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Oxalate mediated nephronal impairment and its inhibition by *c*-phycocyanin: A study on urolithic rats

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Abstract

The assumption of oxidative stress as a mechanism in oxalate induced renal damage suggests that antioxidants might play a beneficial role against oxalate toxicity. An *in vivo* model was used to investigate the effect of C-phycocyanin (from aquatic micro algae; *Spirulina spp.*), a known antioxidant, against calcium oxalate urolithiasis. Hyperoxaluria was induced in two of the 4 groups of Wistar albino rats ($n = 6$ in each) by intraperitoneally injecting sodium oxalate (70 mg/kg body weight). A pretreatment of phycocyanin (100 mg/kg body weight) as a single oral dosage was given, one hour prior to oxalate challenge. An untreated control and drug control (phycocyanin alone) were employed. Phycocyanin administration resulted in a significant improvement ($p < 0.001$) in the thiol content of renal tissue and RBC lysate via increasing glutathione and reducing malondialdehyde levels in the plasma of oxalate induced rats ($p < 0.001$), indicating phycocyanin's antioxidant effect on oxalate mediated oxidative stress. Administering phycocyanin after oxalate treatment significantly increased catalase and glucose-6-phosphate dehydrogenase activity ($p < 0.001$) in RBC lysate suggesting phycocyanin as a free radical quencher. Assessing calcium oxalate crystal retention in renal tissue using polarization microscopy and renal ultrastructure by electron microscopy reveals normal features in phycocyanin – pretreated groups. Thus the study presents positive pharmacological implications of phycocyanin against oxalate mediated nephronal impairment and warrants further work to tap this potential aquatic resource for its medicinal application. (Mol Cell Biochem **284:** 95–101, 2006)

Key words: antioxidant, hyperoxaluria, oxidative stress, phycocyanin

Introduction

Phycocyanin, an accessory photosynthetic pigment present in blue green and red algae, is attached to the chromophore namely phycocyanobilin (PCB) [1]. Phycocyanobilin is a blue tetrapyrrole in which the pyrrole rings are linked by three carbon bridges conferring an "open-chain" configuration resembling mammalian bile pigment [2]. PCB is known to be an important scavenger of reactive oxygen species produced by oxidative stress *in vivo* [3]. Recent reports suggest that

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phycocyanin has anti-inflammatory, hepatoprotective, neuroprotective effects and its therapeutic efficacy has been proved against various disorders [4]. Oxidative stress is associated with various degenerative diseases, including urolithic disease [5], cancer [6], and arteriosclerosis [7]. Renal membrane injury is a prime spot for the deposition of calcium oxalate crystals and subsequent progression to kidney stone, wherein oxalate induces membrane injury through lipid peroxidation (LPO) by generating oxygen free radicals. In recent years, several antioxidant drug therapies have been examined for their ability to prevent calcium oxalate retention by protecting against membrane injury, supplementation of antioxidants either-SH generating amino acid methionine or -SH reagents such as GSH monoester and α-lipoic acid and cysteine, hydroxyl radical scavenger such as mannitol, vitamin E and triterpenes abolished the accumulation of LPO products in plasma and tissue under urolithic conditions [5].

The main goal of the present study was to investigate the efficacy of phycocyanin on oxidative stress after sodium oxalate induction in albino rats, wherein the levels of plasma malondialdehyde (MDA) and renal glutathione were considered as prime factors in determining the lipid peroxidation and cellular thiol status respectively. Erythrocyte antioxidant status and polarization microscopic studies have also been performed to assess the urolithic injury upon pre-treatment with phycocyanin especially under oxalate-induced stress. Also the ultrastructures morphology of renal cell was assessed for urolithic injury, revealed normal cellular features in phycocyanin-pretreated animals, henceforth phycocyanin is presumed to inhibit the oxalate mediated nephronal impairment.

Materials and methods

Male rats (Wistar albino strain) were used in this study conforming to the ethical guidelines by the Institutional Animals Ethics Committee (IAEC). All the reagents not specifically mentioned were purchased from Sigma Chemicals (St. Louis, MO, USA). Phycocyanin was isolated from the microalgae *Spirulina spp* according to the method of Yi-Ming Zhang and Feng Chen [8] and showed a λ_{max} around 620 nm in accordance with an authentic sample.

