Antihyperlipidemic Effect of Microbially Converted Eicosapentaenoic Acid from Rice Bran Oil in Rats

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Abstract

Objective: Eicosapentaenoic acid (EPA) from fish oil is known to have favourable effects on cardiovascular system by lowering the elevated lipid levels. With the intention of producing an alternative vegetarian source to fish EPA, we have microbially synthesized EPA (mEPA) from α- linolenic acid (ALA) isolated from rice bran oil which was spectroscopically analysed. The objective of the present study was to evaluate the effect of mEPA on lipid profile in experimentally induced hyperlipidemic rats.
Methods: Animals were divided into 12 groups of six animals each; control being treated with vehicle, 6 groups received normal diet while 6 groups received high fat diet. Both treatment groups received standard drug Atorvastatin (10 mg/ kg in saline, p.o.); three different doses of EPA (5, 10 and 50 mg/kg) and fish oil (1 g/ kg) for 28 days and were compared with sham hyperlipidemic rats. The hyperlipidemia was induced by high fat diet and lipid profile was estimated.
Results: The elevated levels of LDL, VLDL and triglycerides in high fat diet induced animals were found to be decreased in the treatment groups whereas the decreased levels of HDL in hyperlipidemic group were significantly increased in the mEPA treated groups. The atherogenic index was improved with mEPA treatment.
Conclusion: mEPA has shown promising antihyperlipidemic effect and can fulfil the need of alternative vegetarian source of EPA than fish oil to be used in the hypertensive hyperlipidemic conditions.

Key words: Antihyperlipidemic, Eicosapentaenoic acid, hyperlipidemia, High fat diet, Lipid profile

Introduction

Cardiovascular diseases are a major health concern in recent years throughout the world. Among which ischemic heart disease, stroke are the prominent diseases. Hypercholesterolemia is recognized as a risk factor for ischemic heart disease and coronary mortality[1]. Hyperlipidemia refers to elevated levels of lipids and cholesterol in the blood, and is also identified as dyslipidemia, to describe the manifestations of different disorders of lipoprotein metabolism. Although elevated low density lipoprotein cholesterol (LDL) is thought to be the best indicator of atherosclerosis risk, dyslipidemia can also describe elevated total cholesterol (TC) or triglycerides (TG), or low levels of high density lipoprotein cholesterol (HDL)[2]. Usually this is because one of the coronary arteries that supplies blood to the heart develops a blockage due to an unstable buildup of white blood cells, cholesterol and fat[3]. The known risk factors for cardiovascular disorders include hyperlipi-demia[4]. Hypercholesterolemia is associated with an enhanced platelet thrombus formation on an injured artery, increasing the propensity for acute thrombosis [1]. Cholesterol lowering may therefore reduce the risk of acute coronary events in part by reducing the thrombogenic risk. The CVS protective effects of n-3 PUFAs mainly found in fish oil have been recognized in literature[5,6]. As fish oil or mediterranean diet contains mainly EPA and DHA, the benefits of fish oil can be related with these LCPUFAs (long chain polyunsaturated fatty acids) which are not synthesized in plants. The benefits of fatty fish consumption have been explored in cell culture and animal studies, as well as randomized controlled trials investigating the cardioprotective effects of omega-3 fatty acids[7]. These n-3 PUFAs inhibit the oxidative modifications that are responsible for the development of atherosclerosis, and related cardiovascular diseases[8,9]. These fatty acids also have potent anti-inflammatory effects, and may also be antithrombotic and anti-atherogenic [7].

LCPUFAs, especially EPA in fish oil is shown to stabilize atherosclerotic plaques and inhibit experime-ntal atherosclerosis[8]. Fish oil is the only source of EPA so far which may be unacceptable by the vegetarian population. They have α- linolenic acid (ALA) from plant source as the available source of omega 3 fatty acids. Plant oil rich in omega-3 contents gets converted to EPA in vivo but does not provide the sufficient amount of EPA and also forms arachidonic acid (AA) which is proinflammatory. Hence to avoid these problems, the EPA has been synthesized in our laboratory in 2012[10] by microbial transformation of

ALA isolated from rice bran oil (RBO) and has been confirmed by chromatographic techniques.

