Protective effect of eicosapentaenoic acid-docosahexaenoic acid and α–linolenic acid rich phytosterol ester on brain antioxidant status and brain lipid composition in hypercholesterolemic rats

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With a view to investigate the ameliorative effects of sitosterol esters against degenerative effects of hypercholesterolemia brain antioxidant enzyme assays, brain lipid profile, brain phospholipid compositional change and brain neurotransmitter concentrates (glutamic acid, asparctic acid, glycine) were measured in hypercholesterolemic rats. The results indicated that phytosterol esters have a role in countering hypercholesterolemia-related changes in the brain by decreasing the cholesterol levels, increasing the phospholipid levels and increasing the level of antioxidant enzymes. The results suggest that phytosterol esters may be of therapeutic significance and may offer new and effective options for the treatment of hypercholesterolemia-induced changes in the brain.

Keywords: Alpha-Linolenic acid, Antioxidant enzymes, Docosahexaenoic acid, Eicosapentaenoic acid, Hypercholesterolemia, Lipid profile, Neurotransmitter amino acids, Sterol esters

Cholesterol level is one of the multiple factors, other than familial genetic mutations, that can influence β-amyloid (Aβ) metabolism and accumulate in different neurological disorders. Hypercholesterolemia can result in the damage to endothelial cells of arteries and capillary vessels, a decrease in blood flow, the impairment of metabolism, and the decrease in nutritive and oxygen levels in the brain, thus it increases the possibility of cognitive impairment. Disturbances in cholesterol homeostasis may contribute to the etiology of neurological disorders by promoting Aβ generation. The first indication of a connection between cholesterol level and the accumulation of Aβ plaques was shown in rabbits as a result of a 2% cholesterol-enriched diet administered for 4, 6 and 8 weeks. These data have shed insight into the relationship between onset of dementia and cholesterol level.

Docosahexaenoic acid [DHA, 22:6(n-3)] is the important polyunsaturated fatty acid required in the mammalian brain, and its levels in brain membrane lipids are altered by the type and amount of fatty acids in the diet, and with life stage; increasing with development and decreasing with aging. Mammals obtain DHA either as DHA itself or the precursor α-linolenic acid [ALA,18:3(n-3)], and intermediates between ALA and DHA, including eicosapentaenoic acid [EPA, 20:5(n-3)]. Synthesis of DHA and EPA occurs in phytoplankton and animals, but not in plants. DHA and EPA are absent from all vegetable fats and oils, including nuts, grains, and seeds and are also very low in ruminant fats, including milk and dairy products. The richest dietary sources are fish and sea foods, but poultry and eggs provide lower, but important, sources of EPA and DHA. The major dietary sources of ALA are soybean and canola oils; flax seed oils and some nuts are also high in ALA, but these latter sources are not usually consumed consistently or in large quantities. Once obtained from the diet, ALA can be further metabolized by δ-6 desaturation, elongation, and δ-5 desaturation to EPA on the endoplasmic reticulum. Although the conversion of ALA to DHA appears to be higher in women than in men, and increased in pregnancy, increased dietary intake of ALA does not increase DHA in blood lipids of either pregnant women or their newborn infants. Early studies addressing the low blood (plasma and red blood cell membrane) lipid DHA in infant-formula suggested low

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(immature) activity of fatty acid desaturase enzyme activity in newborns. More recent stable isotope tracer studies have now shown that ALA conversion to DHA is as high in preterm as in term gestation infants and is similar to that in adult men. Thus, low rates of DHA synthesis from ALA appear to be a general characteristic of human metabolism, with the slowest step in (n-3) fatty acid desaturation being at the conversion of EPA to DHA. Dietary DHA, however, is well absorbed and readily incorporated into plasma and blood cell lipids in humans (as shown in many studies relating DHA intake from fish and fish oils to cardiovascular disease risk endpoints and inflammatory mediators). Before birth, DHA is transported across the placenta via pathways involving fatty acid binding, and transport proteins are then released to the fetal circulation. Observational and intervention studies concur that higher dietary intake of DHA during pregnancy results in higher maternal-to-fetal transfer of DHA.

