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Antioxidative Effect of Conjugated Linolenic Acid in Diabetic and Non-Diabetic Blood: an *in vitro* Study

Pubali Dhar[1], Krishna Chattopadhyay[2] ,Debasish Bhattacharyya[2], Anadi Roychoudhury[2], Arundhat Biswas[2] and Santinath Ghosh[2]*

[1] *Department of Physiology, Krishnagar Govt. College (Nadia, West Bengal, INDIA)*
[2] *Department of Chemical Technology, University of Calcutta (92, A.P.C. Road, Kolkata-700 009, INDIA)*

Abstract: The present study examined the *in vitro* antioxidant activity of conjugated octadecatrienoic fatty acid (9*cis*, 11 *trans*, 13 *trans*-18:3), α-eleostearic acid present in karela seed oil (*Momordica charantia*) at about 55% level. The *in vitro* antioxidant properties of α-eleostearic acid are investigated on oxidative modification of human plasma, low-density lipoprotein (LDL) and erythrocyte membrane lipid. Blood samples are collected from diabetic and non-diabetic (normal) healthy individuals. α-eleostearic acid is added at 0.05% and 0.1% concentrations to plasma, LDL and erythrocyte membrane isolated from the respective blood samples and peroxidations are determined against control samples.

A significant increase of respective peroxidation levels has been observed in diabetic control blood than the non-diabetic control blood. α-eleostearic acid has decreased lipid peroxidation level against control samples in a dose dependent manner. The present findings suggest that CLnA, 9*cis*, 11*trans*, 13*trans*-18:3 is a potentially effective antioxidant that can protect plasma, low density lipoprotein and erythrocyte membrane from oxidation which may be effective in reducing the risk of coronary heart disease in diabetes mellitus.

Key words: antioxidant, diabetes mellitus, conjugated linolenic acid

1 INTRODUCTION

The most common problem encountered in diabetes mellitus (DM) is atherosclerotic cardiovascular disease[1], seen frequently associated with dyslipidaemia, including hypertriglyceridaemia and hypercholesterolaemia. Oxidation of lipids accumulated on the arterial wall by endothelial cells, smooth muscle cells and macrophages[2-4] can be amplified by reactive oxygen species (ROS) and reactive nitrogen species generation which play a significant role in the development of atherosclerosis. Oxidant free radicals have been implicated in the pathogenesis of type I DM[5,6]. In addition, diabetic patients have significant defects of antioxidant protection[7,8]. Peroxidation causes impairment of biological membrane functioning, e.g., decreases fluidity, inactivates membrane-bound enzymes and receptors. The more unsaturated a fatty acid side chain, the greater its propensity to undergo lipid peroxidation. Polyunsaturated fatty acids (PUFA) are prone to autooxidation giving rise to peroxyl free-radicals.

The role of free-radical reactions in human disease, biology, toxicology, and the deterioration of food has become an area of interest. Biomedical scientists and clinicians are interested in antioxidants because they protect the body against damage by ROS. Antioxidants scavenge free radicals and reduce the deleterious consequences within the lipid. Conjugated linoleic acid (CLA) has been proved to prevent non-insulin dependent DM[9]. Garlic oil[10], fenugreek[11] sesame lignans, sesamol[12], act as antioxidants and reduce pathological conditions of DM.

Conjugated linolenic acid (CLnA) is one of the highly unsaturated forms of conjugated fatty acids and possibly includes multiple positional and geometric isomers (*cis* and *trans*). CLnA occurs abundantly in some seed oils, such as karela seed, tung oil and pomegranate oil.

Conjugated linolenic acid (CLnA), (α-eleostearic acid:9c, 11t, 13t-18 : 3) commonly found in karela seed (*Momordica charantia*) was nutritionally evaluated in our previous experiment[13]. In another previous study[14] CLnA at 0.5%,

* Correspondence to: Santinath Ghosh, Department of Chemical Technology, University of Calcutta, 92, A.P.C. Road, Kolkata-700 009, West Bengal, INDIA.
E-mail: santinathghosh@yahoo.com.hk
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2%, and 10% levels were fed to rats with sunflower oil and it has been observed that CLnA at 0.5% level significantly lowers plasma lipid peroxidation, erythrocyte membrane lipid peroxidation, liver tissue lipid peroxidation than the control sunflower oil group. The antioxidant property has decreased with the increase of CLnA level in the diet.

In our next *in vivo* study, CLnA has been found to function highly active antioxidant that reduced the production of malonedialdehyde (MDA) in plasma, LDL-cholesterol and erythrocyte membrane in alloxan induced DM in rats[15].

