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Protective Role of Phytosterol Esters in Combating Oxidative Hepatocellular Injury in Hypercholesterolemic Rats

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Abstract: The aim of the present study is to evaluate the effect of eicosapentaenoic acid-docosahexaenoic acid (EPA-DHA) rich sterol ester and α -linolenic Acid (ALA) rich sterol ester on the atherogenic disturbances in hypercholesterolemic atherogenic animals. Six groups of male Wistar rats were employed in this study, wherein five groups were fed with a high cholesterol diet (stock diet supplemented with 1% cholesterol) for 30 days, among which, two groups of rats were also treated with EPA-DHA rich sterol ester in two doses (25 and 50 mg/rat/day, oral gavage) and two groups were treated with ALA rich sterol ester also in two doses (25 and 50 mg/rat/day, oral gavage). The remaining one group served as control. Abnormal increases in the levels of malondialdehyde, as well as depressed antioxidants status, were observed in hepatic tissue of hypercholesterolemic control group. Hypercholesterolemia induced abnormal elevation in the activities of marker enzymes in liver (aspartate transaminase, alanine transaminase and alkaline phosphatase) and was accompanied by increased hepatic cholesterol level and altered fatty changes in the histology of liver. These changes were restored partially in the groups administered with lower doses (25 mg/rat/day) of sterol esters. However, the higher doses (50 mg/rat/day) of sterol esters almost ameliorated the hypercholesterolemic-oxidative changes in the hypercholesterolemic rats. The results of this study present oxidative injury induced by hypercholesterolemic diet and administration of the treatment with higher doses of sterol esters afforded sound protection against lipemic-oxidative injury.

Key words: Phytosterol, alpha linolenic acid, liver, hypercholesterolemia, hypercholesterolemia induced oxidative stress

INTRODUCTION

To study the etiology of hypercholesterolemia related lipemic-oxidative disturbances in liver and plasma feeding a high cholesterol diet has often been used. In diet induced hypercholesterolemia, liver is the primary organ to metabolise the exclusively ingested cholesterol and get affected by subsequent oxidative stress. Development of atherosclerosis is often influenced by the production of endogenous prooxidant conditions in liver cells (Napolitano *et al.*, 2001). An enhanced superoxide production has been demonstrated in hypercholesterolemic animal models (Napoli and Lerman, 2001). Chronic inflammatory responses to hypercholesterolemia and atherosclerosis are often caused by increased oxygen free radical production and decreased nitric oxide generation (Kojda and Harrison, 1999).

Strong evidences have been put forward by various investigators for the involvement of free radicals production and lipid peroxidation in the onset of

atherosclerosis. Thus identification of the antioxidants which can retard the process of lipid peroxidation by blocking the generation of free radical chain reaction, has gained importance in recent years. The antioxidants may act by raising the levels of endogenous defense by upregulating the expression of genes encoding the enzymes such as Superoxide Dismutase (SOD), Catalase, glutathione peroxidase or lipid peroxidase (Surapaneni and Venkataramana, 2007).

Phytosterols include a wide variety of molecules that are structurally similar to cholesterol. Phytosterol contained in vegetable oils is known to exert a hypocholesterolemic function. Phytosterols also chemically acts as an antioxidant, a modest radical scavenger and physically as a stabilizer in the membranes.

Dietary supplementation with polyunsaturated fat is associated with a reduction in the incidence of occlusive vascular diseases including both atherosclerosis and thrombosis (Schierf, 1977; Riediger *et al.*, 2008). However, polyunsaturated fatty acids are susceptible to lipid peroxidation and the product of which may have

deleterious effects leading to tissue damage (Morel and Chisolm, 1989). The change in the dietary lipids may also influence the activity of enzymes involved in the antioxidant defense system. The susceptibility of a cell to oxidative damage is affected by the efficiency of the antioxidant defense enzymes such as catalase, glutathione peroxidase and superoxide dismutase (Leibovitz *et al.*, 1990). It has been seen that antioxidant enzyme activities are enhanced by the incorporation of n-3 PUFA in the diet and thus it is useful in reducing oxidative stress to some extent (Ramprasad *et al.*, 2005; Hunter, 1990).

Reducing cholesterol level and combating oxidative stress are the two major concerns of hypercholesterolemia so, it is reasonable to evaluate the role of sterol esters which combines antilipemic and antioxidant properties. Though the role of sterol ester in reducing cholesterol level is well established, research studies that provide evidence of its lipidemic and antioxidative effects to protect the primary risk organs from hypercholesterolemia are still lacking. The role of the two types of sterol esters namely, eicosapentaenoic acid-docosahexaenoic acid (EPA-DHA) rich sterol ester and Alpha-linolenic Acid (ALA) rich sterol ester in combating oxidative hepatocellular injury were compared in the study.

