Journal of Oleo Science
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doi : 10.5650/jos.ess13211
J. Oleo Sci. **63**, *(5) 439-447 (2014)*

JOURNAL OF OLEO SCIENCE
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Effect of Sterol Esters on Lipid Composition and Antioxidant Status of Erythrocyte Membrane of Hypercholesterolemic Rats

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Abstract: Hypercholesterolemia is a major cause of coronary heart disease. Erythrocyte membrane is affected during hypercholesterolemia. The effect of EPA-DHA rich sterol ester and ALA rich sterol ester on erythrocyte membrane composition, osmotic fragility in normal and hypercholesterolemic rats and changes in antioxidant status of erythrocyte membrane were studied. Erythrocyte membrane composition, osmotic fragility of the membrane and antioxidant enzyme activities was analyzed. Osmotic fragility data suggested that the erythrocyte membrane of hypercholesterolemia was relatively more fragile than that of the normal rats' membrane which could be reversed with the addition of sterol esters in the diet. The increased plasma cholesterol in hypercholesterolemic rats could also be lowered by the sterol ester administration. There was also marked changes in the antioxidant enzyme activities of the erythrocyte membrane. Antioxidant enzyme levels decreased in the membrane of the hypercholesterolemic subjects were increased with the treatment of the sterol esters. The antioxidative activity of ALA rich sterol ester was better in comparison to EPA-DHA rich sterol ester. In conclusion, rat erythrocytes appear to be deformed and became more fragile in cholesterol rich blood. This deformity and fragility was partially reversed by sterol esters by virtue of their ability to lower the extent of hypercholesterolemia.

Key words: hypercholesterolemia, erythrocyte membrane, membrane fragility, membrane lipid profile, antioxidant enzyme assay

1 INTRODUCTION

Atherosclerosis is the primary cause of coronary and cardiovascular diseases. It can generally be viewed as a form of chronic inflammation that is induced and perturbed by lipid accumulation[1]. Hypercholesterolemia is a risk factor for premature atherosclerosis. Oxygen free radicals have been implicated in the pathogenesis of hypercholesterolemic atherosclerosis and antioxidants suppress the development of hypercholesterolemic atherosclerosis[2]. Hypercholesterolemia can increase the cholesterol content of platelets, polymorphonuclear leukocytes and endothelial cells so that endothelial and smooth muscle cells, neutrophils and platelets may be sources of oxygen free radicals[3]. It has been suggested that resistance of erythrocytes to an oxidative stress is decreased in hypercholesterolemic man[4].

Recently, there is growing evidence that ROS contribute to organ injury in many systems including heart, liver and central nervous system[5]. Erythrocytes are permanently in contact with potentially damaging levels of oxygen, but their metabolic activity is capable of reversing this injury under normal conditions. Erythrocytes are equipped by many defense systems representing their antioxidant capacity[6]. This protective system includes superoxide dismutase(SOD), catalase(CAT), reduced glutathione, glutathione peroxidase(GPx), glutathione-S-transferase, and glutathione reductase(GR). However, the cellular antioxidant action is reinforced by the presence of dietary antioxidants.

Abbreviations: ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase; CAT: Catalase; GSH: Reduced glutathione; GPx: Glutathione peroxidase; GR: Glutathione reductase; CHD: Coronary heart disease; LDL: Low density lipoprotein; HDL: High density lipoprotein; TLC: Thin layer chromatography; SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; ALA: Alpha linolenic acid; C:P: Cholesterol:Phospholipid

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Accepted December 24, 2013 (received for review December 2, 2013)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manusriptcentral.com/jjocs

Osmotic fragility, the sensitivity to change in osmotic pressure characteristic of red blood cells, has been found to be altered in various pathological conditions. The integrity of red blood cells may be determined by measuring the changes in erythrocyte osmotic fragility. Measurement of osmotic fragility of erythrocytes has been applied in the diagnosis of membrane permeability studies, alterations in normal body conditions leading to destruction of erythrocytes.