In this study, we investigated the *in vitro* antioxidative effect of CLnA present in karela seed oil by determining the thiobarbituric acid reactive substance (TBARS) in plasma, LDL-cholesterol and erythrocyte membrane ghost lipid peroxidation induced by copper of diabetic and non-diabetic human blood collected from male individuals.

2 MATERIALS AND METHODS

2.1 Preparation and analysis of CLnA

Oil was extracted from authentic karela (*Momordica charantia*) seed, obtained from the local market at Kolkata, India, in a soxhlet apparatus with food grade n-hexane (40-60℃ boiling point range). The solvent was removed under the stream of nitrogen. The oil was completely hydrolyzed by *Candida rugosa* (*cylindracea*) lipase (Activity 7000U/g of lipase obtained from Sigma Chemical Co, St Lous, MO.). About 10 g oil was taken in a stoppered conical flask along with 10 g water containing 0.5% powered enzyme. The mixture was stirred for 8 h at 30℃. The hydrolyzed oil was extracted with hexane and solvent was removed under the stream of nitrogen. The hydrolyzed fatty acid of karela seed oil was stored at −40℃ and used as a source of CLnA. Fatty acid composition was determined by gas−liquid chromatography (GLC) method as reported earlier[15].

2.2 Collection of blood

Normal (non-diabetic) blood was collected from human volunteers of the age group 40−50 years of fasting blood glucose level 90 ± 10 mg/dL. Diabetic blood was collected from patients suffering from diabetes mellitus (oral drug dependent) for at least 5 years with high fasting blood glucose level (200 ± 20 mg/dL) in an uncontrolled manner. Plasma was separated and glucose, total cholesterol and HDL-cholesterol were measured by using enzymatic kit (Ranbaxy Diagnostics Limited, New Delhi, India).

2.3 Scanning electron microscope (SCEM) observation of RBC

After plasma separation, red cells (from human volunteers) were washed and suspended in PBS (0.01M, phosphate buffer solution, pH 7.4). 3% (packed cell volume) cell suspension was then fixed by the addition of glutaraldehyde solution (in PBS) to the test tube containing the cells. Fixation was carried out at 4℃ for about 24 h. The final concentration of glutaraldehyde was 3% in the cell suspensions. The cells were subsequently washed with distilled water and dehydrated through graded series of ethanol. A drop of the cell suspension in 100% ethanol was put on a glass slide (1 × 1 cm), air-dried and coated with 200 Å thick layer of gold. Scanning electron microscopy was done in a Philips Scanning Electron Microscope (PSEM 500) operated at 20 kV.

2.4 Plasma peroxidation

Plasma peroxidation was measured by the assay of thiobarbituric acid-reactive substances (TBARS) according to the standard method[16]. The antioxidants dissolved in dimethyl sulfoxide (DMSO) were added to the plasma samples. In the control sample only DMSO was added. The amount of malone-di-aldehyde (MDA) formed was calculated by taking the extinction coefficient of MDA to be $1.56 \times 10^5 M^{-1}cm^{-1}$.

2.5 Lipoprotein oxidation susceptibility (LOS) test

A 500 μL plasma sample was treated with 50 μL of a solution containing 0.2 mM dextran sulphate (MW 50,000, Genzyme, Cambridge, MA) and 0.5M $MgCl_2.6H_2O$ to precipitate the apo B- containing lipoproteins (LDL and VLDL) according to Bachorik and Albers[17]. After centrifugation at 3000 × g at 20℃ for 10 min, the supernatant was removed, and 1 mL of 6% bovine serum albumin and another 50 μL of the dextran sulfate magnesium solution was added. The solution was briefly vortexed and recentrifuged as above to wash away any HDL or residual serum proteins (except, of course, albumin). The supernatant was removed and washed precipitate (containing LDL and VLDL) was dissolved in 2.5 μL of 4% NaCl. A volume of redissolved precipitate containing 100 μg of non-HDL cholesterol was combined with sufficient 4% NaCl to give a total volume of 500 μL (app. a 1 : 5 dilution). 50 μL of a 0.5 mM $CuCl_2.2H_2O$ solution was added (final copper concentration was 46 μM), and then the samples were incubated at 37℃ in a shaking water bath for 3 h. Next TBARS was measured by adding 2 mL of the TBARS reagent to each tube. The mixture was heated at 100℃ in a water bath for 15 min. After removing and cooling the tubes, 2.5 mL n-butanol was added, the tubes were vortexed and then centrifuged for 15 min at 3000 rpm at room temperature. The pink upper layer was removed and the optical density was determined in a spectrophotometer at 532 nm according to the method described by Phelps and Harris[18].