MATERIALS AND METHODS

Animals and experimental set up: Animal experiments were conducted according to the guidelines of institutional animal ethical committee in the Department of Chemical Technology, University of Calcutta for 32 days in the year 2011. Adult male albino rats of Wistar strain were housed and given food and water ad libitum. The duration of the experimental period was 32 days. The animals were divided into six groups with six rats in each: Group I: Vehicle treated control animals, Group II: Rats were fed with a high cholesterol diet (rat stock diet supplemented with 1% cholesterol) for 32 days, Group III: Rats received EPA-DHA phytosterol ester (25 mg/rat/day, oral gavage) for 25 days alongwith a high cholesterol diet for 32 days, Group IV: Rats received EPA-DHA phytosterol ester (50 mg/rat/day, oral gavage) for 25 days alongwith a high cholesterol diet for 32 days, Group V: Rats fed with high cholesterol diet for 32 days and ALA phytosterol ester (25 mg/rat/day, oral gavage) for the last 25 days, Group VI: rats fed with high cholesterol diet for 32 days and ALA phytosterol ester (50 mg/rat/day, oral gavage) for the last 25 days. Groundnut oil was used as the vehicle and given to all the groups. 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 Eicosapentaenoic 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 and 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% ALA 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 acids were analyzed by GC.

Estimation of hepatic lipid profile: Hepatic lipid was extracted by the method of Folch *et al.* (1951). One gram of tissue 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 liver lipid was used for the estimation of total cholesterol, triglyceride and phospholipid estimation by using standard kits.

Fatty acid compositional changes of hepatic tissue: All the fatty acids of the tissue lipids were analyzed by GC. The fatty acid composition of the tissue phospholipids were analyzed using GC by separating the phospholipid by TLC. The fatty acid composition of the tissue cholesterol esters were also analyzed using GC by separating the cholesterol esters by TLC and then saponifying the esters to yield their respective fatty acids. The GC of the fatty acids was performed by the Metcalfe method (Metcalfe *et al.*, 1966).

Histopathological studies: Permanent preparations were made using routine method (Edem, 2009). The livers were fixed in 10% buffered formalin. The tissues were subsequently dehydrated in upgraded concentrations of alcohol, cleansed in xylene, impregnated and embedded in paraffin wax. Several sections of 3-6 mL were cut using a microtome. The sections were stained with haematoxylin and eosin.

Measurement of MDA: MDA levels in hepatic tissue were estimated as Thiobarbituric Acid (TBA)-reactive substances by the method of Prasad and Kalra (1989). Livers were removed, cleaned of aortic tissue, atria and blood and immersed in formalin buffer. Hepatic tissue was

then added to 10 volumes of phosphate buffer and homogenized with a homogenizer. Homogenate (0.4 mL) was used for the determination of MDA by the assay of TBARS according to the standard method. The amount of Malondialdehyde (MDA) formed was calculated by taking the extinction coefficient of MDA to be $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Dhar *et al.*, 2006).

Estimation of antioxidant enzymes in the hepatic tissue:

Measured amounts of liver tissues were taken and homogenized in different concentrations of phosphate buffer. The samples were then centrifuged and the supernatants were used for enzyme assay. The activity of Catalase (CAT) was determined spectrometrically by the method of Aebi (1984). Superoxide Dismutase (SOD) activity was assayed by measuring the auto oxidation of haematoxylin as described by Martin *et al.* (1987). Reduced Glutathione activity (GSH) was determined by the method of Ellman (1959). Total activity of Glutathione Peroxidase (Gpx) (Gpx EC.1.11.1.9.) was determined in the tissue homogenates according to Flohe and Gunzler (1984). 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.* (1951).

Marker enzymes: Aspartate Transaminase (AST), Alanine Transaminase (ALT) were determined and expressed in terms of U/L. Alkaline phosphatase activity was assayed and expressed as U/L.

Measurement of HMG CoA

Mevalonate ratio: To measure the HMG CoA reductase activity an indirect method was used (Rao and Ramakrishnan, 1975). One gram of fresh liver tissue was homogenized in 10 mL saline-arsenate solution and equal volume of diluted perchloric acid was added and kept for 5 min. After centrifugation at 2000 rpm for 10 min, 1 mL of the filtrate was utilized for HMG Co-A analysis with 0.5 mL alkaline hydroxylamine (pH 5.5) and 1.5 mL ferric chloride and 1 mL of the filtrate was utilized for measuring the mevalonate with 0.5 mL acidic hydroxylamine (pH 2.1) and ferric chloride. The absorbance was measured at 540 nm.