Sitosterol esters produced by enzymatic esterification of sitosterol and omega-3 fatty acids are established to reduce hypercholesterolemia in our previous work[7]. Sitosterol esters are more effective in reducing both total cholesterol and triglyceride concentrations in comparison to free sitosterol because the efficacy of cholesterol lowering is often dependent on the dispersion capability in water and oil. Moreover, the β-sitosterol ester can reduce the susceptibility of omega-3 fatty acids towards peroxidation. β-sitosterol can work against lipid peroxidation and omega-3 fatty acids can enhance the activities of antioxidant enzymes, thus both in combination can be a powerful antioxidant. Omega-3 fatty acids are able to reduce the risk of coronary heart disease (CHD) by decreasing levels of artery-clogging lipids in the blood. Other studies have shown that omega-3 fatty acids offer protection against heart disease by controlling LDL ("bad" cholesterol) levels while raising HDL (the "good" cholesterol) levels.

Thus, the aim of this study is to investigate the role of sterol esters on changes in erythrocyte membrane caused by ingestion of high cholesterol diet.

2 Materials and Methods

2.1 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% 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 acid compositions were analyzed by GC[8].

2.2 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). N_2, H_2 and airflow rates were maintained at 1, 30 and 300 ml/min, respectively. Inlet and detector temperature was kept at 250 and 275℃, respectively, and the oven temperature was programmed at 65–230–280℃ with a 1-min hold at 65℃ and an increase rate of 20℃/min and 1 min hold up to 230 and 8℃/min with 24 min hold up to 280℃. 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 had been previously standardized in GC by preparing esters of β-sitosterol with different fatty acids.

2.3 Animals and experimental set up

Animal experiments were conducted according to the guidelines of Animal Ethical Committee of Dept. of Chemical Technology, University of Calcutta. Adult male albino rats of Wistar strain were housed and given food and water ad libidum. The duration of the experimental period was 32days. The animals were divided into six groups with six rats in each. The dietary pattern of each group is given in Table 1. 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, the rats were anesthetized by chloroform and 5 ml of blood was taken from the heart.

2.4 Osmotic fragility determination

The method described by Dacie and Lewis[9] employed here provided different concentrations of sodium chloride 0.1–0.9% in a series of tubes made from appropriate dilutions of 1% sodium chloride phosphate buffer, pH 7.4 to a final volume of 5 mL. Freshly heparinized blood (20 lL) was pipetted into these tubes containing varying sodium chloride concentration. The contents were gently mixed and allowed to stand for 30 min at room temperature. At the end, the contents of the tubes were mixed again and centrifuged at 500×9 g for 10 min. Absorbance of the supernatant was measured at 540 nm against water blank. The degree of hemolysis was expressed as a percentage, where 100% represents full hemolysis.

2.5 Preparation of erythrocyte membranes

All procedures were done at 0–5℃ (typically on ice), and all centrifugations were performed in a Sorvall SS-34 rotor at 15,000 rpm unless specified. Rat red cells and hemoglobin-free ghosts were prepared as described in the literature[10], except that the hemolysis buffer was 5 mM * NaPi (pH 8), 0.01 mM MgSO4, and the membranes were sus-

Table 1 Dietary pattern of different groups.

Groups	Dietary Pattern
I	Rat fed with groundnut oil as control oil for 32 days
II	Rats made hypercholesterolemic by supplementing the stock diet with 1% cholesterol (HCD diet) for 32 days
III	HCD diet for 32 days alongwith phytosterol (oral gavage) for the last 25 days
IV	HCD diet for 32 days alongwith EPA-DHA rich phytosterol ester (0.25 g/kg body weight/day, oral gavage) for the last 25 days
V	HCD diet for 32 days alongwith EPA-DHA rich phytosterol ester (0.5 g/kg body weight/day, oral gavage) for the last 25 days
VI	HCD diet for 32 days alongwith ALA rich phytosterol ester (0.25 g/kg body weight/day, oral gavage) for the last 25 days
VII	HCD diet for 32 days alongwith ALA rich phytosterol ester (0.5 g/kg body weight/day, oral gavage) for the last 25 days

pended for 10 min in this buffer before each centrifugation to allow hemoglobin to exit fully.