2.6 Preparation and oxidative sensitivity of erythrocyte membrane ghost[19]

After plasma separation, the red blood cells (RBC) were

washed three times by centrifugation at 3,000 × g for 10 min with three volume of a cooled isotonic solution containing 0.15M NaCl and 10^{-5}M EDTA. RBC was hemolized using hypotonic solution and centrifuged at 20,000 × g for 40 min in a cold centrifuge at 4℃. The supernatant was removed carefully with a Pasteur pipette. The process was repeated two more times. After the last wash step, the supernatant was removed as much as possible and the loosely packed milky-looking membrane pellet was re-suspended at the bottom of the tube using 0.89% NaCl solution. Concentrated membrane solution was taken in 2 mL screw cap vial and stored at −40℃.

2.7 Modified 2-thiobarbituric acid test

A modification of the 2-thiobarbituric acid test[20] was used to measure the lipid peroxides. A 0.5 mL aliquot of the red blood corpuscle membrane suspension was mixed with 1.0 mL of 10% trichloroacetic acid and 2.0 mL of 0.67% of 2-thiobarbituric acid. The mixture was heated at 95℃ for 15 min, cooled, and centrifuged. The absorbance of the supernatant was measured at 534 nm in a spectrophotometer (Shimadzu, Tokyo, Japan), and the amount of malonedialdehyde (MDA) formed was calculated by taking the extinction coefficient of MDA to be $1.56 \times 10^5 M^{-1}cm^{-1}$.

2.8 Assay of protein

Erythrocyte membrane protein was estimated by the method of Lowry *et al*.[21].

2.9 Statistical analysis

The results are given as the mean (standard error of mean. For statistical analysis of result, student's t test[22] was performed.

3 RESULTS

Microstructure of RBC of both normal and diabetic blood has been observed and shown in Fig. 1 and Fig. 2 at two different magnifications. Smooth spherical shape of RBC is clearly noted in the non-diabetic human blood (Fig. 1). Crenated sphere shape of diabetic RBC can be easily distinguished from normal shape of non-diabetic RBC. This deformation of cells may be due to glycosylation of haemoglobin. So, ultrastructure (SCEM) of RBC can be used as a tool to observe the diabetic condition.

Fatty acid composition of the hydrolysed karela seed oil is given in Table 1. The fatty acid contains about 57.7% CLnA. The extent of plasma lipid and LDL lipid peroxidations induced by copper was assessed by measuring the formation of TBARS in the blood collected from non-diabetic and diabetic male individuals. Table 2 showed the effect of CLnA at different doses on the oxidative modification of plasma lipid and LDL of non-diabetic and diabetic human blood. Peroxidations were greatly reduced in the CLnA added groups than the control group. 0.1% CLnA exhibited the strongest antioxidative effect, decreasing the TBARS formation in plasma lipid. A significant decline in MDA production had been observed in both non-diabetic as well as diabetic blood for 0.05% and 0.1% CLnA groups. There was no significant change of plasma lipid peroxidation with the increase of CLnA concentration both in non-diabetic and diabetic group. At 0.1% concentration it was most active in both non-diabetic and diabetic groups. Copper induced lipoprotein oxidation susceptibility (LOS) had also been observed and tabulated in Table 2. LOS decreased significantly both in non-diabetic and diabetic blood in presence of CLnA. In case of non-diabetic human blood 0.053 nmole of MDA / mg of non-HDL cholesterol had been observed in 0.05% CLnA treated group from 0.082 nmole of MDA / mg of non-HDL cholesterol in control non-diabetic blood. In diabetic group 0.043 nmole of MDA / mg of non-HDL cholesterol had been observed in 0.05% CLnA group from 0.080 nmole of MDA / mg of non-HDL choles-