Statistical analysis: All results were expressed as the mean Value \pm SEM. Statistical significance of the difference among values was analyzed by one-way ANOVA. Results were considered significant at $p < 0.05$.

RESULTS

Changes in hepatic lipid profile: The liver lipid profile of rats of different groups is shown in Table 1. The type of fat consumed altered the different lipid concentration in plasma. Rats fed with control Groundnut Oil had total cholesterol of 81.71 mg g^{-1} tissue which was significantly increased to 207.93 mg g^{-1} tissue by feeding them high cholesterol diet. Both the doses of sterol ester brought about a decrease in total cholesterol which was much more in case of EPA-DHA rich sterol ester. The higher doses produced better hypocholesterolemic effect than the lower dose. Thus the sterol esters lowered the levels of triacylglycerol significantly ($p < 0.05$). Phospholipid levels in the liver decreased in hypercholesterolemia, but administration of sterol esters increased its level.

Changes in fatty acid composition of liver: Table 2 depicts the fatty acid composition of liver in rats fed with the different dietary lipids. Fatty acid analysis of liver lipids reflected on the type of fat fed to rats. The content of saturated fatty acid increased in hypercholesterolemia with significant decrease in PUFA. Treatment with sterol esters normalized the content of saturated fatty acids, but the content of PUFA increased significantly. EPA-DHA rich sterol ester increased PUFA in much more proportion than ALA rich sterol ester. There was increase in the content of PUFA in the liver tissue with the increase in the dose of the sterol esters.

Histopathological changes: Microscopic examination of the liver cells of the six groups was performed and the histopathological slides are shown in Fig. 1. Figure 1a highlighted the hepatic histology of the control rats. Figure 1b highlighted the abnormal fatty change in the liver due to hypercholesterolemia. Figure 1c and d highlighted the treatment of the livers with EPA-DHA rich sterol ester. The fatty change was fully cured in case of the treatment with higher dose of EPA-DHA rich sterol

Table 1: Liver lipid profile of normal and hypercholesterolemic rats fed with dietary lipids

Parameters (mg g^{-1} tissue)	Group I	Group II	Group III	Group IV	Group V	Group VI
Total Cholesterol	81.71 \pm 2.33	207.93 \pm 4.50 ^a	104.51 \pm 2.00 ^b	70.12 \pm 1.78 ^b	157.32 \pm 3.22 ^{b,c}	107.93 \pm 1.50 ^{b,d}
Triacylglycerol	112.85 \pm 2.50	253.80 \pm 5.90 ^a	118.59 \pm 0.56 ^b	85.02 \pm 1.90 ^b	112.68 \pm 2.30 ^b	104.15 \pm 2.80 ^{b,d}
Phospholipids	172.49 \pm 3.50	61.64 \pm 2.80 ^a	307.83 \pm 1.67 ^b	639.70 \pm 2.89 ^b	117.86 \pm 0.99 ^{b,c}	446.70 \pm 0.50 ^{b,d}

Values are expressed as Mean \pm SEM for six animals, The superscript letters also represent statistical significance at $p < 0.05$ ^aComparisons are made between Groups I and II, ^bComparisons are made between Groups II and III-VI, ^cComparisons are made between Groups III and V, ^dComparisons are made between Groups IV and VI

Table 2: Fatty acid composition (% w/w) of liver in rats fed with different dietary lipids