2.6 Extraction of lipids from erythrocytes and plasma

Blood samples for laboratory analyses were taken into EDTA-containing vacuum tubes. Plasma was separated by centrifugation and stored at −20℃ until analyzed. In the analysis of fatty acid composition of erythrocyte membrane and plasma lipids, 0.5 g of samples were homogenized with 1 mL of 0.74% potassium chloride and 2 mL of chloroform and methanol in the proportion of 1:1 v/v (Chloroform: Methanol) followed by 2 mL of chloroform and methanol in the proportion of 2:1 v/v (Chloroform: Methanol) for 2 min and then centrifuged. Then, the chloroform layer was filtered through a Whatman filter paper (No. 1). The chloroform layer was dried, the lipid contents of the erythrocyte membrane and plasma were measured, and the lipid was used for lipid analysis[11].

2.7 Lipid analysis

The lipid components such as total cholesterol were analyzed using enzyme kits supplied by Merck India Ltd. (Catlog No. 117679), following the method of Allain[12]. Phospholipid was estimated using the method of Chen *et al.*[13]. After obtaining the total cholesterol and phospholipid values, their ratios were also calculated.

2.8 Determination of fatty acid composition of erythrocyte membrane lipids

Erythrocyte membrane lipid extracts were saponified using methanolic KOH, and methyl esters of corresponding fatty acids were made by the standard method[14]. Fatty acid methyl esters were analyzed using gas chromatography. The GC (make: Agilent, model: 6,890 N) instrument used was equipped with FID detector and capillary DB-Wax column (30 mL, 0.32 mm I.D, 0.25 lm FT). N_2, H_2, and airflow rate was maintained at 1 mL/min, 30 mL/min, and 300 mL/min, respectively. Inlet and detector temperature was kept at 250℃, and the oven temperature was programmed as 150–190–230℃ with increase rate of 15℃/min and 5 min hold up to 150℃ and 4℃/min with 10 min hold up to 230℃. The percentage proportions of fatty acids were calculated.

2.9 Determination of fatty acid composition of erythrocyte membrane phospholipids

The presence of phospholipids were first confirmed by thin layer chromatography (TLC) by spotting the lipid mixture after extraction on a Silica Gel G plate (0.2 mm thick) using hexane–diethyl ether–acetic acid (90:10:1) as a developing solvent system. The lipid spots were identified by iodine absorption[15–17]. The phospholipid was then extracted from the TLC plate using chloroform. The fatty acid compositions of the phospholipids were determined by GC by the above mentioned method.

2.10 Scanning electron microscopy

Erythrocyte membrane ghost suspension was made in a cold buffered saline (10 mM NaH_2PO4, pH 7.4) containing 1% glutaraldehyde. After 1 h, the ghosts were washed three times with phosphate buffer and finally fixed with 1% osmic acid in the same buffer. Fixed cells were dehydrated sequentially with ethanol and propylene oxide, spread over a cover glass and air dried. After coating with gold, cells were examined in a scanning electron microscope.

2.11 Antioxidant Enzyme Assay

Measured amounts of liver and brain were homogenized in phosphate buffer. The samples were then centrifuged and the supernatants were used for enzyme assay. The activity of CAT was determined spectrometrically by the method of Aebi[18]. SOD activity was assayed by measuring the auto oxidation of haematoxylin as described by Martin

et al.[19]. GSH JUST GSH was determined by the method of Ellman[20]. Total activity of GPx (EC.1.11.1.9.) was determined in the tissue homogenates and plasma according to Flohe *et al.*[21]. All enzyme activities are expressed as enzyme units per mg protein. Protein was determined using the method of Lowry *et al.*[22].