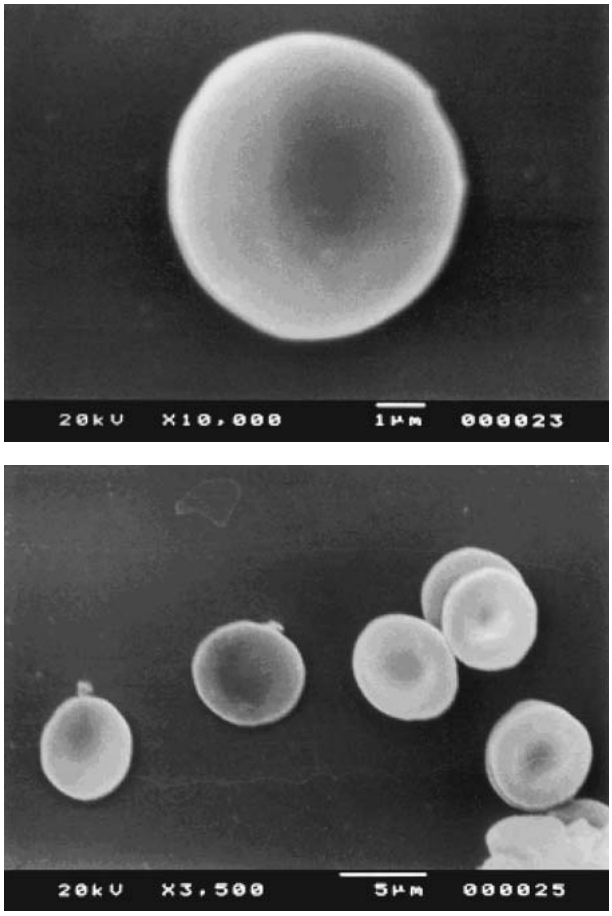

Fig. 1 Smooth Sphere Shape of RBC of Normal Blood (Blood Glucose level of 90 ± 10mg/dL).

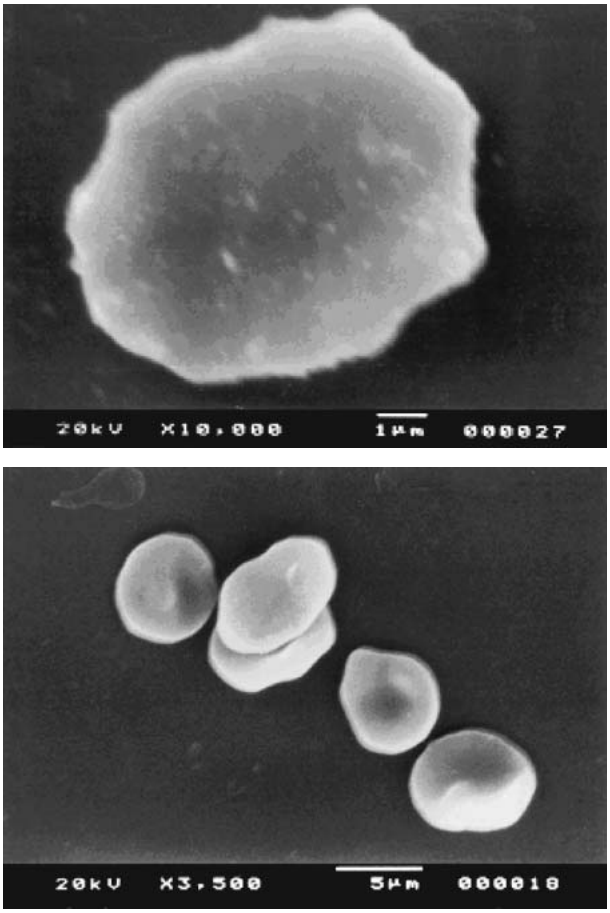

Fig. 2 Crenated Sphere Shape of RBC of Diabetic Blood (Blood Glucose level of 200 ± 20mg/dL).

terol of the control group. With the increase of CLnA concentration from 0.05% to 0.1% the LOS formation decreased but it was not significant.

Table 3 showed the erythrocyte membrane (EM) lipid peroxidation of non-diabetic and diabetic human blood.

Table 1 Fatty Acid Composition of karela Seed Oil.

Fatty Acids	% w/w
16:0	1.4
18:0	31.5
18:1	6.2
18:2	3.2
18:3 CLnA (9c, 11t, 13t, 18:3)	57.7

The erythrocyte membrane lipid peroxidations were significantly restricted in the experimental groups where CLnA was added at 0.05 and 0.1% level. But there was no significant difference of MDA formation both in diabetic and non-diabetic group with the variation of CLnA concentration.

4 DISCUSSION

Clinical and experimental studies have demonstrated that the oxidative modification of LDL is essential for the development of atherosclerosis[23]. It is known that dietetic and pharmacological interventions can modify the susceptibility of lipoproteins to oxidation and consequently the progression of atherosclerosis. Plasma lipid, LDL and EM lipids are susceptible to peroxidation. LDL lipid peroxidation and its inhibition by antioxidants are important in the molecular mechanism of atherosclerosis and in identifying foods that may prevent the disease. Glucose auto oxidation, polyol pathway and protein glycation are biochemical pathways associated with hyperglycaemia and toxic superoxide intermediates of type I diabetes mellitus[24]. This lead to the peroxidation of lipids of plasma, lipoprotein and EM[25]. Oxidized lipoproteins lead to the development of atherosclerosis and diabetic vascular complications. EMs also contain

Table 2 Effect of CLnA on *In Vitro* Plasma and LDL Peroxidation In Non Diabetic and Diabetic Blood.