Fatty Acids	Group I	Group II	Group III	Group IV	Group V	Group VI
14:0	1.13±0.11	1.61±0.08 ^a	0.46±0.06 ^{ab}	0.73±0.01 ^{ab}	1.08±0.12 ^b	0.48±0.09 ^{ab}
16:0	9.89±1.02	12.73±1.01 ^a	10.73±1.01	10.04±1.12	10.56±1.01	10.17±1.11
16:1	4.26±0.44	2.59±0.25 ^a	3.77±0.30 ^b	4.24±0.10 ^b	4.52±0.99 ^b	5.38±0.37 ^{ab}
18:0	4.24±0.50	5.32±1.00	4.86±0.08	4.35±0.11	3.94±0.18	3.79±0.66
18:1	37.27±1.44	40.88±1.20 ^a	36.28±1.00	32.09±1.06 ^{ab}	34.08±0.56 ^{ab}	30.04±0.22 ^{ab}
18:2	22.69±0.20	21.06±0.10	22.80±0.16	22.86±0.10	22.14±0.22	23.61±0.67
18:3	3.69±0.10	1.38±0.23 ^a	2.05±0.34 ^{ab}	4.53±0.32 ^{ab}	7.89±0.45 ^{ab}	10.84±1.20 ^{ab}
20:1	2.06±0.06	0.79±0.08 ^a	1.71±0.02 ^b	1.78±0.03 ^b	2.27±0.06 ^b	2.75±0.11 ^{ab}
20:5	2.84±0.18	1.33±0.05 ^a	6.03±0.04 ^{ab}	7.68±0.16 ^{ab}	3.60±0.19 ^b	3.62±0.09 ^b
22:0	2.67±0.02	2.62±0.02 ^a	1.07±0.10 ^{ab}	0.87±0.08 ^{ab}	2.11±0.01 ^{ab}	2.00±0.10 ^{ab}
22:1	0.62±0.10	1.98±0.02 ^a	1.05±0.02 ^{ab}	1.02±0.01 ^{ab}	0.41±0.02 ^b	0.38±0.02 ^{ab}
22:6	3.38±0.33	0.57±0.03 ^a	3.35±0.11 ^b	5.76±0.56 ^{ab}	2.63±0.12 ^{ab}	2.37±0.10 ^{ab}
24:0	1.00±0.01	2.36±0.10 ^a	2.28±0.30 ^a	2.16±0.16 ^{ab}	0.71±0.01 ^b	0.70±0.01 ^b
24:1	4.26±0.10	4.78±0.08 ^a	3.56±0.22 ^{ab}	1.89±0.03 ^{ab}	4.06±0.56	3.87±0.05 ^b
ΣSFA	18.93±1.98	24.64±2.09 ^a	19.40±0.22 ^b	18.15±0.20 ^b	18.40±0.10 ^b	17.14±0.19 ^b
ΣMUFA	48.47±1.22	51.02±0.83 ^a	46.37±0.99 ^{ab}	41.02±1.20 ^{ab}	43.07±0.67 ^{ab}	42.42±0.22 ^{ab}
ΣPUFA	32.60±1.09	24.34±2.30 ^a	34.23±0.50 ^b	40.83±1.00 ^{ab}	38.53±0.45 ^{ab}	40.44±0.87 ^{ab}

Values are expressed as Mean±SEM for six animals. The superscript letters represent statistical significance at p<0.05, ^aComparisons are made between Groups I and II, ^bComparisons are made between Groups II and III-VI

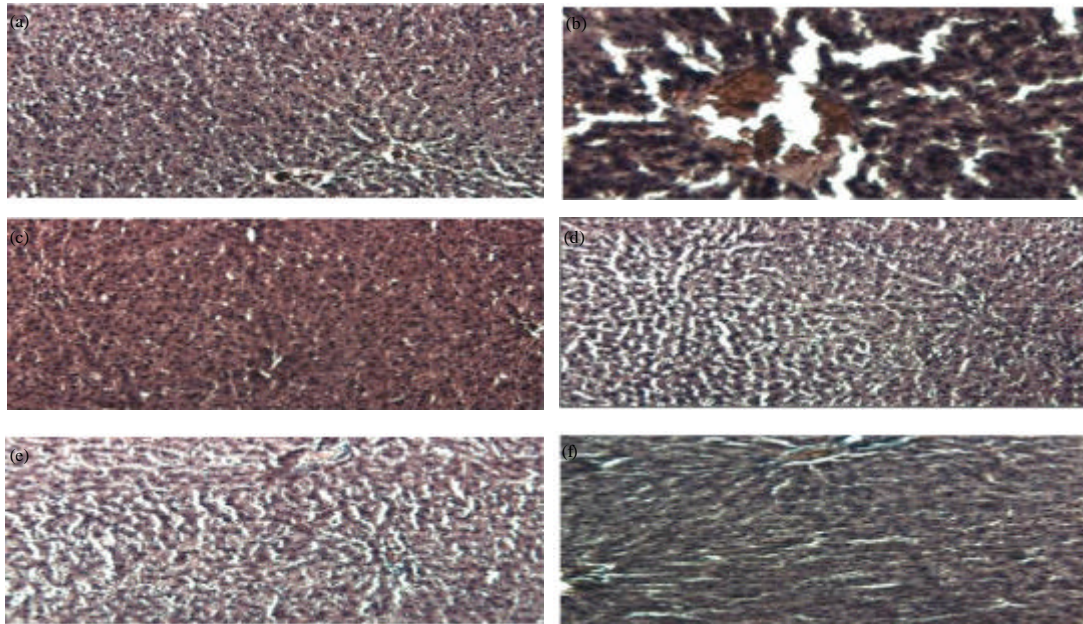


Fig. 1(a-f): Pictomicrographs of liver tissues of (a) Normal rats fed with control groundnut oil, (b) Hypercholesterolemic rats fed with groundnut oil, (c) Hypercholesterolemic rats fed with low dose of EPA-DHA ester, (d) Hypercholesterolemic rats fed with high dose of EPA-DHA ester, (e) Hypercholesterolemic rats fed with low dose of ALA ester and (f) Hypercholesterolemic rats fed with high dose of ALA ester

