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Attenuation of diabetic complications by C-phycoerythrin in rats: antioxidant activity of C-phycoerythrin including copper-induced lipoprotein and serum oxidation

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In the present study, the protective role of purified C-phycoerythrin (C-PE) against diabetic complications and Cu-mediated lipoprotein oxidation was evaluated. C-PE (25 and 50 mg/kg body weight per d) was administered to experimental streptozotocin–nicotinamide-induced type 2 diabetic male rats for 28 d. C-PE treatment successfully ameliorated diabetic complications by decreasing food intake, organ weights, serum concentrations of glucose, cholesterol, TAG, VLDL-cholesterol, creatinine, uric acid and thiobarbituric acid-reactive substances (TBARS), with increases in body weight, Hb, total protein, bilirubin and ferric-reducing ability of plasma values. Hepatic and renal tissues demonstrated significant decreases in TBARS, lipid hydroperoxide and conjugated diene contents, with increases in superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, vitamin E and vitamin C levels. Furthermore, the 4-week *ex vivo* and *in vitro* administration of C-PE (0·5 and 1·0 mg/ml) indicated a decrease in Cu-mediated serum oxidation. The kinetics of the LDL oxidation profile showed significant prolongation of the lag phase with declines in oxidation rate, conjugated dienes, lipid hydroperoxide and TBARS. Results indicated the involvement of C-PE in the amelioration of diabetic complications by significant reductions in oxidative stress and oxidised LDL-triggered atherogenesis.

Phycoerythrin: Diabetes: Antioxidants: Low-density lipoprotein oxidation

Blue-green algae (cyanobacteria) are among the most primitive life forms on Earth. *Nostoc*, *Spirulina* and *Aphanizomenon* species are considered as a nutrient-dense food, because of the presence of carotenoids, biliprotein, minerals, essential amino acids, fatty acids and many other bioactive compounds[1,2]. Bilins, linear tetrapyrrole molecules, are important cofactors of phytochrome and phycobiliproteins[1]. Phycoerythrin harvests light in the photosynthetic apparatus of cyanobacteria, allowing the organism to efficiently absorb light in the region of the visible spectrum that is poorly covered by chlorophyll a. Through resonance energy transfer the absorbed light energy is transferred to the photosynthetic reaction centre[1]. Because of the unique colour, fluorescence and antioxidant properties of phycobiliproteins, they possess a wide range of promising applications in the food and cosmetics, diagnostics, biomedical and therapeutic industries[2,3]. A significant body of data suggests that the immunoenhancing properties of cyanobacteria can be useful in the adjunct treatment of various diseases involving (a) a suppressed or exhausted immune system and (b) inappropriate immune responses such as allergies, autoimmune diseases and chronic inflammatory conditions. Phycobiliproteins from various species of cyanobacteria and red algae are widely reported for their anti-inflammatory, anti-hepatotoxicity, anti-carcinogenic, antiplatelet and nutritious values[2,4]. Recently, we reported the ameliorative action of C-phycoerythrin (C-PE) against hepato- and renal toxicity in rats[5].

The pathogenesis of type 2 non-insulin-dependent diabetes mellitus is complex, involving the progressive development of insulin resistance and a defective insulin secretion which gradually leads to overt hyperglycaemia. Hyperlipidaemia is a feature frequently observed in diabetes and certainly contributes to the high prevalence of the atherosclerosis- and CHD-associated metabolic disorder[6,7]. There is considerable evidence that hyperglycaemia causes many of the major complications of diabetes including nephropathy, retinopathy, neuropathy as well as macro- and microvascular damage[6,8]. Insulin-resistant patients with and without type 2 diabetes are at increased risk of developing the metabolic syndrome, a major cause of heart disease, hypertension and dyslipidaemia[7,8]. The relationship among anaemia, diabetes, chronic kidney disease and risk of CHD has also been reported in several studies[9,10]. Diabetics have a higher risk of developing coronary artery disease and peripheral atherosclerosis than the general population. High blood sugar is commonly associated with increased oxidative changes in LDL by elevated

Abbreviations: BW, body weight; C-PE, C-phycoerythrin; TBARS, thiobarbituric acid-reactive substances.

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polyol pathway flux, increased oxygen free radical formation and advanced glycosylation which appear to accelerate plasma LDL oxidation[11,12]. Elevated levels of glucose cause oxidative stress due to an increased production of mitochondrial reactive oxygen species, non-enzymic glycation of proteins, glucose auto-oxidation and oxidative modification of LDL depleting the appropriate compensatory response from the endogenous antioxidants[6,8,11].

Therefore, hyperglycaemia-derived reactive oxygen radicals are considered as the main mediator of diabetic complications, and a treatment designed to reduce oxidative stress could be a major approach in the reduction of diabetes complications. In the present investigation, we used experimental streptozotocin–nicotinamide-induced type 2 diabetic male albino rats and evaluated the potency of purified C-PE on various diabetes-related clinical and oxidative stress markers. The effect was also emphasised by *ex vivo* and *in vitro* kinetic studies of Cu-induced LDL and serum oxidation.