After birth, the infant is provided with DHA in mother’s milk. However, human milk levels of DHA vary from 0.1 to 1.0 g/100 g milk fatty acids, a result of differences in the amount of DHA in the mother’s diet. As in adults, increasing the intakes of DHA, but not ALA, from human milk or milk substitutes results in higher blood lipid levels of DHA in the recipient infant. Analyses of human infant autopsy tissue have shown lower brain cortex DHA; 15% lower, in infants fed formula with no DHA than in breast-fed infants. Chronic dietary restriction of (n-3) fatty acid in developing animals results in reduced brain DHA, increased brain levels of (n-6) fatty acids, including DPA, and deficits in behavioral tasks of learning. Animal models addressing the role of DHA in the developing brain use dietary restriction of all (n-3) fatty acids, including ALA, EPA, and DHA, to overcome efficient conversion of ALA to DHA, particularly in rodents. Typically, brain DHA levels are 50–80% lower in (n-3) fatty acid–deficient animals than control animals, and this, together with usual issues relating to species differences, needs to be considered in extrapolating from studies with animals to humans.

Li et al. concluded that "β-sitosterol confers protection against thymocytes by regulation of the intracellular redox balance which is carried out via the scavenging of ROS and maintenance of mitochondrial membrane stability." They tested the effect of β-sitosterol on cultured thymus cells. They found that phytochemical reduced irradiation-induced cell death, DNA damage and intracellular reactive oxygen species, but increased antioxidant enzymes.

Esterification of β-sitosterol with fish oil, a rich source of EPA and DHA and with linseed oil, a rich source of ALA incurs the beneficial effects of β-sitosterol as well as the different fatty acids. Moreover sitosterol ester has much better solubility and acceptability than individual sitosterol. The aim of our study is to investigate the protective effects of EPA-DHA rich sterol ester and ALA rich sterol ester on brain antioxidant status and brain lipid and amino acid composition in hypercholesterolemic rats.

Materials and Methods

Preparation of β-sitosterol esters—A standard β-sitosterol sample was procured from Fluka Chemicals and analyzed at the laboratory by gas chromatography (GC). Fish oil (Mega-Shelcal capsules from Elder Pharmaceuticals, India) was used as the source of eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA), and the GC analysis of the fish oil showed that the oil contained 32% EPA and 22% DHA. Refined, bleached linseed oil procured from V.K.V.K. Oil Limited, Kolkata, India, was used as the source of alpha linolenic acid (ALA), and the GC analysis of the oil showed the presence of 54% ALnA in the oil. Thermomyces lanuginosus lipase (Lipozyme TLIM), used as biocatalyst, was a generous gift from Novozyme India, Ltd., Bangalore, India. Phytosterol esters were formed by enzymatic transesterification reactions in a packed bed reactor and their fatty acid compositions were analyzed by GC.

Fatty acid compositional analysis of phytosterol esters—The percent compositions of various sterol esters according to fatty acid compositions were determined by GC. The GC instrument (Agilent, model 6890 N) used was equipped with a FID detector and capillary HP 5 column (30 mL, 0.32 mm I.D, 0.25 µm FT). N2, H2 and airflow rates were maintained at 1, 30 and 300 mL/min, respectively. Inlet and detector temperature was kept at 250 and 275 °C, respectively, and the oven temperature was programmed at 65–230–280 °C with a 1-min hold at 65 °C and an increase rate of 20 °C/min and 1 min hold up to 230 and 8°C/min with 24 min hold up to 280 °C. Sterol esters were fractionated according to the fatty acid composition from which the amount of each fatty acid incorporated in the ester was calculated. The retention time (Rt) of each sterol ester was then released to the fetal circulation.
had been previously standardized in GC by preparing esters of β-sitosterol with different fatty acids.