Induction and treatment

The experimental animals were divided into four groups, having six animals in each group. Group I-control rats were given 0.5 ml of saline. Group II-rats were administered with single dosage of sodium oxalate (70 mg/kg dissolved in 0.5 ml of saline) as intraperitoneal injection (i.p). Group III-rats were administered phycocyanin orally (100 mg/kg dissolved in 0.5 ml of saline) 1 h prior to sodium oxalate challenge. Group IV-rats were administered with phycocyanin alone (100 mg/kg dissolved in 0.5 ml of saline). Twenty-four hours later, urine specimens were collected from the rats in a plastic container with sodium azide as preservative for the quantification of urinary marker protein. At the end of the experimental period, the animals were anaesthetized with pentothal sodium (40 mg/kg; i.p) and blood samples were collected via intracardiac puncture using heparin anticoagulant. Plasma and buffy coat were removed by centrifugation at 3000 rpm for 10 min. The RBCs were washed three times with an equal volume of cold saline. The kidney was rapidly removed and frozen in liquid nitrogen for further analysis.

Determination of lipid peroxidation and glutathione

The levels of plasma LPO [9], erythrocyte & kidney glutathione [10] were estimated using high performance liquid chromatography (HPLC). Redox index, calculated as ([GSH] +2[GSSG/2GSSG*100]) provides an index for the redox state of the cells [11].

Antioxidant enzymes

The RBC catalase activity was determined spectrophotometrically [12] by using hydrogen peroxide as substrate. G6PD activity was determined using glucose-6-phosphate as substrate and $NADP^+$ [13].

Visualization of crystal deposition

Renal tissue slices were stained using Haematoxylin & Eosin to evaluate pathological changes and examined by a Carl Zeiss Jena polarizing microscopy (Jenaval, Germany) to detect calcium oxalate crystals in the kidneys.

Electron microscopic analysis of kidney sections

Rat kidneys were immersed in 4% paraformaldehyde/2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 h at 4 °C. The kidneys were cut to 300 μm slices and post-fixed with 1% osmium tetroxide for 2 h. Subsequently it was dehydrated with ethanol, and embedded in Araldite resin. Also, 80–100 nm sections cut from the same resin embedded block was stained with uranyl acetate and lead citrate for electron microscopic (LEO 912AB, LEO, Germany) analysis.

Statistical analysis

Data are expressed as mean ± S.D for six animals in each group. Statistical analysis of variance (ANOVA) and Student-Newman-Keul multiple comparison test were applied to determine the significant differences among the groups. *P* values less than 0.05 were considered significant.

Results

Effect of phycocyanin on LPO

The level of LPO was ascertained using HPLC by monitoring the changes in MDA absorbance at 267 nm and values recorded as nmoles of MDA/ml. Only the numeric data has been presented in this article, for facilitating accurate interpretation and statistical comparison (Fig. 1). The plasma MDA content was increased 5-fold in the urolithic rats ($p < 0.001$). The MDA levels were significantly controlled in phycocyanin-pretreated group (1.2 nmole ml^{-1}), where it was found closer to the control rats (1.0 nmole ml^{-1}) and only a negligible difference was seen between control and phycocyanin alone (drug control).

Effect of phycocyanin on GSH

Oxalate induction significantly depleted the levels of GSH in RBCs lysates and renal tissues ($P < 0.001$). Nevertheless this has been stabilized in phycocyanin-pretreated groups (Table 1) in contrast to the low reduced glutathione (GSH) levels as seen in untreated oxalate-induced rats.