Materials and methods

Cultivation of *Phormidium tenue* and purification of C-PE was done as detailed in our previous report[5]. The molecular weight of purified C-PE was 12·5 kDa. UV-visible spectroscopy suggested 542 nm as the absorbance maximum (λ_{max}) of purified C-PE.

Animals and housing conditions

Male albino rats, aged 3 months (Charles Foster), were obtained from the animal house, Department of Biochemistry, M. S. University of Baroda, Vadodara, India and acclimatised for 10 d before commencement of the experiments at BRD School of Biosciences, S. P. University, Vallabh Vidyangar. Animals were housed individually with *ad libitum* access to water and fed on commercial chow (Pranav Agro Industries Ltd, Pune, India) in a well-ventilated animal unit (26 ± 2°C, humidity 60 %, 12 h light–12 h dark cycle). The commercial chow (basal diet) contained carbohydrates (56 g/100 g), proteins (22 g/100 g), fat (4 g/100 g), fibre (4 g/100 g) and mineral mixture (6 g/100 g). Care and procedures adopted for the present investigation were in accordance with the approval of the Institutional Animal Ethics Committee.

Induction of non-insulin-dependent diabetes mellitus

The non-insulin-dependent diabetes mellitus model was induced in overnight fasted animals by a single intraperitoneal injection of streptozotocin (65 mg/kg body weight (BW); Sigma Aldrich, Deisenhofen, Germany) dissolved in citrate buffer (pH 4·5)[10]. After 15 min, nicotinamide (110 mg/kg BW; Qualigens Fine Chemicals, Mumbai, India) dissolved in normal saline was intraperitoneally administrated. Hyperglycaemia was confirmed by the elevated glucose level in the blood, as determined at 72 h and then on day 7 after injection. The animals with fasted overnight blood glucose concentration more than 1600 mg/l were used for the study.

Experimental design

On day 8 after streptozotocin–nicotinamide injection, the C-PE treatment was started in the male albino rats (245–275 g). A total of twenty-four rats (eighteen diabetic, six normal) were divided into four groups. Group I (normal control) received the vehicle of normal saline by oral administration at the level of 1 ml/d for 28 d. Group II (diabetic control) received the vehicle of normal saline by oral administration at the level of 1 ml/d for 28 d. Groups III (diabetic + 25 mg C-PE) and IV (diabetic + 50 mg C-PE) served as the diabetic treatment groups and received purified C-PE dissolved in distilled water by oral administration at the dose levels of 25 and 50 mg/kg BW per d, respectively, for 28 d.

Daily food intake was determined. Rats were weighed at the beginning and at the end of the experimental period. At end of the 28 d treatment period, rats were fasted for 18 h and killed under mild (diethyl ether) anaesthesia. Blood was collected by cardiac puncture and immediately analysed for Hb content. For clinical chemistry parameters, blood samples were kept at room temperature for 1 h, and centrifuged at 1000 ***g*** for 10 min to obtain serum. Serum, liver and kidney were stored at − 20°C until assayed.

Clinical chemistry

Biochemical parameters measured using standard kits (Eve's Inn Diagnostics, Vadodara, India) were: Hb, total protein, glucose, total cholesterol, TAG, total bilirubin, creatinine, blood urea N and uric acid. HDL-cholesterol was estimated by a standard kit containing phosphotungstate-magnesium chloride reagent. LDL-cholesterol and VLDL-cholesterol were calculated according to the Friedewald formula[13]. The ferric-reducing ability of plasma assay was performed to measure the concentration of total antioxidants. The method is based on the measurements of absorption changes that appear when the 2,4,6-tri-pyridyl-s-triazine (TPTZ)–Fe^{3+} complex is reduced to the TPTZ–Fe^{2+} form in the presence of antioxidants. Results were expressed as a concentration of antioxidants having a ferric-reducing ability equivalent to that of 1 μM-$FeSO_4$[14]. Measurement of thiobarbituric acid-reactive substances (TBARS) as an index of lipid peroxidation was assayed following the method of Buege & Aust[15].

Determination of oxidative stress and antioxidant biomarkers in tissues

Preparation of post-mitochondrial fraction. Liver and kidney were homogenised in chilled potassium chloride (1·17 %, w/v) using a glass homogeniser[16]. Homogenate was centrifuged at 800 ***g*** for 5 min at 4°C in order to separate nuclear debris. The supernatant fraction was again centrifuged at 10 500 ***g*** for 20 min at 4°C to obtain the post-mitochondrial fraction which was used to assay lipid peroxidation (TBARS, conjugated dienes and lipid hydroperoxide), catalase, superoxide dismutase, glutathione peroxidase, reduced glutathione, vitamin E and vitamin C.

Assay of lipid peroxidation. The TBARS and conjugated diene contents were assayed following the method of Buege & Aust[15]. The lipid hydroperoxide content was measured according to Jiang *et al.*[17].