2.12 Statistical analysis

All the data were presented as mean ± S.E.M. Significance was calculated using two-way ANOVA. Differences were considered significant at $p > 0.05$.

3 Results

3.1 Osmotic fragility of erythrocytes

The effect of dietary intake of different oils on the osmotic fragility of erythrocytes of the rats fed with different lipids is presented in Table 2. In normal control rats, the mean cell fragility (50% haemolysis) was being evident at 0.5% NaCl concentration. In hypercholesterolaemic control rats, the mean cell fragility (50% haemolysis) was at 0.9% NaCl concentration. Thus, the osmotic fragility data suggested that the red blood cells of high cholesterol diet fed animals were relatively fragile.

The increased osmotic fragility of red blood cells evidenced in hypercholesterolemic rats was partially reversed by dietary phytosterol and sterol esters as shown in Table 2. The mean cell fragility of red blood cells was reduced to 0.8% NaCl concentration by administration of phytosterol. The mean cell fragility of red blood cells was further reduced to 0.6 and 0.4% NaCl concentration under lower and higher doses of EPA-DHA ester respectively. On the other hand the mean cell fragility of red blood cells was reduced to 0.7 and 0.5% NaCl concentration under lower and higher doses of ALA ester respectively. This showed that EPA-DHA ester was more useful in decreasing membrane fragility.

3.2 Changes in erythrocyte membrane lipid profile

Lipid profiles of erythrocyte membranes of different rats fed with different dietary oils are presented in Table 3. The amount of membrane cholesterol was lower and phospholipid was higher in the normal control rats fed groundnut oil than in the hypercholesterolemic rats. All the experimental oils significantly lowered the alteration in cholesterol and increased slightly the phospholipid content in hypercholesterolemic condition. Therefore the experimental oils also decreased the C:P ratio to a significant level ($p < 0.05$).

3.3 Changes in fatty acid composition of erythrocyte membrane lipids

The fatty acid composition of erythrocyte membrane lipids of normal and hypercholesterolemic rats is presented in Table 4. The lipid of erythrocyte membrane of normal rats fed with experimental oils showed a significant decrease in SFA and MUFA levels and a significant increase in the PUFA levels. The erythrocyte membrane lipid of hypercholesterolemic subjects showed a significant rise in SFA

Table 2 Osmotic fragility of erythrocytes in normal and hypercholesterolemic rats maintained on different sterol esters.

Animal Groups	NaCl concentration(%) causing 50% haemolysis
I	0.5 ± 0.01
II	0.9 ± 0.03[a]
III	0.8 ± 0.03[d]
IV	0.6 ± 0.01[b]
V	0.4 ± 0.02[b]
VI	0.7 ± 0.01[b, c]
VII	0.5 ± 0.04[b, c]

Values are expressed as % are mean ± S.E.M of 6 animals per group. The superscript letters represent statistical significance at $p<0.05$ [I: Control; II: Hypercholesterolemic Control; III: Phytosterol; IV: Low dose of EPA-DHA ester; V: High dose of EPA- DHA ester; VI: Low dose of ALA ester; VII: High dose of ALA ester]

[a] Comparisons are made between Groups I and II

[b] Comparisons are made between Groups II and IV, V, VI, VII

[c] Comparisons are made between EPA-DHA ester and ALA ester Groups

[d] Comparisons are made between Groups II and III

Table 3 The effect of dietary sterol esters on lipid profile of erythrocyte membrane of different rats.