The objective of present study was to observe and elucidate the possible mechanism for the effect of our microbially converted EPA (mEPA) in experimentally induced hyperlipidemic rats by evaluating its action on lipid profile.

Materials and Methods

Drugs and Chemicals: The kits for the lipid profile estimation were obtained from Span Diagnostics and Autopak Siemens. Atorvastatin was obtained from Sun Pharmaceuticals Industries Ltd, Mumbai. Other chemicals used were of analytical grade.

Experimental animals and treatment: Adult male wistar rats weighing around 150-200 g were divided into seven groups of six animals each. They were maintained under normal laboratory conditions of temperature $24 \pm 2\ ^0$C and natural light-dark cycle and had free access to drinking water and standard pellet diet. The protocols of animal studies were approved by Institutional Animal Ethical Committee of Sharad Pawar College of Pharmacy, Nagpur (Reg. No. 536/02/CPCSEA, dated 20.01.02).

Based on the results of toxicity studies, three doses were selected for mEPA as 5, 10 and 50 mg/ kg and fish oil as 1 g/ kg and were administered by oral route in Tween 80 as vehicle.
The animals were treated as follows:

Group 1   : Normal diet- control vehicle group p.o.
Group 2   : Normal diet + (Standard) Atorvastatin (10 mg/ kg in saline, p.o.)
Group 3   : Normal diet+ mEPA (5 mg/kg, p.o.)
Group 4   : Normal diet + mEPA (10 mg/kg, p.o.)
Group 5   : Normal diet + mEPA (50 mg/kg, p.o.)
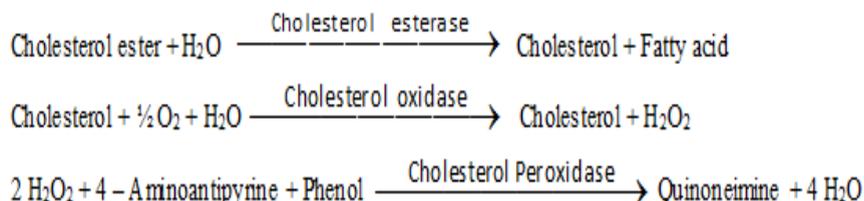Group 6   : Normal diet + Fish oil (1 g/ kg, p.o.)

Group 7   : High Fat Diet (HFD)-control vehicle group p.o.
Group 8   : HFD + (Standard) Atorvastatin (10 mg/ kg, in saline, p.o.)
Group 9   : HFD + mEPA (5 mg/kg, p.o.)
Group 10   : HFD + mEPA (10 mg/kg, p.o.)
Group 11   : HFD + mEPA (50 mg/kg)
Group 12   : HFD + Fish oil (1 g/ kg)

Group 1 to 6 were fed with normal pellet diet while groups 7 to 12 were fed with 12 g of high fat diet [11] for 28 days, i. e. throughout the study with water ad libitum. The animals were treated with respective treatments i.e. mEPA, fish oil, standard drug atorvastatin or vehicle daily by oral route for 28 days from day 1.

After the treatments of 28 days, the blood samples were taken from the retro-orbital plexus and centrifuged at 1500 rpm for 10 min in Remi's cooling centrifuge and serum thus obtained was used for biochemical analysis.

Estimation of lipid profile: The serum samples were analyzed for total cholesterol (TC), triglycerides (TG) and high density lipoprotein (HDL) by CHOD-PAP, GPO-PAP and PEG-CHOD-PAP methods respectively using commercial kits provided by Biosystems Diagnostics and Precision Biotech with autoanalyzer. Other types of lipoproteins and their ratios were calculated using the values of TC, TG and HDL [12].

1. Very low density lipoprotein (VLDL) cholesterol = TG/5
2. Low density lipoprotein (LDL) = LDL (mg/100 ml) = TC _ (HDL + VLDL)
3. Atherogenic Index = log (TG / HDL)

The analytical procedures are briefly described as follows.