Assay of antioxidant enzymes. Superoxide dismutase (*EC* 1.15.1.1). The assay mixture contained 100 μl of post-mitochondrial fraction, 1·2 ml sodium pyrophosphate buffer (0·052 M; pH 8·3), 100 μl phenazine methosulfate (186 μM)

and 300 μl nitro blue tetrazolium (300 μM). The reaction was started by the addition of 200 μl NADH (750 μM). After incubation at 30°C for 90 s, the reaction was stopped by the addition of 100 μl glacial acetic acid. The reaction mixture was stirred vigorously with 4·0 ml *n*-butanol. The colour intensity of the chromogen in the butanol was measured at 560 nm and the concentration of superoxide dismutase was expressed as U/mg protein[18].

*Catalase (*EC *1.11.1.6) activity*. A volume of 100 μl of the post-mitochondrial fraction was added to a cuvette containing 1·9 ml phosphate buffer (50 mM; pH 7·0). The reaction was started by the addition of 1·0 ml freshly prepared H_2O_2 (30 mM). The rate of H_2O_2 decomposition (nmol H_2O_2 decomposed/s per g) was measured at 240 nm[19].

*Glutathione peroxidase (*EC *1.11.1.9) activity*. The reaction mixture consisted of 400 μl potassium phosphate buffer (250 mM; pH 7·0), 200 μl post-mitochondrial fraction, 100 μl reduced glutathione (10 mM), 100 μl NADPH (2·5 mM) and 100 μl glutathione reductase (6 U/ml). The reaction was started by adding 100 μl H_2O_2 (12 mM) and the absorbance was measured for 5 min at 366 nm (at an interval of every 1 min). The concentration of glutathione peroxidase was calculated using the absorbance value and the molar extinction coefficient of $6{\cdot}22 \times 10^3$/M per cm. Values were expressed as U/mg protein[20].

Assay of non-enzymic antioxidants. Reduced glutathione estimation. An equal quantity of the post-mitochondrial fraction was mixed with 10 % TCA and centrifuged to separate proteins. To 100 μl of supernatant fraction, 2 ml phosphate buffer (250 mM; pH 8·4), 500 μl 5,5′-dithiobis 2-nitrobenzoic acid (10 mM) and 400 μl double distilled water were added. The mixture was vortexed and the absorbance read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as μg/g tissue[21].

Vitamin E was analysed using colour reagent (2,4,6-tri-pyridyl-s-triazine (0·12 %) and $FeCl_3$ (0·12 %)) after absolute ethanol and xylene extraction. The absorbance was recorded at 600 nm and concentration was expressed as μg/g tissue[22].

Vitamin C estimation. A volume of 500 μl of the post-mitochondrial fraction was mixed with 1·5 ml TCA (6 %) to separate proteins. To 500 μl of the supernatant fraction, 500 μl 2,4 dinitro phenyl hydrazine reagent was added and mixed well. Tubes were allowed to stand at room temperature for 3 h and then placed in ice-cold water. To this, 2·5 ml sulfuric acid (85 %) were added and allowed to react for 30 min. The colour developed was read at 530 nm and concentration was expressed as μg/g tissue[23].

Lipoprotein and serum oxidation

Isolation of low-density lipoprotein. LDL was isolated from the serum of normal control, diabetic control and treatment groups by the heparin-citrate buffer precipitation method[24]. The protein concentration of the LDL was measured with the Lowry method[25] using bovine serum albumin as standard.

Copper-induced low-density lipoprotein oxidation ex vivo. LDL (100 μg) was diluted to 1 ml with PBS and oxidation was initiated by the addition of 10 μl of freshly prepared 0·167 mM-$CuSO_4$ (final concentration of $CuSO_4$ was 1·67 μM) in 1 cm quartz cuvettes. The method was essentially the same as described by Palomäki *et al.*[26]. The oxidation kinetics were determined according to the method of Estebauer *et al.*[27]. Production of conjugated dienes was determined by monitoring the change in absorbance at 234 nm every 10 min at 37°C for 3 h on a diode array UV-visible spectrophotometer (model 8452A; Hewlett Packard Instruments Inc., Palo Alto, CA, USA). Lag time (min) was determined from the intercept of lines drawn through the linear portions of the lag phase and propagation phase. The rate of oxidation (nmol/min) was determined from the slope of the propagation phase. The maximum concentration of dienes formed was calculated from the difference in absorbance at zero time (at diene peak) and expressed as nmol/mg protein. The concentrations of conjugated dienes in the samples were calculated using the molar extinction coefficient of $2{\cdot}95 \times 10^4$/M per cm[27]. Two sets of 100 μl sample were obtained from the above reaction mixture after 3 h and added to 10 μl of EDTA (10 mM) to stop further oxidation and subsequently used for measurement of TBARS and lipid hydroperoxide.