Parameters	I	II	III	IV	V	VI	VII
Total Cholesterol(g/l)	2.2 ± 0.02	6.1 ± 0.05[a]	5.2 ± 0.05[e]	3.9 ± 0.04[b]	1.9 ± 0.01[b]	4.2 ± 0.11[b, c]	2.3 ± 0.12[b, d]
Phospholipid(g/l)	8.5 ± 0.12	3.2 ± 0.18[a]	3.7 ± 0.06[e]	4.9 ± 0.10[b]	9.1 ± 0.01[b]	4.1 ± 0.19[b, c]	7.6 ± 0.09[b, d]
C/P ratio	0.3 ± 0.01	1.9 ± 0.03[a]	1.4 ± 0.06[e]	0.8 ± 0.02[b]	0.2 ± 0.07[b]	1.0 ± 0.22[b, c]	0.3 ± 0.09[b, d]

Values are expressed as mean ± S.E.M of 6 animals per group. The superscript letters represent statistical significance at $p<0.05$ [I: Control; II: Hypercholesterolemic Control; III: Phytosterol; IV: Low dose of EPA-DHA ester; V: High dose of EPA- DHA ester; VI: Low dose of ALA ester; VII: High dose of ALA ester]

[a] Comparisons are made between Groups I and II

[b] Comparisons are made between Groups II and IV, V, VI, VII

[c] Comparisons are made between Groups IV and VI

[d] Comparisons are made between Groups V and VII

[e] Comparisons are made between Groups II and III

Table 4 The effect of dietary oils on fatty acid composition of erythrocyte membrane.

Dietary Groups	Fatty Acids(% w/w)		
	SFA	MUFA	PUFA
I	37.7 ± 0.21	30.2 ± 1.01	32.1 ± 0.32
II	53.5 ± 0.56[a]	24.6 ± 0.98[a]	21.8 ± 0.45[a]
III	40.6 ± 0.46[e]	28.6 ± 0.38[e]	32.8 ± 0.56[e]
IV	30.9 ± 0.09[b]	20.8 ± 0.26[b]	45.3 ± 0.09[b]
V	20.5 ± 0.07[b, c]	25.2 ± 1.02[b, c]	54.3 ± 0.21[b, c]
VI	38.9 ± 0.15[b]	19.6 ± 0.24[b]	41.4 ± 0.44[b]
VII	32.6 ± 0.22[b, d]	23.9 ± 0.97[b, d]	43.6 ± 0.12[b, d]

Values are expressed as mean ± S.E.M of 6 animals per group. The superscript letters represent statistical significance at $p<0.05$ [I: Control; II: Hypercholesterolemic Control; III: Phytosterol; IV: Low dose of EPA-DHA ester; V: High dose of EPA- DHA ester; VI: Low dose of ALA ester; VII: High dose of ALA ester]

[a] Comparisons are made between Groups I and II

[b] Comparisons are made between Groups II and IV, V, VI, VII

[c] Comparisons are made between Groups IV and VI

[d] Comparisons are made between Groups V and VII

[e] Comparisons are made between Groups II and III

and MUFA levels and a significant lowering of PUFA levels in comparison with the normal subjects. The changes were reversed in case of administration with experimental oils to hypercholesterolemic subjects. The ameliorative effect of sterol esters was much better in comparison to phytosterol alone. The table depicts that the changes were more pronounced in case of EPA-DHA rich sterol ester than ALA rich sterol ester.

3.4 Changes in fatty acid composition of erythrocyte membrane phospholipids

The fatty acid compositions of phospholipids of different dietary groups are presented in Table 5. The table depicts that the normal control rats showed 34.4% SFA, 33.06% MUFA and 32.54% PUFA in membrane phospholipids. On the other hand the hypercholesterolemic control rats showed 40.14% SFA, 37.10% MUFA and 22.76% PUFA in their membrane phospholipids. A significant decrease in SFA and MUFA levels and significant increase in PUFA levels was observed by feeding the hypercholesterolemic rats with experimental oils. The increase in PUFA was much more in the rats fed with case of EPA-DHA rich sterol ester than ALA rich sterol ester. The ameliorative effect of sterol esters was much better in comparison to phytosterol alone.