Estimation of Total Cholesterol (CHOD-PAP method)
Chemicals
Reagent: Sodium cholate, phenol cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine; Cholesterol Standard: 200 mg/dL (5, 18 mmol/L)

Method
1.0 ml of reagent was mixed thoroughly with 10 µL of standard and serum sample and the tubes were incubated for 10 minutes at room temperature (16-25^{0}C) or for 5 minutes at 37^{0}C. The absorbance of the Standard and Sample was measured at 500 nm against the Blank (1.0 ml of reagent) using spectrophotometer. Free and esterified cholesterol in the sample forms a coloured complex by means of the coupled reactions described below.

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + \tfrac{1}{2}O_2 + H_2O \xrightarrow{\text{Cholesterol oxidase}} \text{Cholesterol} + H_2O_2$$

$$2\,H_2O_2 + 4-\text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{Cholesterol Peroxidase}} \text{Quinoneimine} + 4\,H_2O$$

Estimation of triglycerides (GPO-PAP method)
Chemicals
Reagent: Triglyceride standard, sodium phosphate buffer, hydrogen peroxide, aminoantipyrine and triglyceride enzyme mixture; Triglyceride standard

Method
   The GPO-PAP method is based on colourimetric enzymatic test as described below. In the test tubes, 1 ml reagent was mixed with 10 µL sample and standard. The tubes were incubated for 5 minutes at 37^0C or 10 minutes at room temperature, and absorbance (ΔA) of standard / sample against reagent blank was read at 505 nm (505-520).

$$Triglycerides \xrightarrow{UPL} Glycerol + fatty\ Acids$$

$$Glycerol + ATP \xrightarrow{GK} Glycerol\text{-}3\text{-}Phosphate + ADP$$

$$Glycerol + 3\,Phosphate + O_2 \xrightarrow{GFO} Dihydroxy\ Acetone\ Phosphate + H_2O_2$$

$$2H_2O_2 + Amino\,antipyrine \xrightarrow{POD} Quinonimine + 4\ H_2O$$
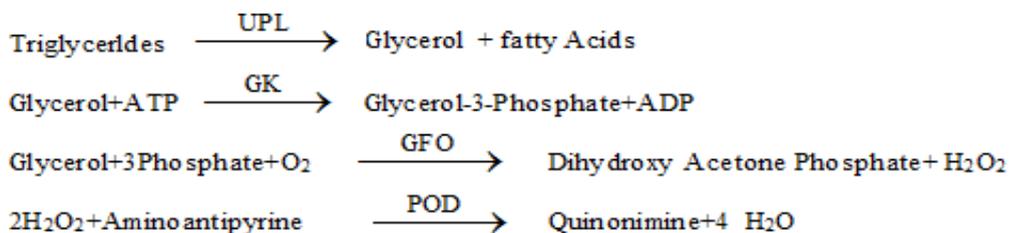
Estimation of High Density Lipoprotein (HDL)
Chemicals
HDL Cholesterol enzyme mixture, disodium N, N-bis (4-sulfobutyl)-m-toluidine, accelerator, 4-aminoantipyr-ine, restrainer, ascorbate oxidase, PEG 6000, stabilizer

Method
   In this method, 0.2 ml plasma sample was mixed with 0.2 ml precipitating reagent (PEG 6000, stabilizer and N, N- bis (4-sulphobutyl) –m- toludine disodium salt as preservative), followed by 10 minutes incubation at room temperature. The lipoproteins like LDL, VLDL and chylomicrons were precipitated. The mixture was centrifuged at 2000 rpm for 15 minutes. The supernatant containing HDL was separated and mixed with working cholesterol reagent. The HDL cholesterol was estimated by CHOD-PAP method of cholesterol estimation.

Statistical analysis: All the data is expressed in Mean ± SD. The statistical significance between more than one groups were tested by one way ANOVA using Graph Pad Prism software Version 6.04 or unless specified. The level of significance used are * $p \le 0.05$; ** $p \le 0.01$; *** $p \le 0.001$; **** $p \le 0.0001$.