Copper-induced serum oxidation ex vivo. Cu-induced serum oxidation was estimated using diluted serum (0·67 % in 100 mM-PBS; pH 7·4)[28]. For the oxidation study, a pooled serum sample of each group was used and $CuSO_4$-initiated oxidation was performed in a manner similar to that of LDL oxidation. The maximum conjugated dienes, lipid hydroperoxide and TBARS were estimated.

Copper-induced serum and low-density lipoprotein oxidation in vitro. LDL and serum of the normal and diabetic control groups were oxidised separately using $CuSO_4$ in the presence of C-PE (0·5 and 1 mg/ml). The parameters studied were conjugated dienes, lipid hydroperoxide and TBARS. Serum and LDL samples of the normal and diabetic controls served as the common control model for *ex vivo* and *in vitro* studies. The estimation of pooled samples from each group was performed in triplicate.

Statistical analysis

Results are given as mean values and standard deviations. Significant differences among the groups were determined by one-way ANOVA using SPSS (version 10; SPSS Inc., Chicago, IL, USA) with Duncan's test as *post hoc* analysis. Differences were considered significant if $P<0{\cdot}05$.

Results

Body-weight gain, organ weight and food intake

Induction of diabetes with streptozotocin–nicotinamide showed a slower rate of BW gain with increased food intake. The administration of C-PE normalised both parameters in a dose-dependent manner. There was no significant effect of 25 mg C-PE/kg BW (diabetic +25 mg C-PE group) on the liver and kidney weights. However, 50 mg C-PE/kg BW (diabetic +50 mg C-PE group) showed significantly lowered ($P<0{\cdot}05$) liver and kidney weights (data not presented).

Clinical chemistry

A 28 d dose-dependent treatment with C-PE significantly decreased ($P<0{\cdot}05$) serum glucose, total lipid, total cholesterol, TAG, VLDL-cholesterol, creatinine, blood urea N,

uric acid and TBARS concentrations in both of the diabetic + C-PE treatment groups as compared with the diabetic control group. The C-PE treatment significantly elevated ($P<0·05$) serum Hb, total protein, total bilirubin and ferric-reducing ability of plasma values when compared with the diabetic control group. Serum concentrations of HDL-cholesterol and ceruloplasmin were significantly decreased while LDL-cholesterol and the atherogenic index were significantly increased ($P<0·05$) in the diabetic control group as compared with the normal control group. The treated groups showed no significant change in these parameters (Table 1).

Oxidative stress and antioxidant biomarkers in tissues

Tables 2 and 3 show oxidative stress and antioxidant biomarkers of liver and kidney tissues respectively. The diabetic control group showed significant increases ($P<0·05$) in TBARS, lipid hydroperoxide and conjugated diene contents of liver and kidney tissues as compared with the normal control group. However, both of the diabetic + C-PE treatment groups showed dose-dependent decreases ($P<0·05$) in these parameters as compared with the diabetic control group. Further, antioxidant biomarkers revealed remarkable declines ($P<0·05$) in superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, vitamin E and vitamin C of liver and kidney tissues in the diabetic control group compared with the normal control group, while these parameters were significantly ($P<0·05$) raised in both of the diabetic + C-PE treatment groups in comparison with the diabetic control group.

Copper-induced low-density lipoprotein and serum oxidation ex vivo

A dose-dependent administration of C-PE showed significant effects ($P<0·05$) on the *ex vivo* Cu-induced serum and LDL oxidation. The kinetic study of *ex vivo* LDL oxidation (Fig. 1 and Table 4) showed a significant ($P<0·05$) decrease in lag time along with increases in oxidation rate, and maximum conjugated diene, TBARS and lipid hydroperoxide concentrations in the diabetic control group as compared with the normal control group. However, these parameters were significantly ($P<0·05$) normalised after 28 d dose-dependent C-PE treatments in both of the diabetic + C-PE treatment groups. An insignificant difference was observed in the rate of LDL oxidation. Similar results were observed in *ex vivo* Cu-induced serum oxidation parameters. Decreases in maximum conjugated diene, TBARS and lipid hydroperoxide levels were observed in both treatment groups as compared with the diabetic control group (Table 4).

Copper-induced low-density lipoprotein and serum oxidation in vitro

Table 5 shows that treatment with C-PE (0·5 and 1·0 mg/ml) on LDL, isolated from normal or diabetic serum, followed by Cu-mediated oxidation, resulted in a significantly ($P<0·05$) prolonged lag time along with decreased oxidation rate, conjugated diene, TBARS and lipid hydroperoxide concentrations as compared with respective normal or diabetic control LDL. C-PE also showed antioxidant effects on *in vitro* Cu-induced serum oxidation by decreased ($P<0·05$) conjugated diene, TBARS and lipid hydroperoxide concentrations in C-PE-treated normal or diabetic serum as compared with the respective controls (Table 5).