Table 5 Effect of dietary oils on fatty acid composition of erythrocyte membrane phospholipids.

Dietary Groups	Fatty Acids(% w/w)		
	SFA	MUFA	PUFA
I	34.4 ± 0.54	33.1 ± 1.34	32.5 ± 0.45
II	40.1 ± 0.76[a]	37.1 ± 1.00[a]	22.7 ± 0.78[a]
III	35.6 ± 0.23[e]	32.5 ± 1.06[e]	31.9 ± 0.52[e]
IV	25.4 ± 1.09[b]	29.8 ± 1.12[b]	44.8 ± 0.46[b]
V	27.6 ± 1.22[b, c]	31.6 ± 0.89[b, c]	40.7 ± 2.34[b, c]
VI	37.2 ± 0.98[b]	35.0 ± 0.78[b]	27.8 ± 2.11[b]
VII	36.1 ± 0.12[b, d]	33.2 ± 0.67[b, d]	30.7 ± 0.99[b, d]

Values are expressed as mean ± S.E.M of 6 animals per group. The superscript letters represent statistical significance at $p<0.05$ [I: Control; II: Hypercholesterolemic Control; III: Phytosterol; IV: Low dose of EPA-DHA ester; V: High dose of EPA- DHA ester; VI: Low dose of ALA ester; VII: High dose of ALA ester]

[a] Comparisons are made between Groups I and II

[b] Comparisons are made between Groups II and IV, V, VI, VII

[c] Comparisons are made between Groups IV and VI

[d] Comparisons are made between Groups V and VII

[e] Comparisons are made between Groups II and III

3.5 Changes in erythrocyte membrane holes as seen by scanning electron microscope

The holes in hemoglobin-free ghosts generated by osmotic lysis of erythrocytes of normal and hypercholesterolemic rats were characterized. The pore formation in the erythrocyte membrane was confirmed by scanning electron microscopy as shown in Fig. 1. The control rats showed an uneven surface texture of the hole. It can be seen from the figures that the texture of the surface of hypercholesterolemic control rats were more uneven than normal control and the texture was smoothened by administration of different experimental oils. The texture of the surface of the membrane ghost holes of rats treated with EPA-DHA rich sterol esters was smoother in comparison with rats treated with ALA rich sterol ester. The higher dose showed better effects in comparison to lower dose. The effect of sterol ester was better than phytosterol only.

3.6 Antioxidant enzyme activities

The same pattern of effect of the diets was seen on antioxidant enzyme activities in erythrocyte membrane (Table 6). Adding cholesterol to the diet decreased the activities of all enzymes, although the effect was not significant in all cases. However, all enzyme activities were higher in erythrocyte membrane when rats received the phytosterol, sterol esters and groundnut oil plus cholesterol compared with those compared the control groundnut oil plus cholesterol (Table 6). The highest enzyme activities were always seen in the group receiving ALA rich sterol ester in higher dose.

4 Discussion

Alterations in membrane fluidity are determined by the amount of cholesterol and cholesterol/phospholipid molar ratio. The fluidity of the erythrocyte membrane is determined by a number of factors among which cholesterol content and fatty acid composition have significant influences[23, 24]. The interactions of these factors seem to affect the physiological properties of the membranes to a varying degree. Cholesterol enrichment in red cells resulting in loss of membrane fluidity has been reported in literature[15]. Accumulation of cholesterol in erythrocyte membrane may alter the red cell surface area and cause distortion in the shape of red cells. Results indicated that hypercholesterolemic subjects showed an increase in cholesterol and C:P ratio in erythrocyte membrane. In hypercholesterolemic situation, where the cholesterol content of plasma is increased, the concomitantly higher C:P ratio in the blood plasma will have a direct influence on cholesterol transfer from plasma to erythrocytes, resulting in the accumulation of cholesterol in the erythrocyte membrane[25]. The hypercholesterolemic situation achieved in these animals by maintaining on cholesterol enriched diet also resulted in a significant enrichment of erythrocyte membranes with cholesterol (nearly 64% more than the control). There was a decrease in the membrane phospholipid concentration in