ester. Figure 1e and f highlighted the treatment of the livers with ALA rich sterol ester. Here again the effect of the higher dose was much better. The effect of treatment of the rats with EPA-DHA rich sterol ester was greater in comparison with ALA rich sterol ester.

Changes in antioxidant enzyme levels: Table 3 reveals the effect of two different doses (25 and 50 mg/rat/day) of two different phytosterol esters (EPA-DHA rich sterol ester

and ALA rich sterol ester) on hepatic antioxidant enzymes on hypercholesterolemic groups (HCD). It was seen from the result that hypercholesterolemia decreased the levels of antioxidant enzymes in Group II significantly. Treatment with the two sterol esters increased the antioxidant enzyme levels. The effect of ALA rich ester was more antioxidative compared to EPA-DHA rich ester and the higher dose produced better effect than the lower one.

Table 3: Effect of phytosterol esters on hepatic antioxidant enzyme status on HCD fed groups compared with the control animals

Antioxidant enzymes U/min/mg protein	Group I	Group II	Group III	Group IV	Group V	Group VI
CAT	6.07±0.07	4.26±0.77 ^a	7.97±0.27 ^b	13.12±0.28 ^b	11.69±1.22 ^{b,c}	15.03±0.23 ^{b,d}
SOD	3.70±0.11	1.32±0.08 ^a	3.43±0.12 ^b	4.31±0.20 ^b	4.15±0.12 ^{b,c}	6.13±0.57 ^{b,d}
GSH	41.69±0.45	29.34±1.29 ^a	35.39±0.11 ^b	48.40±0.18 ^b	58.44±2.22 ^{b,c}	66.27±2.37 ^{b,d}
GPx	6.39±0.56	4.95±0.78 ^a	5.92±0.02 ^b	6.87±0.59 ^b	7.40±0.97 ^{b,c}	9.03±0.65 ^{b,d}

Values are expressed as Mean±SEM for six animals, The superscript letters also represent statistical significance at p<0.05, CAT: Catalase; SOD: Superoxide Dismutase; GSH: Reduced Glutathione; GPx: Glutathione Peroxidase, ^aComparisons are made between Groups I and II, ^bComparisons are made between Groups II and III-VI, ^cComparisons are made between Groups III and V, ^dComparisons are made between Groups IV and VI

Table 4: Alterations in hepatic tissue enzyme activities of HCD fed groups and the effect of sterol ester treatments compared with controls

Marker Enzymes	Group I	Group II	Group III	Group IV	Group V	Group VI
Aspartate transaminase	7.45±1.23	24.61±0.78 ^a	15.36±1.78 ^b	4.75±1.20 ^{b,c}	16.15±0.29 ^b	5.41±0.99 ^{b,d}
Alanine transaminase	10.21±0.56	29.94±2.17 ^a	13.15±2.90 ^b	6.37±0.50 ^{b,c}	13.52±1.67 ^b	6.60±1.00 ^{b,d}
Alkaline phosphatase	30.16±3.45	71.64±3.89 ^a	60.92±3.50 ^b	39.82±4.12 ^{b,c}	44.55±3.00 ^b	25.93±2.65 ^{b,d}

Values are expressed as Mean±SEM for six animals, The superscript letters represent statistical significance at p<0.05, ^aComparisons are made between Groups I and II, ^bComparisons are made between Groups II and III-VI, ^cComparisons are made between Groups III and IV, ^dComparisons are made between Groups V and VI

