is a source of MUFA, sunflower oil is source of n-6 PUFA, and garden cress seed oil is a source of n-3 PUFA.