Discussion

To the best of our knowledge, the present investigation is the first report to demonstrate the protective role of purified C-PE against streptozotocin–nicotinamide-induced diabetic

Table 1. Effect of C-phycoerythrin (C-PE) on the clinical chemistry parameters of rats (n 6)
(Mean values and standard deviations)

Group...	Normal control		Diabetic control		Diabetic + C-PE (25 mg/kg BW per d)		Diabetic + C-PE (50 mg/kg BW per d)	
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hb (g/l)	143[a]	9	94[c]	7	101[b,c]	8	108[b]	7
Glucose (mg/l)	910[d]	47	2109[a]	54	1859[b]	53	1397[c]	62
Total lipids (mg/l)	2853[d]	122	5517[a]	126	4535[b]	96	3507[c]	92
TAG (mg/l)	700[d]	48	1728[a]	89	1376[b]	64	1056[c]	77
Cholesterol (mg/l)	625[d]	43	1378[a]	57	1275[b]	63	1198[c]	65
HDL-cholesterol (mg/l)	250[a]	21	142[b]	23	142[b]	26	143[b]	27
LDL-cholesterol (mg/l)	243[b]	31	895[a]	55	858[a]	69	844[a]	60
VLDL-cholesterol (mg/l)	140[d]	9	346[a]	18	275[b]	13	211[c]	15
Atherogenic index	2·5[b]	0·2	10·0[a]	1·8	9·2[a]	2·1	8·7[a]	1·9
Total protein (g/l)	57[a]	3	35[d]	2	39[c]	2	42[b]	2
Total bilirubin (mg/l)	2[c]	0·2	2[c]	0·2	3[b]	0·3	3[a]	0·3
Creatinine (mg/l)	3[d]	0·3	7[a]	0·4	5[b]	0·3	4[c]	0·3
Blood urea N (mg/l)	155[c]	20	230[a]	19	203[b]	22	180[b]	21
Uric acid (mg/l)	15[d]	0·4	30[a]	1	25[b]	1	19[c]	0·4
TBARS (nmol/ml)	2·3[c]	0·1	4·3[a]	0·1	3·4[b]	0·1	2·4[c]	0·1
FRAP (μmol/l)	291·7[a]	5·9	148·2[d]	5·0	200·7[c]	5·8	267·2[b]	5·9
Ceruloplasmin (mg/l)	183[a]	16	98[b]	13	07[b]	9	110[b]	15

BW, body weight; TBARS, thiobarbituric acid-reactive substances; FRAP, ferric-reducing ability of plasma.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

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Table 2. Effect of C-phycoerythrin (C-PE) on liver oxidative stress and antioxidant parameters of rats (*n* 6)
(Mean values and standard deviations)

Group...	Normal control		Diabetic control		Diabetic + C-PE (25 mg/kg BW per d)		Diabetic + C-PE (50 mg/kg BW per d)	
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TBARS (nmol/g)	27·1^{d}	3·8	62·0^{a}	5·3	51·7^{b}	4·3	44·8^{c}	4·6
Lipid hydroperoxide (μmol/g)	258·7^{d}	6·7	502·2^{a}	11·1	418·3^{b}	17·9	311·3^{c}	47·8
Conjugated dienes (μmol/g)	224·0^{d}	7·9	422·8^{a}	9·2	342·2^{b}	7·1	241·0^{c}	5·2
Superoxide dismutase (U/mg protein)	4·1^{a}	0·2	1·1^{c}	0·2	2·5^{b}	0·1	4·0^{a}	0·1
Catalase (nmol H_2O_2 decomposed/s per g)	19·8^{a}	1·7	8·6^{c}	0·9	9·6^{b,c}	0·7	10·7^{b}	0·7
Glutathione peroxidase (U/mg protein)	3·6^{a}	0·2	1·5^{d}	0·1	1·8^{c}	0·1	2·1^{b}	0·1
Reduced glutathione (μg/g)	406·2^{a}	11·9	153·0^{d}	6·8	182·8^{c}	6·8	208·7^{b}	6·2
Vitamin E (μg/g)	45·8^{a}	3·3	31·0^{c}	2·5	33·7^{b,c}	2·5	35·7^{b}	2·2
Vitamin C (μg/g)	150·8^{a}	7·4	86·2^{c}	5·9	92·0^{b,c}	6·8	98·8^{b}	7·5

BW, body weight; TBARS, thiobarbituric acid-reactive substances.
^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

Table 3. Effect of C-phycoerythrin (C-PE) on kidney oxidative stress and antioxidant parameters of rats (*n* 6)
(Mean values and standard deviations)