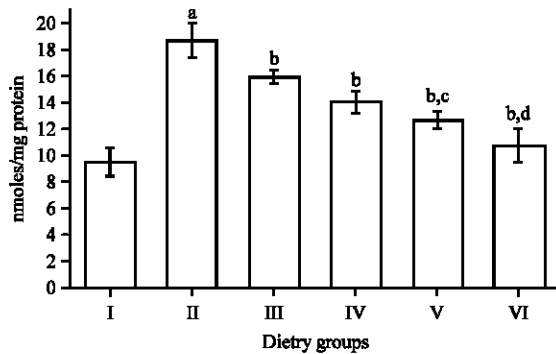


Fig. 2: Changes in MDA level in the different groups [I: normal rats fed with control groundnut oil; II: hypercholesterolemic rats fed with groundnut oil; III: hypercholesterolemic rats fed with low dose of EPA-DHA ester; IV: hypercholesterolaemic rats fed with high dose of EPA-DHA ester; V: hypercholesterolaemic rats fed with low dose of ALA ester; VI: hypercholesterolaemic rats fed with high dose of ALA ester.]. Each bar is expressed as overall Mean±SEM for six animals, The superscript letters represent statistical significance at p<0.05, ^aComparisons are made between Groups I and II, ^bComparisons are made between Groups II and III-VI, ^cComparisons are made between Groups III and V, ^dComparisons are made between Groups IV and VI

Changes in MDA level: Figure 2 depicts the changes in MDA level which corresponds to peroxidation, in the different dietary groups. MDA level increased significantly in hypercholesterolemia. Both the doses of ALA rich sterol ester normalized the MDA levels, but the second dose was more effective. EPA-DHA rich sterol ester caused no significant change in the MDA levels.

Table 5: Liver total cholesterol and HMG CoA reductase activity in different dietary groups

Dietary groups	Total cholesterol (mg g ⁻¹ m tissue)	HMG CoA: Mevalonate
Group I	81.71±2.33	0.89±0.06
Group II	207.93±4.50 ^a	0.38±0.02 ^a
Group III	104.51±2.00 ^b	0.79±0.05 ^b
Group IV	70.12±1.78 ^{b,c}	0.98±0.05 ^{b,c}
Group V	157.32±3.22 ^b	0.55±0.01 ^b
Group VI	107.93±1.50 ^{b,d}	0.75±0.09 ^{b,d}

HMG CoA- 3-hydroxy-3-methylglutaryl-coenzyme A, Values are expressed as Mean±SEM for six animals. The superscript letters represent statistical significance at p<0.05, ^aComparisons are made between Groups I and II, ^bComparisons are made between Groups II and III-VI, ^cComparisons are made between Groups III and IV, ^dComparisons are made between Groups V and VI

Changes in marker enzymes levels: Table 4 reveals alterations in hepatic tissue enzyme activities of HCD fed groups and the effect of phytosterol ester treatments compared with controls. In the experimental model of early phase atherogenic fatty changes, the above enzymes exhibit significant elevation in their activities (p<0.05). Treatment with sterol esters restored their activities to normal level.

Correlation between HMG-CoA/ Mevalonate Ratio and liver cholesterol: The total cholesterol levels increased in cholesterol stressed groups. With the increase in the total cholesterol level there was a decrease in the HMG CoA: Mevalonate ratio. In the treated groups the level of total cholesterol decreased with the increase in the HMG CoA: Mevalonate ratio (Table 5).

DISCUSSION

High plasma cholesterol represents a major risk factor for atherogenesis. Total serum cholesterol is really an indicator of the amount of the free radical damage in the body. Higher the free radical level, higher the body needs

to produce cholesterol internally from liver to act as an antioxidant and free radical scavenger. Liver being the major organ responsible for cholesterol transport, metabolism and excretion, it is reasonable to study hepatic lipemic-oxidative disturbances in hypercholesterolemic diet induced atherogenesis. It was previously reported that feeding High Cholesterol Diet (HCD) for 32 days resulted in increased serum lipid levels and oxidative injury to heart tissue (Kumar *et al.*, 2005). In exogenous hypercholesterolemia, increased accumulation of cholesterol in the serum and tissue exhibit enhanced production free radicals (Keaney *et al.*, 1995) and the produced free radicals itself having capability to enhance the activities of HMG CoA reductase results in increased endogenous production of cholesterol. These findings support the importance of combined supplementation of hypocholesterolemic agent and antioxidant in the treatment of hypercholesterolemia. The present investigations reveal the protective role played by EPA-DHA ester and ALA ester in hypercholesterolemic condition.

The hallmark of atherosclerosis is the accumulation of cells containing excessive lipids and this is notable in the histopathological observation of the present study where, there was an increased lipid infiltration in hepatocytes of HCD fed rats. Furthermore, the cholesterol levels in the liver of HCD fed rats were also increased. It is reported that supplementation of EPA-DHA ester and ALA ester reduced the lipid infiltration in hepatocytes. The ameliorative effect of EPA-DHA ester was more than ALA ester. This was because supplementation of EPA and DHA present in EPA-DHA ester results in increased excretion of cholesterol from animal, by increasing the transfer of cholesterol into bile. Phytosterol present in EPA-DHA ester also decreases the cholesterol excretion by decreasing its absorption by tissues. Thus, phytosterol in combination fish oil efficiently reduced the increased lipid infiltration and cholesterol level in hepatic tissue of hypercholesterolemic rats.