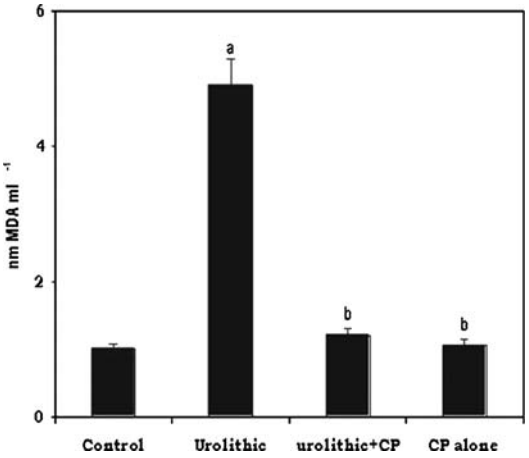

Fig. 1. Effect of phycocyanin on plasma lipid peroxidation in the experimental animals. Values are mean ± S.D for six animals. Group I-control; Group II-sodium oxalate (Hyperoxaluria); Group III-phycocyanin + sodium oxalate; Group IV-phycocyanin alone (Drug control). 'a' compared with Group I; 'b' compared with Group II. Different superscripts within a bar show significant variation between groups, $p < 0.05$. *Values are the average of triplicates, determined by HPLC.

Antioxidant enzymes

Oxalate induction causes significantly diminished catalase activity in RBCs lysates (Fig. 2, $P < 0.001$). This enzyme activity reverted to near control level in the membranes of phycocyanin-pretreated animals. The G6PD activity measured in the RBCs lysates of control, sodium oxalate-induced and phycocyanin-pretreated rats was observed to be decreased with oxalate administration ($P < 0.001$). But, phycocyanin-pretreated rats showed increased G6PD activity ($P < 0.001$) as shown in Fig. 2.

Table 1. Effect of phycocyanin on the level of glutathione in sodium oxalate induced rat erythrocyte lysate and kidney tissues determined by HPLC

	RBC			Kidney		
	Redox index	GSH	GSSG	Redox index	GSH	GSSG
Control	0.071 ± 0.006	18.3 ± 1.40	1.54 ± 0.126	0.076 ± 0.007	0.25 ± 0.020	0.020 ± 0.0017
Hyperoxaluria	0.038 ± 0.004[a]	10.2 ± 0.80[a]	1.95 ± 0.154[a]	0.033 ± 0.003[a]	0.110 ± 0.009[a]	0.026 ± 0.0021[a]
Hyper + CP	0.062 ± 0.006[a,b]	16.4 ± 1.30[b]	1.67 ± 0.132[b]	0.063 ± 0.006[a,b]	0.23 ± 0.0183[b]	0.021 ± 0.0017[b]
CP alone	0.074 ± 0.007[b,c]	18.7 ± 1.48[b,c]	1.48 ± 0.14[b,c]	0.078 ±0.008[b,c]	0.26 ± 0.022[b,c]	0.018 ± 0.0016[b,c]

Values are mean ± S.D for six animals.
[a]compared with Group I.
[b]compared with Group II.
[c]compared with Group III.
Different superscripts within a row show significant variation between groups, $p < 0.05$.
Glutathione values are expressed in nmoles/ mg protein.

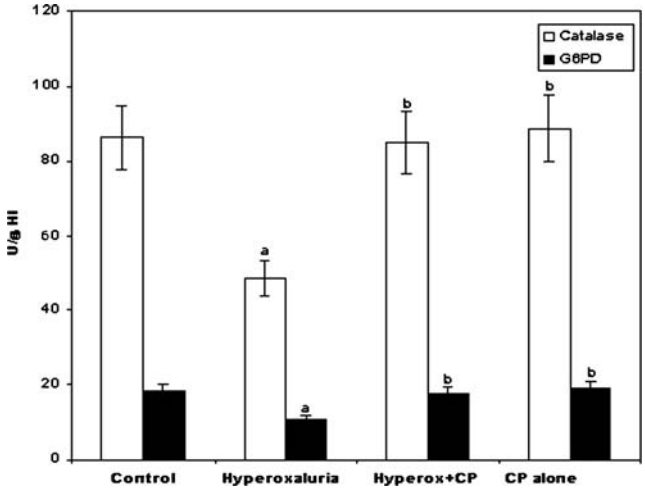

Fig. 2. Effect of phycocyanin on the levels of catalase and G6PD activities in sodium oxalate induced rat erythrocytes. Values are mean ± S.D for six animals. 'a' compared with Group I; 'b' compared with Group II, Different superscripts within a bar show significant variation between groups, $p < 0.05$.