Results
Effect of HFD on lipid profile of rats: The high-fat diet resulted in significant increase of plasma lipids including the total cholesterol (from 92.20 ± 8.1 mg/ dl to 266.0 ± 11.45 mg/ dl; 288.5 %), triglycerides (94.17 ± 7.9 mg/ dl to 230.2 ± 18.69 mg/ dl; 244.45 %), LDL (49.70 ± 15.4 mg/ dl to 201.3 ± 14.0 mg/ dl; 405.03 %)

and VLDL (18.83±1.6 to 46.04±3.73mg/dl; 244.50%) as compared to the control. These significant rises were accompanied by significant declines of plasma HDL by % as compared to the control (17.71±2.27 mg/dl to 12.84±2.4 mg/dl; 72.50%). The LDL:HDL and TC:HDL ratios were significantly increased in hypercholesterolemic rats by +526.83% (2.422±1.4 to 12.76±6.7) and +389.71% (4.280±1.7 to 16.68±8.2) respectively, as compared with the control. But atherogenic index (AI) was not significantly changed from 0.615±0.22 to 1.121±0.22; 182.27%). (Table 1, 2, 3)

Effect of mEPA on lipid profile of hypercholesterolemic rats: The oral administration of 50mg/kg dose of mEPA for 28 days to hypercholesterolemic rats resulted in significant declines in plasma total cholesterol, TG, LDL and VLDL-cholesterol by 62.78%, 63.03%, 84.57% and 69.05%, respectively, as compared to the hypercholesterolemic animals. The HDL, AI, TC:HDL and LDL: HDL ratios were significantly improved by + 276.48%, 62.71%, 81.5% and 87.77% respectively; as compared to hypercholesterolemic animals. The dose of 10 mg/kg also showed significant actions on lipid profile parameters however antihyperlipidemic effect exhibited by mEPA at the oral dose of 50 mg/kg/day was more pronounced and comparable to standard drug atorvastatin and fish oil (Table 1, 2, 3).
   The effects of mEPA on lipid profile parameters of rats receiving normal diet were not different from untreated control rats (Results not included).

Table 1: Effect of mEPA, standard drug atorvastatin and fish oil in hypercholesterolemic rats on total cholesterol (TC) and triglycerides (TG)

Groups	TC (mg/ dl)	TG (mg/ dl)
Control	92.20±8.1	94.17±7.9
HFD induced Sham control [a]	266.0±11.45 ***	230.2±18.69 **
Standard (Atorvastatin 10mg/ kg)	104.8±4.42 **	97.01±9.23 **
mEPA (5mg/kg)	200.0±9.22 *	198.8±19.8
mEPA (10mg/kg)	102.3±11.4 **	118.6±6.12 *
mEPA (50mg/kg)	99.0±22.4 **	85.10±8.0 **
Fish oil (1g/kg)	100.0±9.7 **	105.5±12.82 **

Table 2: Effect of mEPA, standard drug atorvastatin and fish oil in hypercholesterolemic rats on VLDL, LDL and HDL

Groups	VLDL (mg/ dl)	LDL (mg/ dl)	HDL (mg/ dl)
Control	18.83±1.6	49.70±15.4	17.71±2.27
HFD induced Sham control[a]	46.04±3.73 **	201.3±14.0 **	12.84±2.4*
Standard (Atorvastatin 10mg/kg)	19.40±1.85 **	52.39±5.31 **	26.50±5.15
mEPA (5 mg/kg)	39.77±4.0	133.0±20.83	26.80±9.8
mEPA (10 mg/kg)	29.96±2.0 *	54.02±18.07 *	28.40±6.0
mEPA (50 mg/kg)	14.25±1.4 **	31.06±8.8 **	35.5±3.60 **
Fish oil (1 g/kg)	21.10±2.6 **	53.30±10.75**	34.43±3.5 **

Table 3: Effect of mEPA, standard drug atorvastatin and fish oil in hypercholesterolemic rats on LDL: HDL, TC: HDL ratios and atherogenic index