Lipids, proteins and DNA are important targets of Reactive Oxygen Species (ROS) and their oxidative products have a variety of biological effects. Proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells, resulting in the formation of carbonyl groups on protein side chains by ROS. 8-OHdG is one of the major abundant degradation products of DNA, generated by oxidative stress. In the present study, HCD fed rats showed an increase in the level of malondialdehyde (MDA) which may react with a variety of nucleophilic sites in DNA and protein, generating various types of adducts that represent markers of oxidative stress. It has been reported that MDA modified

LDL is notable in atherosclerotic lesions (Tamura *et al.*, 2003). Increased MDA, protein carbonyl and 8-OHdG levels in the hepatic tissue of HCD fed rats might result from an enhanced production of superoxide in hypercholesterolemic condition which shows build up oxidative stress as evident from diminished antioxidant defense system in this study. MDA concentration detected in the present study showed that an increase in lipid peroxidation occur in animals fed on HCD when compared to the control. Significant decrease of MDA levels in the rats treated with ALA ester.

The activities of antioxidant enzymes like CAT, SOD, GSH and GPx form the first line of defense against ROS and decrease in their activities contribute to the oxidative stress. Increased hepatic oxidative damage to the major biomolecules was accompanied by deteriorating antioxidant enzyme status in the HCD fed groups of this study, signifying heightened oxidative injury. Restoration of activities of these enzymes in the liver tissue of drug treated HCD fed groups were seen due to fish oil and phytosterol present in EPA-DHA ester and linseed oil and phytosterol in ALA ester. Hypolipidemic activity of EPA present in fish oil reduces hypercholesterolemia induced superoxide formation which may improve the antioxidant defense system in HCD fed rats.

Hypercholesterolemia inactivates cell constituents by oxidation or cause oxidative stress by undergoing radical chain reaction ultimately leading to loss of membrane integrity. It is reported that rats fed atherogenic diet results in increased activities of hepatic marker enzymes (Deepa and Varalakshmi, 2004). This is consistent with these findings in HCD fed rats of present study where we observed increase in the activities of these enzymes in hepatic tissue. This might result from increased oxidative stress as evidenced from oxidation of major biomolecules and diminished antioxidant defense system. Both the phytosterol esters diminished the activities of these marker enzymes. Fisher *et al.* (1986) reported that n-3 fatty acids present in fish oil can reduce superoxide anion production by inflammatory cells (Fisher *et al.*, 1986). Thus free radical scavenging potential of both EPA present in fish oil, ALA present in linseed oil and phytosterol prevents ROS induced inflammation of hepatocytes in HCD fed rats. Therefore as these results show that administration of EPA-DHA ester and ALA ester derivative to HCD fed rats attenuated oxidation of major biomolecules and collapse of antioxidant system, thereby prevents oxidative injury and subsequent inflammation of hepatic tissue and results in restoration of normal activities of aminotransferases and ALP. This scheme of combining a phytosterol with fish oil and linseed oil, demonstrating anti-inflammatory and

hepatoprotective properties, has improved the efficacy of the individual components of the derivative phytosterol, fish oil and linseed oil.

CONCLUSION

The aforesaid discussion concludes that EPA-DHA rich sterol ester provides greater hypolipemiant effects in comparison with ALA rich sterol ester while ALA rich sterol ester provides more antioxidative effect in comparison to EPA-DHA rich sterol ester. The optimum dose of esters should be given in the dosage of 0.5 g kg⁻¹ body weight/day for the treatment of hepatocellular injury caused due to hypercholesterolemia.

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REFERENCES

Aebi, H., 1984. Catalase *in vitro*. Methods Enzymol., 105: 121-126.

Deepa, P.R. and P. Varalakshmi, 2004. Protective effects of certoparin sodium, a low molecular weight heparin derivative, in experimental atherosclerosis. Clin. Chem. Acta, 339: 105-115.

Dhar, P., D. Bhattacharyya, D.K. Bhattacharyya and S. Ghosh, 2006. Dietary comparison of conjugated linolenic acid (9 cis, 11 trans, 13 trans) and alpha-tocopherol effects on blood lipids and lipid peroxidation in alloxan-induced diabetes mellitus in rats. Lipids, 41: 49-54.

Edem, D.O., 2009. Haematological and histological alterations induced in rats by palm oil-containing diets. Europ. J. Scientific Res., 32: 405-418.