Group...	Normal control		Diabetic control		Diabetic + C-PE (25 mg/kg BW per d)		Diabetic + C-PE (50 mg/kg BW per d)	
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TBARS (nmol/g)	39·7^{d}	3·3	81·2^{a}	5·6	62·8^{b}	6·5	50·5^{c}	5·1
Lipid hydroperoxide (μmol/g)	286·8^{d}	7·6	527·2^{a}	12·4	428·0^{b}	10·8	329·0^{c}	9·3
Conjugated dienes (μmol/g)	273·2^{c}	6·7	493·5^{a}	11·1	387·7^{b}	9·8	283·3^{c}	6·6
Superoxide dismutase (U/mg protein)	6·2^{a}	0·2	2·8^{c}	0·1	4·4^{b}	0·2	6·1^{a}	0·2
Catalase (nmol H_2O_2 decomposed/s per g)	12·1^{a}	1·0	4·6^{d}	0·9	5·8^{c}	0·6	6·9^{b}	0·7
Glutathione peroxidase (U/mg protein)	3·1^{a}	0·2	1·1^{d}	0·1	1·5^{c}	0·1	1·6^{b}	0·1
Reduced glutathione (μg/g)	360·8^{a}	11·6	133·7^{d}	8·5	165·2^{c}	7·1	189·7^{b}	9·1
Vitamin E (μg/g)	34·3^{a}	2·7	19·7^{c}	2·7	22·8^{b,c}	3·1	26·0^{b}	3·6
Vitamin C (μg/g)	114·0^{a}	7·5	54·2^{c}	3·3	57·3^{b,c}	3·7	60·5^{b}	3·9

BW, body weight; TBARS, thiobarbituric acid-reactive substances.
^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

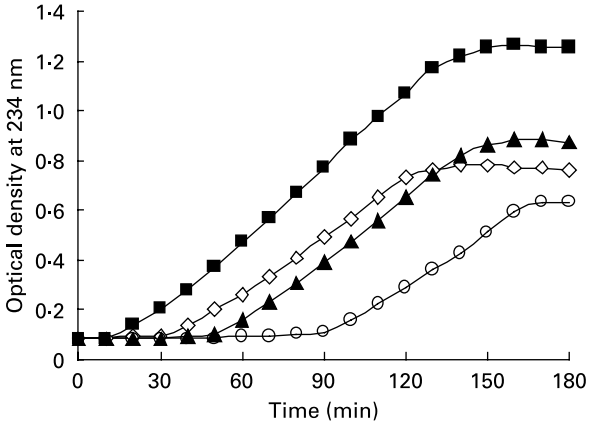

Fig. 1. Effect of C-phycoerythrin (C-PE) on Cu-induced LDL oxidation kinetics *ex vivo*. LDL was subjected to 0·167 μM-$CuSO_4$-mediated oxidation at 37°C in PBS (pH 7·4). Production of conjugated dienes was determined by monitoring the change in absorbance at 234 nm every 10 min on a diode array UV-visible spectrophotometer. Values are means of three independent sets of experiments. (–◇–), Group I (normal controls); (–■–), group II (diabetic controls); (–▲–), group III (diabetic control rats treated with C-PE at the dose level of 25 mg/kg body weight per d for 28 d); (–○–), group IV (diabetic control rats treated with C-PE at the dose level of 50 mg/kg body weight per d for 28 d).

complications. By streptozotocin–nicotinamide administration to adult rats, Masiello *et al.* induced a diabetic syndrome with stable metabolic alterations and reduced pancreatic insulin storage which mimicked some features of type 2 diabetes mellitus[29]. Streptozotocin acts as a diabetogenic agent owing to its ability to destroy pancreatic β-cells, possibly by a free radical mechanism[30]. In the present study, we used a similar streptozotocin–nicotinamide-induced diabetic rat model, which showed abnormal glucose, lipids and renal function tests as well as oxidative and antioxidant parameters with significant BW loss.

We observed increases in food consumption, organ weights and serum blood urea N with decreased BW and serum protein in streptozotocin-induced diabetic rats. This indicated dehydration and catabolism of fats and proteins. However, these parameters were significantly restored upon treatment with C-PE for 28 d, demonstrating ameliorative actions of C-PE against the streptozotocin-induced diabetic complications. As C-PE is composed of a variety of amino acids, it may have became a direct source of protein for the rats and produced various metabolic effects. Severe dyslipidaemia in the streptozotocin-induced diabetic rats was observed in the present study. The present study demonstrated a significant decline

Table 4. Effect of C-phycoerythrin (C-PE) on copper-induced LDL and serum oxidation *ex vivo*
(Mean values and standard deviations)

Group...	Normal control		Diabetic control		Diabetic + C-PE (25 mg/kg BW per d)		Diabetic + C-PE (50 mg/kg BW per d)	
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lag phase time (min)	37·0^{c}	4·0	17·0^{d}	4·0	53·3^{b}	6·5	91·3^{a}	7·5
Oxidation rate (nmol conjugated dienes/min per mg LDL protein)	2·2^{b}	0·2	3·0^{a}	0·5	2·4^{a,b}	0·3	2·2^{b}	0·2
Maximum conjugated dienes (nmol/mg LDL protein)	264·7^{c}	9·5	427·3^{a}	16·6	298·0^{b}	10·5	213·7^{d}	6·0
LDL lipid hydroperoxide (nmol/mg protein)	627·0^{d}	24·6	971·9^{a}	30·2	891·0^{b}	24·6	791·5^{c}	20·5
LDL TBARS (nmol/mg LDL protein)	55·5^{c}	5·8	83·9^{a}	7·2	70·3^{b}	8·5	56·3^{c}	6·1
Serum maximum conjugated dienes (nmol/ml)	100·3^{c}	8·0	149·4^{a}	11·2	120·3^{b}	8·5	88·0^{c}	8·0
Serum lipid hydroperoxide (nmol/ml)	18·2^{c}	3·3	38·0^{a}	3·0	33·2^{a,b}	2·4	30·8^{b}	3·0
Serum TBARS (nmol/ml)	12·8^{c}	2·4	30·4^{a}	2·5	26·0^{a,b}	2·6	21·5^{b}	2·5