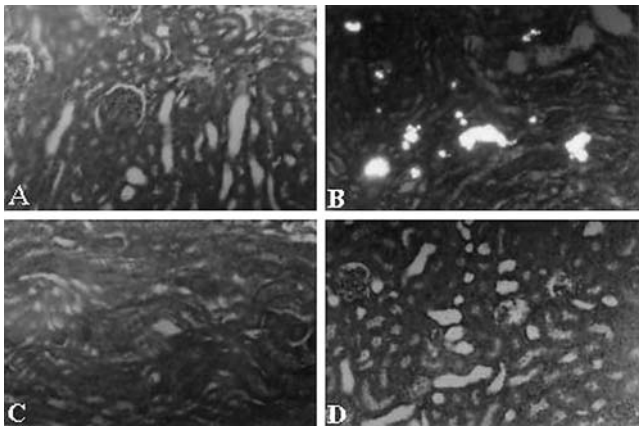

Fig. 3. Crystal studies under Polaroid microscopy. Crystal studies on hyperoxaluric and phycocyanin-pretreated rat kidneys photographed under partially polarizing microscopy. A: Sections from control group showing normal glomeruli and tubules without crystal deposition. B: Sections from hyperoxaluric groups, showing calcium crystals oxalate crystals occupying almost the whole of tubular lumina. C: Phycocyanin-pretreated group-No calcium oxalate crystals in lumen of renal tubules were visualized by polarized light. D: Phycocyanin alone-There was no calcium oxalate crystal deposition, which was similar to that of control (H&E, X 200).

Crystal analysis

Histological section of control group shows normal glomeruli and tubules without crystal deposition (Fig. 3A). Hyperoxaluric rat kidney, when viewed under polarized light microscope (Fig. 3B) showed the presence of calcium oxalate crystals adhering to the tubular lumen, whereas, in rats with phycocyanin-pretreatment, no crystal deposition was noticed (Fig. 3C).

Ultrastructural study to reveal the nephropathology of oxalate induction and the counteraction by phycocyanin

Sub-cellular morphology to determine any degenerative changes in the oxalate affected cells with respect to the controls showed that the degeneration observed in oxalate treated samples was apoptotic in character.

Electron microscopic pictures of cells not treated with oxalate were observed for nucleo-cytoplasmic morphology. The nucleus appears round with a regular membrane envelope (Fig. 4A).

Figure 4B section clearly demonstrates degenerative changes in the nucleus such as the nuclear membrane hyperchromasia seen initially followed by a clear gap between the nuclear membrane and the nuclear contents. The cells were observed for the same parameters as applied to the oxalate-induced cells. Interestingly, phycocyanin-pretreated kidneys nuclear membrane appeared unruffled and the nucleus was round and regular (Fig. 4C). There was no nuclear hyperchromasia, the membrane and the nuclear contents appeared to be in-flush without any gap (Fig. 4C). The nuclear pyknosis can be appreciated and the membrane shows blebbing (Figs. 4D and 4E). The membrane blebbing and ruffling indicate apoptosis. The chromatin material seems to be marginated and condensed (Fig. 4F). The hyperchromasia of the cytoplasm could be seen at a later stage and the nucleo-cytoplasmic demarcation becomes inconspicuous (Figs. 4D and 4F). No evidence of inflammation in the regions showing degenerative changes was found.

Figure 4G no treated with oxalate kidney section shows clear cytoplasm, and the sub-cellular organelles are well appreciated.

The changes in the cytoplasm (Fig. 4E) show that initially, the cytoplasm, as such develops dense hyperchromasia and the nucleo-cytoplasmic boundary disappears. The endoplasmic reticula appear hazy and are not discernible (Fig. 4B). The mitochondria in the oxalate-affected cells appear swollen and the cristae are fractured (Fig. 4H). Several electron-dense bodies could be appreciated in the cytoplasm (Figs. 4D and 4H) representing the characteristic apoptotic bodies. The cytoplasmic degenerative changes were absent and all organelle can be well appreciated in the phycocyanin-pretreated groups. The mitochondria demonstrate a rounded structure and very few broken cristae (Fig. 4I).