Ellman, G.L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys., 82: 70-77.

Fisher, M., K.S. Upchurch, P.H. Levine, M.H. Johnson, C.H. Vaudreuil, A. Natale and J.J. Hoogasian, 1986. Effects of dietary fish oil supplementation on polymorphonuclear leukocyte inflammatory potential. Inflammation, 10: 387-392.

Flohe, L. and W.A. Gunzler, 1984. Assay of glutathione peroxidase. Method Enzymol., 105: 114-121.

Folch, J., I. Ascoli, M. Lees, J.A. Meath and N.L. Baron, 1951. Preparation of lipid extracts from brain tissue. J. Biol. Chem., 191: 833-841.

Hunter, J.E., 1990. n-3 fatty acids from vegetable oils. Am. J. Clin. Nutr., 51: 809-814.

Keaney, Jr. J.F., A. Xu, D. Cunningham, T. Jackson, B. Frei and J.A. Vita, 1995. Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation. J. Clin. Invest., 95: 2520-2529.

Kojda, G. and D. Harrison, 1999. Interactions between NO and reactive oxygen species: Pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovascular Res., 43: 652-671.

Kumar, S.A., V. Sudhakar and P. Varalakshmi, 2005. Attenuation of serum lipid abnormalities and cardiac oxidative stress by eicosapentaenoate-lipoate (EPA-LA) derivative in experimental hypercholesterolemia. Clin. Chem. Acta, 355: 197-204.

Leibovitz, B., Hu, M.L. and A.L. Tappel, 1990. Lipid peroxidation in rat tissue slices: Effect of dietary vitamin E, corn oil, lard and menhaden oil. J. Nut., 120: 97-104.

Lowry, O.H., N.J. Rosendrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with folin-phenol reagent. J. Biol. Chem., 193: 265-275.

Martin, jr. J.P., M. Dailey and E. Sugarman, 1987. Negative and positive assays of superoxide dismutase based on hematoxylin autoxidation. Arch. Biochem. Biophys., 255: 329-336.

Metcalf, L.D., A.A. Schmitz and J.R. Pelka, 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. Anal. Chem., 38: 514-515.

Morel, D.W. and G.M. Chisolm, 1989. Antioxidant treatment of diabetic rats inhibits lipoprotein oxidation and cytotoxicity. J. Lipid. Res., 30: 1827-1834.

Napoli, C. and L.O. Lerman, 2001. Involvement of oxidation-sensitive mechanisms in the cardiovascular effects of hypercholesterolemia. Mayo Clin. Proc., 76: 619-631.

Napolitano, M., R. Rivabene, M. Avella, L. Amicone, M. Tripodi, K.M. Botham and E. Bravo, 2001. Oxidation affects the regulation of hepatic lipid synthesis by chylomicron remnants. Free Radical Biol. Med., 30: 506-515.

Prasad, K. and J. Kalra, 1989. Experimental atherosclerosis and oxygen free radicals. Angiology., 40: 835-845.

Ramprasad, T.R., V. Baskaran, T.P. Krishnanantha and B.R. Lokesh, 2005. Modulation of antioxidant enzyme activities, platelet aggregation and serum prostaglandins in rats fed spray-dried milk containing n-3 fatty acid. Mol. Cell. Biochem., 277: 19-26.

- Rao, A.V. and S. Ramakrishnan, 1975. Indirect assessment of hydroxymethylglutaryl-coa reductase(NADPH) activity in liver tissue. *Clin. Chem.*, 21: 1523-1525.
- Riediger, N.D., R. Othman, E. Fitz, G.N. Pierce, M. Suh and M.H. Moghadasein, 2008. Low n-6:n-3 fatty acid ratio, with fish- or flaxseed oil, in a high fat diet improves plasma lipids and beneficially alters tissue fatty acid composition in mice. *Europ. J. Nut.*, 47: 153-160.
- Schieff, G., 1977. Polyunsaturated Fatty Acid. In: American Oil Chemist's Society, Kunau, W.H. and R.T. Holman (Eds.). University of Illinois Press, Champaign, pp: 183-191.
- Surapaneni, K.M. and G. Venkataramana, 2007. Status of lipid peroxidation, glutathione, ascorbic acid, vitamin E and antioxidant enzymes in patients with osteoarthritis. *Indian J. Med. Sci.*, 61: 9-14.
- Tamura, A., T. Watanabe and M. Nasu, 2003. Effects of atorvastatin and pravastatin on malondialdehyde-modified LDL in hypercholesterolemic patients. *Circ. J.*, 67: 816-820.