**Animals**—Animal experiments were conducted according to the guidelines of Animal Ethical Committee of Department of Chemical Technology, University of Calcutta. Adult male albino rats of Wistar strain were housed and given balanced amount of food and water. The duration of the experimental period was 32 days. The animals were divided into the following 6 groups of six rats each: Gr. I: vehicle treated control animals, Gr. II: rats were fed with a high cholesterol diet (rat stock diet supplemented with 1% cholesterol) for 32 days. These rats served as hypercholesterolemic rats. Gr. III-VI were hypercholesterolemic rats given appropriate treatments; Gr. III: rats received EPA-DHA phytosterol ester (0.25 g/kg body weight/day, oral) for 25 days alongwith a high cholesterol diet for 32 days, Gr. IV: rats received EPA-DHA phytosterol ester (0.5 g/kg body weight/day, oral) for 25 days along with a high cholesterol diet for 32 days, Gr. V: rats received ALA phytosterol ester (0.25 g/kg body weight/day, oral) for 25 days along with a high cholesterol diet for 32 days, Gr. VI: rats received ALA phytosterol ester (0.5 g/kg body weight/day, oral) for 25 days along with a high cholesterol diet for 32 days. Groundnut oil was used as the vehicle and given to all the groups. At the end of the experiment the feeding of rats was stopped and after 12 h fasting, the rats were anesthetized by chloroform and 5 mL of blood was taken from the heart. Brain tissue was extracted from all the groups and stored at -20 °C.

**Brain lipid extraction**—Brain lipids were extracted as per Folch et al. Tissue (1 g) was homogenized with 1 mL of 0.74% potassium chloride and 2 mL of different proportions of chloroform and methanol for 2 min and then centrifuged. The mixture was left overnight and the chloroform layer was filtered through a Whatman filter paper (no.1). The chloroform layer was dried, the tissue lipid contents were measured and the lipid was used for lipid analysis. The extracted lipids were used for the estimation of different lipid components of brain.

**Brain fatty acid compositional analysis**—All the fatty acids of the brain lipids were analyzed by GC. Fatty acid methyl esters (FAME) were prepared by the method described by Metcalfe and the compositions were determined by GC analysis using an analytical gas chromatography (Agilent 6890 Series Gas chromatograph) equipped with a Flame Ionization Detector (FID) and HP-Wax capillary column (J & W Scientific Columns From Agilent Technologies) of 30 m length with 0.25 mm (id) and 0.25 mm (film thickness). The GC inlet temperature and FID detector temperature was maintained at 250 °C and oven temperature was maintained at 250 °C for 2 min, then temp was increased at 10 °C /min, up to 280 °C, then 20 min hold at 280 °C. The gas flow was 30 mL/min, 300 mL/min and 29 mL/min for hydrogen, air and nitrogen respectively.

**Brain amino acid assay**—About 0.1 g of brain tissue was taken and digested for 6 h with 6 N HCl. The digested product was filtered and converted to N-[2,2-bis(ethoxy carbonyl) vinyl] derivatives by for easy spectrophotometric detection at 280 nm. Amino acid profile was determined by HPLC (Waters 2487 with UV detector, column; Nova-pak C18, 3.9 × 150 mm) analysis after hydrolysis of protein and derivatizing the amino acids with diethyl-ethoxy-methylenemalonate.

**Column chromatographic separation of different lipid components of brain**—The column chromatographic procedure for the isolation of different brain lipid components was employed on silicic acid column for fractionation of lipids on the basis of their ionic charge. A portion of the total lipid extracted from each tissue was evaporated to dryness and its constituents were subsequently fractionated on a 20 × 2.5 cm silicic acid column. Cholesterol was eluted from this column with chloroform-hexane 1:1 (200 mL); ceramide with chloroform-methanol 97:3 (150 mL); cerebroside with chloroform-methanol 4:1 (200 mL) and phospholipid with methanol (200 mL).

**Analysis of different components of brain phospholipids**—The brain phospholipids were separated by TLC (Hexane:diethyl ether-8:2) and extracted with diethyl ether. The extracted phospholipids were separated by reversed-phase HPLC.

**Brain phospholipids fatty acid compositional analysis**—The fatty acid composition of the brain phospholipids were analyzed using GC by separating the phospholipids by thin layer chromatography (TLC).