Groups	Plasma Lipid Peroxidation (nmole of MDA/mL of Plasma		Lipoprotein Oxidation Susceptibility (LOS) (nmole of MDA/mg of non-HDL Cholesterol)	
	Non-Diabetic	Diabetic	Non Diabetic	Diabetic
Control	$10.64 \pm 0.132^{\#\#}$	$34.46 \pm 5.38^{\#}$	$0.082 \pm 0.023^{\#}$	$0.080 \pm 0.01^{\#\#}$
CLnA (0.05%)	$2.77 \pm 0.56^{\#\#}$	$5.48 \pm 1.17^{\#}$	$0.053 \pm 0.019^{\#}$	$0.043 \pm 0.007^{\#\#}$
CLnA (0.1%)	$2.04 \pm 0.715^{\#\#}$	$4.9 \pm 0.679^{\#}$	$0.044 \pm 0.001^{\#}$	$0.039 \pm 0.006^{\#\#}$

Values are Mean ± SEM, n = 6
$^{\#}p < 0.05$, $^{\#\#}p<0.001$

Table 3 Effect of CLnA on *In Vitro* Erythrocyte Membrane Lipid Peroxidation In Non Diabetic and Diabetic Blood.

Groups	Erythrocyte Membrane Lipid Peroxidation (nmole of MDA/mg of protein)	
	Non Diabetic	Diabetic
Control	$4.41 \pm 0.24^{\#}$	$6.91 \pm 0.298^{\#\#}$
CLnA (0.05%)	$1.69 \pm 0.22^{\#}$	$2.54 \pm 0.383^{\#\#}$
CLnA (0.1%)	$1.63 \pm 0.17^{\#}$	$2.36 \pm 0.43^{\#\#}$

Values are Mean ± SEM, n = 6, $^{\#}p < 0.05$, $^{\#\#}p < 0.001$

high amount of polyunsaturated fatty acids, which are prone to oxidation by free radicals generated in diabetes mellitus. The SCEM study has revealed that the EM is clearly affected in the diabetic mellitus individuals (with uncontrolled diabetes). The erythrocytes are decamped, with rigid membrane structure.

In the present study, overall plasma, LDL and EM obtained from blood of diabetic and non-diabetic individuals were used to investigate the lipid peroxidations and also to elucidate the antioxidative activity of CLnA at two different concentrations. In non-diabetic group the peroxidation produced in plasma, and EM lipid was much less than that produced in diabetic group. Hence in diabetic group 0.1% CLnA was more effective than 0.05% concentration. In non-diabetic group plasma peroxidations are effectively reduced by 0.05%CLnA. Similarly, EM lipid peroxidations were high in diabetic group in comparison to the non-diabetic group. The scavenging action of CLnA was most effective at 0.1% level in diabetic group.

Suzuki *et al*.[26] observed that the oxygen consumption of bitter gourd oil containing CLnA is much faster than the other non conjugated fatty acids. The oxidations products of CLnA are mainly dimmers and polymers, where as hydroperoxides are the common oxidation products of other fatty acids. So the protecting ability of CLnA is mainly due to fast oxygen absorption to form dimmers and polymers. However, the oxidation of CLnA is still not clear and less is known about the effect of the oxidation products of CLnA.

The present study had verified the remarkable ability of CLnA to function as a protective agent against plasma and EM lipid peroxidation when present in a small concentration in human blood. Our *in vivo* experiment[14] on CLnA had shown that CLnA at 0.05% in the diet reduced the peroxidation levels of plasma, LDL and EM lipid. In alloxan induced diabetes mellitus rats[15] CLnA at 0.5% level functioned as the more effective antioxidant than 0.15% α-tocopherol. The *in vivo* results of our previous experiments clearly supported the *in vitro* results. So presence of CLnA in a very small concentration in the blood stream would be very much effective in reducing the risk of CHD not only in normal conditions but also in diabetic conditions.

5 CONCLUSIONS

Conjugated octadecatrienoic fatty acid (9*cis*, 11*trans*, 13*trans*-18 : 3), α-eleostearic acid acts as effective antioxidants in *in vitro* study. In *in vivo* condition CLnA may possibly function in reducing the risk of coronary heart disease in diabetes mellitus.

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