Discussion

The mechanisms by which oxalate causes its deleterious effects to kidneys, liver and the hematological system have yet to be determined. In recent studies, however, some of toxic effects of oxalate have been attributed to oxalate induced oxidative stress [14]. The oxalate induced-membrane peroxidation

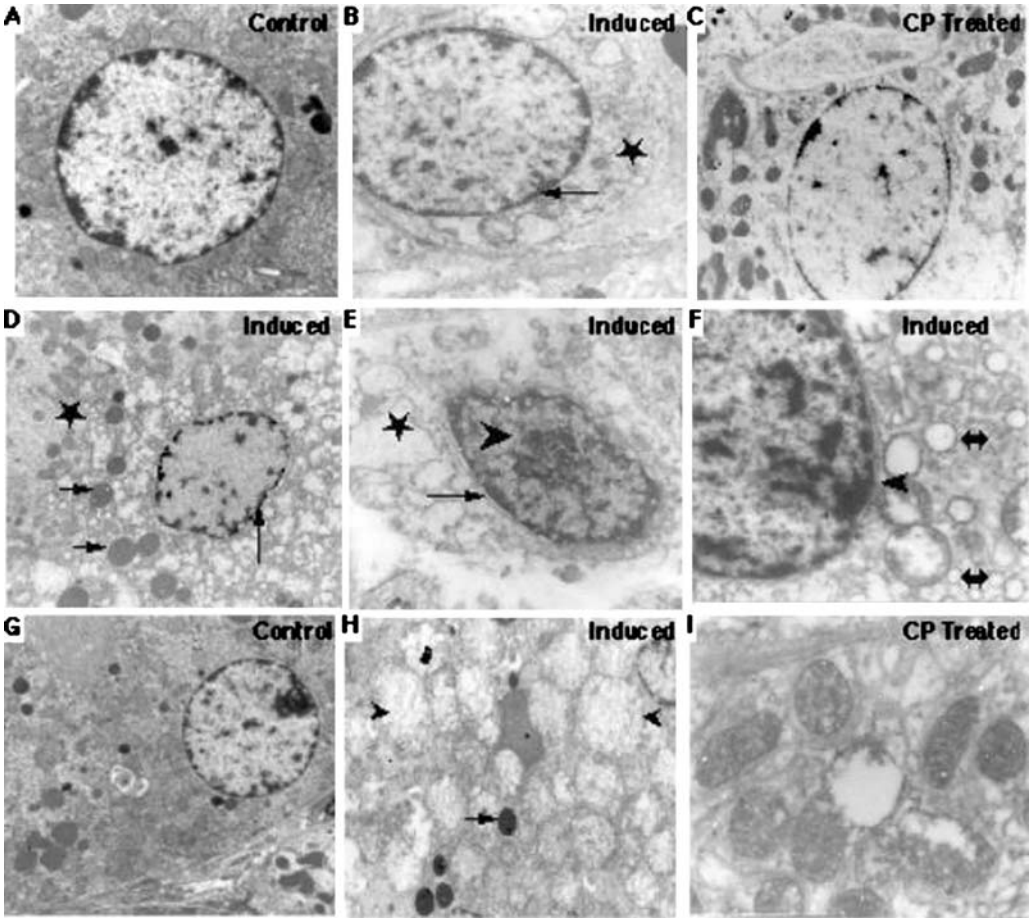

Fig. 4. Ultrastructural studies. Electron-micrographs of control (Figs. A, G), oxalate induced (Figs. B, D, E, F and H) and C-Phycocyanin-pretreated (Figs. C and I) renal sections. (B, D, E) A gap separating the nuclear membrane and the interior of the nucleus is seen (long arrow). Cytoplasmic organelles are also displaced (star). (D, E, F) Abnormal nuclear structure (arrow head), a number of apoptotic bodies (small arrow), condensation of cytoplasm (star), membrane blebs and hyperchromatic nuclear membrane are also apparent with pyknotic nuclei (arrow head), number of vacuoles are present (double arrow). (G) Mitochondria in controls show normal morphology. (H) Mitochondria in oxalate-induced cell sections show abnormal structure, broken cristae and swollen appearance (small arrowhead). (C, I) Nucleo-cytoplasmic features on CP-pretreatment showing stabilization of normal nuclear structure and mitochondria with very few features of altered structure.

leads to membrane integrity loss renal cell damage and, finally, calcium oxalate crystal deposition. In this study, the phycocyanin pretreatment decreased the LPO and reversed the effects of oxalate on oxidative stress parameters.