**Brain antioxidant enzyme assay**—Measured amounts of brain tissues were taken and then homogenized in different concentrations of phosphate buffer. The activity of catalase (CAT) was determined spectrometrically by the method of Aebi. Superoxide dismutase (SOD) activity was assayed by measuring the auto oxidation of haematoxylin as described by Martin et al. Reduced Glutathione (GSH) was
determined by the method of Ellman. Total activity of glutathione peroxidase (GPx) was determined according to Flohe et al. All the enzyme activities were expressed in terms of enzyme units per mg protein. Protein was determined using the standard method of Lowry et al.

Brain MDA analysis—MDA levels in brain tissue were estimated as thiobarbituric acid (TBA)-reactive substances by the method of Prasad et al.

Statistical analysis—All results were expressed as the mean value ± SE. Statistical significance of the difference among values was analyzed by one-way ANOVA followed by Tukey test. Results were considered significant at $P<0.05$.

Results

Fatty acid composition of phytosterol esters—The transesterification reaction was carried out in the packed bed reactor. In the first set of reaction fish oil was used as a source of EPA and DHA fatty acids and in the second set, flaxseed oil was used as a source of ALA fatty acid. Approximately 32% EPA and 22% DHA was present in fish oil and 54% ALA in flaxseed oil. Analysis of fatty acid composition of sterol-esters by GC showed that almost all the fatty acids present in different oils (in TAG form) were incorporated in the corresponding esters. Table 1 shows the fatty acid profile of the two sitosterol esters produced in packed bed bioreactor.

Effect of phytosterol esters on brain lipid profile—The levels of both cerebroside and ceramide decreased with the administration of cholesterol in the diet (Fig. 1A and B). However, treatment of hypercholesterolemic rats with EPA-DHA rich and ALA rich sterol esters resulted in an increase in the level of the two important lipids (cerebroside and ceramide) of brain. The lower therapeutic dose of ALA rich ester (25 mg/d/rat) however did not cause any significant increase in the level of ceramide. Thus, when the effect of EPA-DHA rich ester was compared with that of ALA rich ester, it was seen that EPA-DHA rich ester produced greater ameliorative effect. Administration of sterol esters normalized the cholesterol and phospholipid levels. Here also the effect of EPA-DHA rich ester was greater. The higher dose produced qualitatively greater effect than the lower dose.

Effect of phytosterol esters on brain fatty acid composition—The dietary phytosterol esters influenced the fatty acid composition of the brain (Table 2). The fatty acid composition of the brain was significantly influenced in rats fed with n-3 PUFA rich phytosterol esters. The level of SFA was significantly decreased in the brain tissues of rats given EPA-DHA rich ester compared to ALA rich ester and control group. The amount of polyunsaturated fatty acids were maximum in the lipid of the rat fed with EPA-DHA rich sterol ester and least in the lipid of the rats fed with control groundnut oil only.

Changes in phospholipid composition of brain—Treatment with the two phytosterol esters increased the levels of phospholipids (Table 3). The effect of the higher dose of EPA-DHA rich sterol ester was greater than the lower dose of ALA rich sterol ester.

Changes in brain phospholipid fatty acid composition—The fatty acid composition of phospholipids of brain (Table 4) in the two experimental groups (in dosages) showed an increase in the polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA) and decrease in the saturated fatty acids (SFA) in comparison with the hypercholesterolemic control group. Therefore the changes in the fatty acids due to hypercholesterolemia were reversed by the treatment with phytosterol esters. The increase in PUFA was greater in EPA-DHA rich sterol ester. The rats of Group VI showed pronounced EPA-DHA effects.