Groups	LDL: HDL ratio	TC: HDL ratio	Atherogenic Index (AI)
Control	2.422±1.4	4.280±1.7	0.615±0.22
HFD induced Sham control [a]	12.76±6.7	16.68±8.2	1.121±0.22
Standard (Atorvastatin 10mg/kg)	1.936±0.77	3.745±0.94	0.569±0.12 *
mEPA (5mg/kg)	5.637±2.5	8.266±3.02	0.89±0.14
mEPA (10mg/kg)	2.068±1.07	2.782±1.29	0.552±0.05
mEPA (50mg/kg)	1.561±0.87 *	3.087±0.87 *	0.418±0.05 *
Fish oil (1g/kg)	2.303±1.039*	4.172±1.25 *	0.629±0.11 *

N= 6; Values are expressed in Mean ± SD. [a] control group compared with sham control group; treated groups are compared with sham control group. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.
TC-Total Cholesterol, TG- Triglycerides, LDL- Low Density Lipoprotein, VLDL- Very Low Density Lipoprotein, HDL- High Density Lipoprotein

Discussion

The present study has been undertaken to evaluate and confirm the antihyperlipidemic action exerted by microbially synthesized EPA. As fish oil LCPUFAs are well known to have beneficial effects on heart diseases and hypercholesterolemia, it was necessary to prove the similar actions of EPA synthesized in our laboratory. High fat diet induced hyperlipidemia was employed to study the effect of mEPA on various lipid profile parameters.

The effect of mEPA on the lipid profile of normal and hypercholesterolemic rats was evaluated. Hyperlipidemia was induced by high fat diet. The animals were given the respective treatments for 28 days; blood samples were withdrawn after completion of treatment period and subjected to estimation of lipid profile. The serum levels of TC, TG, VLDL, LDL were significantly elevated in hyperlipidemic rats while there was significant decline in HDL level. In the treatment groups, these elevated levels were found to be reduced dose dependently, the results being maximum with 50 mg/kg dose of mEPA. The HDL (good cholesterol) level was increased with mEPA indicating its protective role in hyperlipidenia. The results were non-significant in normal rats. The dose of 10 mg/kg also showed significant actions on lipid profile parameters however antihyperlipidemic effect exhibited by mEPA at the oral dose of 50mg/kg was more pronounced and comparable to standard drug atorvastatin and fish oil (Table 19, 20 and 21).

Except for the HDL cholesterol, high level of all lipids in the blood is arguably a high risk factor in the onset of cardiovascular disorders. High serum

concentrations of cholesterol, triglycerides and LDL have adverse effects on human health.

They are reportedly a major cause of cardiovascular derangements such as atherosclerosis, myocardial infarction and coronary heart diseases[13].

mEPA has shown antihyperlipidemic activity as seen from the results. This observation is similar to the study of Moore et al[14], who has reported the reducing action of oily fish on serum triglycerides in humans. LDL and VLDL carry and deposit cholesterol in the peripheral tissues whereas HDL transports cholesterol from peripheral tissues to liver and aids its excretion. Thus increase in LDL and VLDL is atherogenic and LDL/HDL ratio is often used as an index for cardiovascular disorders[15]. In this study the LDL/HDL ratio in all the mEPA treated groups was less than the control and high fat diet sham group, thus further strengthening the antihyperlipidemic properties of mEPA. As HDL level was increased, atherogenic index also improved with mEPA.

There is a possibility that mEPA possess the ability to facilitate the transport of cholesterol and triglycerides from the blood into tissues. This may have probably occurred through the induction or suppression of certain enzymes critical to the metabolism of these lipids[13].

Conclusion

EPA has been synthesized by our team by microbial transformation of ALA isolated from rice bran oil. This avoids the fish oil as EPA source and hence fulfils the need of LCPUFAs of vegetarian population. mEPA has also shown the promising effect on antihyperlipidemic effect as indicated by alteration of lipid profile parameters. Hence it may serve as an alternative source of EPA for the treatment of hyperlipidemia, atherosclerosis and cardiovascular disorders.

Conflict of Interest: None

Source of Support: Nil

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