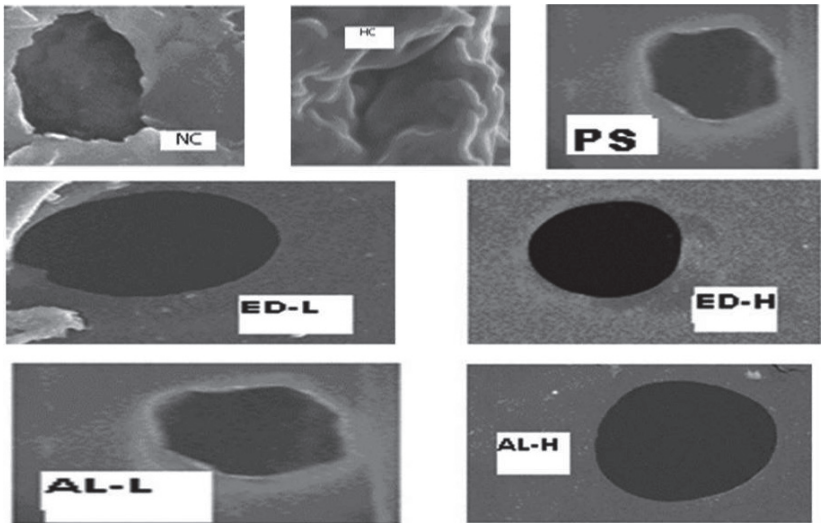

Fig. 1 Erythrocyte membrane ghost hole of different rats (NC-normal control, HC-hypercholesterolaemic control, PS- hypercholesterolemic fed with phytosterol, ED-L- hypercholesterolemic fed with Eicosapentaenoic acid-docosahexaenoic acid-rich sterol ester low dose, ED-H- hypercholesterolaemic fed with Eicosapentaenoic acid-docosahexaenoic acid rich sterol ester high dose, AL-L- hypercholesterolemic fed with alpha-linolenic acid-rich sterol ester low dose, AL-H- hypercholesterolemic fed with alpha-linolenic acid- rich sterol ester high dose).

Table 6 Changes in antioxidant enzyme activities.

Parameters	I	II	III	IV	V	VI	VII
CAT	3.2 ± 0.02	1.1 ± 0.03[a]	1.5 ± 0.06[e]	1.9 ± 0.14[b]	2.1 ± 0.11[b]	2.2 ± 0.01[b, c]	3.2 ± 0.02[b, d]
SOD	1.6 ± 0.12	0.7 ± 0.01[a]	0.8 ± 0.03[e]	0.8 ± 0.10	1.0 ± 0.01[b]	0.9 ± 0.03[b, c]	1.6 ± 0.04[b, d]
GSH	12.3 ± 0.01	5.9 ± 0.13[a]	6.3 ± 0.16[e]	6.8 ± 0.12[b]	8.8 ± 0.12[b]	9.0 ± 0.22[b, c]	11.9 ± 0.29[b, d]
GPx	0.9 ± 0.01	0.2 ± 0.01[a]	0.3 ± 0.01	0.3 ± 0.02[b]	0.4 ± 0.02[b]	0.5 ± 0.01[b, c]	0.9 ± 0.04[b, d]

Values are expressed as mean ± S.E.M of 6 animals per group. The superscript letters represent statistical significance at $p<0.05$ [I: Control; II: Hypercholesterolemic Control; III: Phytosterol; IV: Low dose of EPA-DHA ester; V: High dose of EPA- DHA ester; VI: Low dose of ALA ester; VII: High dose of ALA ester]

[a] Comparisons are made between Groups I and II

[b] Comparisons are made between Groups II and IV, V, VI, VII

[c] Comparisons are made between Groups IV and VI

[d] Comparisons are made between Groups V and VII

[e] Comparisons are made between Groups II and III

hypercholesterolemia resulting in a significant increase in the C:P ratio. With the administration of sterol esters the cholesterol content decreased and phospholipid content increased in the membrane resulting in a decrease in C:P ratio.