Free radicals are generally short lived and thus inflict damage only in the local environment where they are produced. Recent findings indicate that lipid peroxidation also results in the production of a great variety of stable, diffusible saturated and unsaturated aldehydes like malondialdehyde. The cytotoxic aldehydes are extremely active, they can diffuse within or even escape from the cell and attack targets far from the site of the original free radical initiated event, resulting in cell damage and therefore act as 'cytotoxic second messengers' [15]. Malondialdehyde is a by-product of lipid peroxidation and its measurement serves as an indicator of free radical damage. Recently, HPLC techniques have been developed for the determination of free MDA in biological samples. The MDA standard added to normal and other experimental groups shows sensitive results and offers a feasible way of its detection and determination in animal plasma. It is suggested from the analysis that high level of MDA is seen only in urolithic rats and low or negligible level of its kind is observed in control and drug control plasma, while a controlled level of MDA is noted as an interesting observation only in drug pretreated groups, suggesting the potential ability of phycocyanin to reduce the greater impact of free radical damage by interacting with hydroxyl radical and hence the suppression of free radical mediated lipid peroxidation.

Further evidence of the efficacy of phycocyanin in relieving oxalate induced oxidative stress includes rebalancing the GSH content, catalase and G6PD activity in oxalate treated animals. Glutathione is the most prevalent low molecular

weight antioxidant within cells. Reduced glutathione protects cellular constituents from oxidative damage by reacting directly with oxidants or as the substrate for glutathione peroxidase to scavenge peroxides [16]. GSH also promotes the antioxidant properties of vitamin C and vitamin E by maintaining these nutrients in a reduced state [17], notably, decreased tissue GSH concentration have been associated with cell damage [18], and depressed immunity [19]. Moreover, GSH depletion increases the susceptibility of cells to stress induced cell death [20]. We have shown that nutritional antioxidant provides potential protection on oxalate induced tissue damage in which GSH levels are compromised [21]. In the present study, we observed that, in phycocyanin pretreated rats, the stable level of cellular GSH was maintained suggesting that the antioxidant ability of phycocyanin is potent enough to quench the oxalate induced free radical reaction in the cell.

G6PD is an enzyme in the pentose phosphate pathway that provides most of the extramitochondrial NADPH to cells. The pathway is more important for RBCs because they lack mitochondria. The turnover of the pathway is shown to decrease under oxidative stress conditions where demand for NADPH increases [22]. Under oxidative stress conditions, formation of GSSG would be expected to increase consumption of hydrogen peroxide via glutathione peroxidase. Glutathione disulfide will then be reduced to GSH by glutathione reductase using NADPH as a substrate. In the present study the decrease in catalase activity in oxalate induced animals may indicate further depletion of NADPH. Therefore, inhibition of G6PD activity in oxalate toxicity thereby prevents NADPH production through G6PD. Phycocyanin pretreatment hyperoxaluric rats restored to control levels of G6PD activity that can be explained by the increased need for NADPH.

The EM observation and ultrastructural characterization of degenerating nephrons in the renal tissue of the rat model induced with the oxalate and subsequent visualization of the effect of phycocyanin on the normalization of cell morphology vindicates the beneficial effects of phycocyanin in countering the negative effects of oxalate. The hallmark of our EM study is the unequivocal demonstration of a degenerative cytopathology in all the oxalate-induced cells. This when considered in the background of a normalization of the morphology of cells pretreated with phycocyanin lends ample support to our additional findings of the presence of excess oxidative stress in the oxalate-induced cells which is reversed by the antioxidant effects of phycocyanin.

Conclusions

The present study confirms the involvement of free radicals in inflicting cell death mechanisms by a programmed fashion. Cytotoxic aldehydes play a key role in initiating the steps that lead to functional impairment of the urothelium following sodium oxalate administration. Phycocyanin was found to have marked ameliorative effects corroborated by ultrastructural evidence as well as biochemical determination of an activated antioxidant system.

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