Effect of phytosterol ester on brain amino acid status—Sterol ester treatment resulted in an increase in glutamic acid content and aspartic acid content and

<table>
<thead>
<tr>
<th>Sample</th>
<th>C_{14:0}</th>
<th>C_{16:0}</th>
<th>C_{18:0}</th>
<th>C_{18:1}</th>
<th>C_{18:2}</th>
<th>C_{18:3}</th>
<th>C_{20:0}</th>
<th>C_{20:1}</th>
<th>C_{20:5}</th>
<th>C_{22:0}</th>
<th>C_{22:1}</th>
<th>C_{24:0}</th>
<th>C_{22:6}</th>
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<tr>
<td>Phytosterol-DHA-EPA</td>
<td>0.10</td>
<td>2.44</td>
<td>1.23</td>
<td>1.0</td>
<td>1.90</td>
<td>3.05</td>
<td>2.75</td>
<td>5.12</td>
<td>39.20</td>
<td>5.65</td>
<td>2.19</td>
<td>3.84</td>
<td>31.98</td>
</tr>
<tr>
<td>Ester</td>
<td>±0.00</td>
<td>±0.04</td>
<td>±0.02</td>
<td>±0.15</td>
<td>±0.27</td>
<td>±0.34</td>
<td>±0.64</td>
<td>±0.83</td>
<td>±1.10</td>
<td>±0.37</td>
<td>±0.08</td>
<td>±0.19</td>
<td>±1.63</td>
</tr>
<tr>
<td>Phytosterol-ALA ester</td>
<td>-</td>
<td>12.48</td>
<td>1.89</td>
<td>28.28</td>
<td>12.83</td>
<td>44.52</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>±0.60</td>
<td>±0.17</td>
<td>±1.09</td>
<td>±1.01</td>
<td>±1.76</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

Table 1—Fatty acid profiles of EPA-DHA rich and ALA rich sterol esters used as Nutraceutical [Values are expressed as mean ± SD]
a decrease in the glycine content (Fig. 2). The lower dose of ALA rich sterol ester did not produce any significant change. EPA-DHA rich sterol ester provided ameliorative effects in both the dosages. In comparison to ALA rich sterol ester EPA-DHA rich sterol ester produced better effect.

**Changes in brain antioxidant enzyme activity**—For studying the effect of phytosterol esters on antioxidant status, the activities of antioxidant enzymes, superoxide dismutase (SOD), catalase, glutathione reductase (GSH) and glutathione peroxidase (GPx) were investigated. The enzyme activities were increased significantly in
hypercholesterolemic rats treated with both sterol esters (in both dosages). The effect of ALA rich ester was better in comparison to EPA-DHA rich ester (Fig. 2).

Changes in MDA level—MDA content was 0.20 nmoles/mg protein in normal control group. The administration of ALA rich sterol ester (Groups V and VI) caused significant decrease in the MDA content (Fig. 3). There was no significant change in the MDA content when the rats were treated with EPA-DHA rich sterol ester (Groups III and VI).

Table 4—Fatty acid composition (% w/w) of brain phospholipid in rats fed with different dietary lipids [Values are as mean ± SE from 6 animals in each group]

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Gr. I</th>
<th>Gr. II</th>
<th>Gr. III</th>
<th>Gr. IV</th>
<th>Gr. V</th>
<th>Gr. VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA</td>
<td>25.88±0.11</td>
<td>43.49±0.15</td>
<td>27.30±0.10</td>
<td>17.22±1.02</td>
<td>29.36±0.27</td>
<td>20.65±0.11</td>
</tr>
<tr>
<td>MUFA</td>
<td>34.62±1.00</td>
<td>31.28±0.22</td>
<td>37.89±0.23</td>
<td>42.30±0.77</td>
<td>35.83±0.50</td>
<td>41.02±0.78</td>
</tr>
<tr>
<td>PUFA</td>
<td>39.49±0.29</td>
<td>25.23±0.19</td>
<td>34.80±0.09</td>
<td>40.48±0.23</td>
<td>34.81±0.19</td>
<td>38.33±0.10</td>
</tr>
<tr>
<td>SFA:UFA</td>
<td>1:2.8</td>
<td>1:1.3</td>
<td>1:2.6</td>
<td>1:4.8</td>
<td>1:1.2.4</td>
<td>1:3.8</td>
</tr>
</tbody>
</table>

P values: <0.05; between a Gr. I and II; b Gr. II and Gr. III-VI
SFA= saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA=polyunsaturated fatty acids

Fig. 2—Effect of phytosterol ester on amino acid neurotransmitter level and brain antioxidant enzyme activities in rat brain. [Values are mean ± SE for 6 animals, \( P < 0.05 \)] compared between a Groups I and II; b Groups II and III-VI; c Groups IV and VI; d Groups III and V
Discussion

The results of the present study showed that hypercholesterolemia decreased the phospholipid level and increased the cholesterol levels in the brain. The phytosterol esters increased the phospholipid concentration in the brain and normalized the cholesterol levels. Normalization of cholesterol levels will contribute to improvement in neuronal membrane functions.