The present data on osmotic fragility have revealed the relatively higher fragility of erythrocytes in hypercholesterolemic rats. Possibly because of the increased C:P ratio, the cells have become more fragile which is a contributory factor towards cell deformity. The ability of the erythrocyte to deform, one of the most important determinants for their survival in the circulation involves several factors including the deformability of the membrane itself, the surface area per volume ratio of the cell, and the internal viscosity[26]. In this study, the sterol esters were evidenced to effectively counter the increased mean cell fragility of erythrocytes by lowering the membrane cholesterol and C:P ratio. EPA-DHA rich ester produced better effect against membrane fragility than ALA rich ester. The higher doses of sterol esters produced better effects.

The present study was also designed to examine the levels of erythrocyte antioxidant enzyme activities in hypercholesterolemic and treated groups. Several enzymes have evolved in aerobic cells to overcome the damaging effects of reactive oxygen species (ROS). They are significantly used to maintain the redox balance during oxidative

stress and are collectively called as endogenous antioxidative enzymes. Superoxide dismutase (SOD), glutathione peroxidase (GPx), reduced glutathione (GSH) and catalase (CAT) are the main endogenous enzymatic defense systems of all aerobic cells[27]. They give protection by directly scavenging superoxide radicals and hydrogen peroxide, converting them to less reactive species[28]. SOD catalyzes the dismutation of superoxide radical ($\cdot O_2$) to hydrogen peroxide (H_2O_2). Although H_2O_2 is not a radical, it is rapidly converted by fenton reaction into $\cdot$OH radical which is very reactive. Among various antioxidant mechanisms in the body, SOD is thought to be one of the major enzymes that protect cells from ROS. Glutathione peroxidase (GPx) neutralizes hydrogen peroxide by taking hydrogens from two GSH molecules resulting in two H_2O and one GSSG. The enzyme glutathione reductase then regenerates GSH from GSSG with NADPH as a source of hydrogen. Another important part of the enzymatic defense system is catalase. CAT is one of the most active catalysts produced by nature. CAT is largely, but not exclusively, localized in peroxisomes, wherein many H_2O_2 producing enzymes reside. Thus CAT, which exhibits a high Km for H_2O_2, can act upon H_2O_2 produced before it diffuses to other parts of the cell. CAT is a tetrameric heme containing enzyme that is found in all aerobic organisms. Because of its wide distribution, evolutionary conservation and capacity to rapidly degrade hydrogen peroxide, it has been proposed that CAT plays an important role in systems which have evolved to allow organisms to live in aerobic environments. Therefore activity of CAT is one of the important biomarker of oxidative stress. Results showed that antioxidant enzyme activities were lowest in hypercholesterolemia due to excessive production of ROS. Sterol esters increased the enzyme activities thus lowering the oxidative stress induced by hypercholesterolemia. ALA rich sterol ester in higher dose produces the highest effect against hypercholesterolemia.

5 Conclusion

In conclusion, rat erythrocytes appear to be deformed and become more fragile in cholesterol-rich blood. This deformity and fragility were partially reversed by sterol and esters by virtue of their ability to lower the extent of hypercholesterolemia. High dose EPA-DHA rich sterol ester produced the best results.

Acknowledgements

Financial support obtained from University of Calcutta (through UPE projects) is gratefully acknowledged by the authors. Authors are sincerely thankful to Dr. S. Ghosh, Dept. of Chemical Technology for his help and encouragement.

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