BW, body weight; TBARS, thiobarbituric acid-reactive substances.
^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

in serum total cholesterol levels by C-PE treatment, suggesting inhibition of cholesterol absorption from the intestinal wall. Nagaoka *et al.* revealed a significant decrease in cholesterol levels by *Spirulina platensis* concentrate-derived phycocyanin (C-PE analogue)-treated rats where phycocyanin showed a greater bile acid-binding capacity *in vitro* as well as inhibitory effects on micellar cholesterol solubility and cholesterol uptake by Caco-2 cells[31]. This effect could be due to the structural similarities among C-PE, Hb and bilirubin. Here, the tetrapyrrole molecule of C-PE may have served as an analogous structure for the pyrrole ring present in Hb and bilirubin. Increased Hb concentration opens a possibility that the tetrapyrrole molecule of C-PE participated in Hb formation by donating the preferable substrate for Hb synthesis. Recently, a similar hypothesis has been raised by Dammeyer & Frankenberg-Dinkel[1].

In the present study, diabetic rats also show renal dysfunction, i.e. increased serum creatinine and urea, reflecting a decline in the glomerular filtration rate. A high uric acid concentration is also associated with gout and CVD[32]. Rats treated with C-PE showed significant decreases in serum creatinine and uric acid concentrations. Jenkins *et al.* [33] revealed that a protein-rich diet in hyperlipidaemic subjects significantly decreased serum creatinine and uric acid concentrations. Khan *et al.* [34] reported that crude phycocyanin from *Spirulina* could reduce plasma creatinine and urea concentrations and protect rats from cyclosporine-induced nephrotoxicity. Our previous work on C-PE showed kidney-protective action against carbon tetrachloride-induced nephrotoxicity, by decreasing serum creatinine levels[5].

Diabetes mellitus is associated with the generation of reactive oxygen species leading to oxidative damage particularly in the liver and kidney, and coexists with a decrease in the antioxidant status[6]. In the present study, C-PE showed a potent ability to work as an antioxidant agent against streptozotocin-induced oxidative stress in rats. C-PE ameliorated lipid peroxidation and improved the antioxidant enzymes as well as non-enzymic markers in serum, liver and kidney.

Table 5. Effect of C-phycoerythrin (C-PE) on copper-induced LDL and serum oxidation in vitro
(Mean values and standard deviations)

Group...	Normal control		Normal control + C-PE (0·5 mg/ml)		Normal control + C-PE (1·0 mg/ml)		Diabetic control		Diabetic control + C-PE (0·5 mg/ml)		Diabetic control + C-PE (1·0 mg/ml)	
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lag phase time (min)	37·0^{c}	4·0	76·0^{b}	6·0	125·7^{a}	9·5	17·0^{d}	4·0	47·0^{c}	5·0	85·3^{b}	6·5
Oxidation rate (nmol conjugated dienes/min per mg LDL protein)	2·2^{b,c}	0·2	2·0^{b,c}	0·3	1·7^{c}	0·3	3·0^{a}	0·5	2·9^{a}	0·4	2·5^{a,b}	0·2
Maximum conjugated dienes (nmol/mg LDL protein)	264·7^{c}	9·5	210·3^{d}	6·8	132·0^{c}	4·4	427·3^{a}	16·6	346·7^{b}	11·9	264·0^{c}	9·0
LDL lipid hydroperoxide (nmol/mg LDL protein)	627·0^{d}	24·6	499·3^{e}	24·0	374·8^{f}	24·8	971·9^{a}	30·2	900·1^{b}	29·8	797·3^{c}	26·1
LDL TBARS (nmol/mg LDL protein)	55·5^{c}	5·8	42·3^{d}	5·5	30·0^{e}	5·0	83·9^{a}	7·2	72·6^{a,b}	8·4	62·7^{b,c}	6·8
Serum maximum conjugated dienes (nmol/ml)	100·3^{c}	8·0	72·4^{d}	7·5	53·2^{e}	6·8	149·4^{a}	11·2	129·5^{b}	9·3	94·1^{c}	8·6
Serum lipid hydroperoxide (nmol/ml)	18·2^{c}	3·3	14·7^{c,d}	3·2	10·7^{d}	3·2	38·0^{a}	3·0	35·3^{a,b}	2·5	32·0^{b}	3·0
Serum TBARS (nmol/ml)	12·8^{c}	2·4	8·1^{d}	2·1	5·0^{d}	1·8	30·4^{a}	2·5	28·3^{a,b}	2·5	24·5	2·5

TBARS, thiobarbituric acid-reactive substances.
$^{a-f}$ Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).