Phospholipids are an important component of brain membrane and changes in their membrane composition lead to neurological disorders. The present study also showed that there was a decrease in the levels of different types of phospholipids due to hypercholesterolemia. Phytosterol esters showed an increase in the level of different phospholipids. The present study also measured the fatty acid composition of total phospholipids present in brain tissue. There was no effect of diet on MUFA levels. The SFA content of phospholipids increased in hypercholesterolemic condition which was normalized by phytosterol ester treatment. On the other hand the PUFA content of phospholipids decreased in hypercholesterolemia, but the level of PUFA increased with the administration of phytosterol esters. This was due to the presence of EPA and DHA in EPA-DHA rich sterol esters and ALA in ALA rich sterol ester.

The effects of a diet rich in phytosterol esters on the fatty acid composition of brain in adult hypercholesterolemic rats was examined. The SFA level was increased and the MUFA and PUFA levels were decreased by hypercholesterolemia. On administration of phytosterol esters in the diet the levels of SFA and MUFA were normalized, but that of PUFA were increased. The increase of PUFA content could be due to the presence of PUFA in the phytosterol esters. The increase in PUFA content was greater in EPA-DHA rich sterol esters due to the presence of two PUFA namely EPA and DHA in that ester whereas in ALA rich sterol ester there was only one major PUFA that is ALA.

Amino acid neurotransmitters play important roles in controlling the learning and memory process. Glutamic acid and aspartic acid are two of the most abundant amino acids in the central nervous system (CNS) including the cerebral cortex, the dentate gyrus of the hippocampus and striatum, where they correlate with the mediation of cognitive performance as excitatory neurotransmitters. These amino acids play important roles in cognitive functions including memory formation. Glycine on the other hand is an inhibitory neurotransmitter which decreases memory performance. The present findings suggest that hypercholesterolemia decrease the levels of Glu and Asp and increase the level of Gly. Thus the phytosterol esters increased the levels of excitatory neurotransmitter and decreased the levels of inhibitory neurotransmitters and this may be significance for memory and learning processes.

Evidence that the brain affiliated with disorders such as Alzheimer is subjected to a critical oxidative stress load and that an initial source of oxidative stress such as high cholesterol may initiate amyloid formation is increasing. High cholesterol diet provides a relevant example of endogenous chronic oxidative stress due to the resulting hypercholesterolemia. The present study confirms the role of cholesterol-enriched diet in producing a state of oxidative stress with biochemical and biological characteristics of hypercholesterolemia. The effect of phytosterol esters on brain oxidative stress induced by a diet high in cholesterol was also studied. The data demonstrate that the diet high in cholesterol triggered a drop in antioxidant enzyme levels in brain and an increase in lipid peroxidation. The present data show that the administration of phytosterol esters exerts a neuroprotective effect by increasing the levels of the key antioxidant enzymes in brain. This is due to the combined effect of n-3 fatty acids and phytosterols. But the lipid peroxidation did not change significantly by administration of EPA-DHA rich sterol esters due to the presence of long chain fatty acids.

In conclusion the present study indicated that phytosterol esters have a role in countering...
hypercholesterolemia-induced changes in the brain by decreasing the cholesterol levels, increasing the phospholipid levels and increasing the level of antioxidant enzymes. Phytosterol esters increased the brain levels of excitatory amino acid neurotransmitters. The EPA-DHA rich sterol ester produced quantitatively greater effects than and thus may enhance excitatory neuronal functions ALA rich sterol ester. The results indicate that EPA-DHA enriched sterol ester may be of therapeutic significance in treatment of hypercholesterolemia.

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