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C-PE was found to be a more potent antioxidant in renal tissue. Our recent study indicated restoration of the redox imbalance in liver and kidney tissues by C-PE against carbon tetrachloride-mediated oxidative damage in rats[5]. A number of studies indicated cyanobacterial proteins as the molecules of high potency to work as antioxidants by scavenging peroxyl, hydroxyl, peroxynitrite, superoxide radicals, and as inhibitors of lipid peroxidation[2,35]. In the present study, the administration of C-PE in diabetic rats increased the overall antioxidant status by increasing bilirubin, superoxide dismutase, catalase, glutathione peroxidase concentrations and the non-enzymic antioxidant system (Tables 2 and 3). Stocker *et al.* suggested bilirubin as a powerful biological chain-breaking antioxidant that scavenges peroxy radicals[36]. Correlating with earlier reports, increased bilirubin levels after C-PE treatment could be involved in scavenging α-tocopheroxyl radicals and increasing reduced glutathione concentration[37,38]. While an increase in tissue ascorbic acid (vitamin C) level indicates an added advantage against free radical generation, the actual mechanism of C-PE on ascorbic acid remains unidentified. For a toxicity point of view, an increased level of serum bilirubin reflects the depth of jaundice as well as kernicterus[39]. However, the combined evidence from animal and human studies indicates bilirubin as a major physiological cytoprotectant[37,40]. We found (B Soni, N Dalwadi, N Visavadiya and D Madamwar, unpublished results) that C-PE was non-toxic during 14 d acute and 90 d subchronic oral safety studies in rats.

In the present study, a dose-dependent administration of C-PE showed the ability to protect *ex vivo* and *in vitro* Cu-mediated serum and LDL oxidation in normal and diabetic rats (Tables 4 and 5). This was confirmed by a significant prolongation of lag phase time along with declines in oxidation rate, conjugated diene, TBARS and lipid hydroperoxide contents. Cu-mediated LDL oxidation begins first with a lag phase, during which protective endogenous antioxidants are consumed and produce free radical species. After the consumption of all endogenous antioxidants, a lipid radical-propagated peroxidation chain reaction is initiated in which the PUFA content of the LDL is rapidly oxidised to lipid hydroperoxide[41]. In the present study, the serum oxidation and kinetics of LDL oxidation from diabetic rats differed significantly from non-diabetic rats. In 28 d of administration, C-PE may become biotransformed into bilirubin and, thereby, significantly decrease serum and LDL oxidation in diabetic rats. This observation is consistent with the protective role of bilirubin in *in vitro* models of liposomal lipid peroxidation through a synergistic effect in the presence of α-tocopherol[37,42]. Schuster *et al.* demonstrated that in order to display resistance to oxidative stress *in vivo*, LDL should exist in the core lipid structure, i.e. cholesteryl ester and TAG molecules organised in two concentric layers[43]. Since bilirubin is amphiphilic, it may dissolve in the lipid components of LDL and assist apolipoprotein to rearrange the disrupted isotropic state of LDL (at 37°C) into the core lipid structure, protecting LDL oxidation. A similar observation has also been noted by Wu *et al.* [44]. Thus, the antioxidant defence provided by bilirubin for serum and LDL is physiologically significant. A recent report also suggested an inverse correlation between serum bilirubin levels and the risk of atherosclerosis[45]. On the other hand, our *in vitro* study showed that in a dose-dependent manner C-PE could decrease the susceptibility of serum and LDL towards oxidation.

We have found (B Soni, N Dalwadi, N Visavadiya and D Madamwar, unpublished results) that C-PE has the ability to scavenge superoxide, NO and hydroxyl radicals as well as possessing an anti-lipid peroxidative activity *in vitro*. The resonance energy transfer system of C-PE could provide electrons if suitable electron acceptors or donors are available and in this manner could scavenge free radicals during Cu-induced *in vitro* LDL oxidation and thereby could improve endogenous antioxidant status as well as decline the propagation of radical chain reactions in lipid peroxidation. This observation is consistent with that of Zhou *et al.* [46], that the tetrapyrrole molecule is the main compound involved in scavenging free radicals and contributes significantly to the antioxidant activity.

In conclusion, this is the first report on the protective effects of C-PE against streptozotocin-induced diabetes in rats. Streptozotocin–nicotinamide treatment induces type 2 diabetic complications in male albino rats, which are associated with major alterations in the values of clinical chemistry, oxidative stress, antioxidant biomarkers and lipid peroxidation profiles. The C-PE dose-dependent treatment ameliorated most of these parameters. C-PE also exhibited an important ability to protect LDL (*ex vivo* and *in vitro*) against Cu-mediated oxidation in normal and diabetic rats.

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