Protective Effects of Phycocyanin on Galactosamine-induced Hepatitis in Rats

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ABSTRACT

This study was performed to determine the potential hepatoprotective and anti-inflammatory effects of the microalgae pigment phycocyanin in galactosamine-induced acute liver damage in rats. Phycocyanin (50-200 mg kg^{-1} i.p) was administered one hour before the galactosamine (600 mg kg^{-1} i.p) and the blood was extracted 24 hours later to determine alanine amino transferase (ALT), aspartate amino transferase (AST) and malondialdehyde (MDA) in the serum. The livers were removed to evaluate damage using optic and electron microscopy. Phycocyanin reduced ALT and AST activities as well as MDA concentrations in the serum. It also decreased necrosis and inflammation in the liver compared to the controls treated only with galactosamine. These results indicate that phycocyanin exerts hepatoprotective and antiinflammatory effects in this human hepatitis animal model.

Key words: Phycocyanin, Hepatoprotective, Galactosamine, Hepatitis, Anti-inflammatory

Biotecnología Aplicada 2003;20:107-110

RESUMEN

Efecto hepatoprotector de ficocianina en hepatitis inducida por galactosamina en ratas. Este estudio fue realizado para determinar el posible efecto anti-inflamatorio y hepatoprotector del pigmento de microalga ficocianina en el daño hepático agudo inducido por la galactosamina en la rata. La ficocianina (50–200 mg Kg^{-1} i.p) fue administrada una hora antes que la galactosamina (600 mg Kg^{-1} i.p) y 24 horas más tarde la sangre de las ratas fue obtenida para las determinaciones de la alanina aminotransferasa (ALT), la aspartato aminotransferasa (AST) y el malondialdehído (MDA) en el suero. Los hígados fueron extirpados para la evaluación del daño utilizando la microscopía óptica y electrónica. La ficocianina redujo las actividades de la ALT y AST así como las concentraciones de MDA en el suero. La ficocianina también disminuyó la necrosis y la inflamación en los hígados con respecto a los controles solamente tratados con la galactosamina. Estos resultados indican que la ficocinina ejerce efecto anti-inflamatorio y hepatoprotector en este modelo animal de la hepatitis humana.

Palabras Claves: ficocianina, hepatoprotector, galactosamina, hepatitis, antinflamatorio

*I*ntroduction

Phycocyanin is a biliprotein found in blue green algae such as Spirulina and it constitutes more than 20% of its dry weight. Recently we demonstrated that phycocyanin exerts scavenging actions against reactive oxygen species (ROS) as well as anti-inflammatory activity in various in vitro and in vivo experimental models such as zymosan activated human polymorphonuclear leukocytes, arachidonic acid-induced mouse ear oedema, acetic acid-induced colitis and zymosan-induced arthritis among others. These phycocyanin effects are due to its scavenging action against oxygen radicals and its inhibitory effects on arachidonic acid metabolism [1-4]. Thus, taking into account the former findings we decided to determine if the biliprotein exerts a hepatoprotective activity on galactosamine-induced liver damage in rats, an animal model of hepatitis that histologically resembles human viral hepatitis [5] and in which the roles in its pathogenesis of ROS and of the metabolites of arachidonic acid have been well documented.

*M*aterials and Methods

Animals

Female Wistar rats (180-200 g) were used in these experiments. The animals were purchased from the National Center for Laboratory Animal Production (CENPALAB), Havana, Cuba. The animals were housed in a room with temperature (t=25 ºC) and air humidity (60%) control with a 12h light-dark cycle. They were reared on a standard laboratory ration and drinking water was offered *ad libitum*. The experiments were conducted in accordance with the ethical guidelines for research in laboratory animals and were approved by the Ethics Committee for Animal Experimentation of the National Center for Scientific Research (CNIC).

Chemicals.

D-Galactosamine hydrochloride was obtained from Fluka (Germany). Other chemicals of analytical grade were obtained from normal commercial sources.

Preparation of phycocyanin extract.

Phycocyanin was extracted from the microalgae *Spirulina* as described in a Cuban patent [6]. The blue powder thus obtained showed a single peak at 620 nm in the visible absorption spectrum, which is very close to the one reported for c-phycocyanin [7].

Galactosamine (Gal N)-induced acute liver damage in rats

The animals were randomly distributed in groups of six. Food was withdrawn 12 h before the administra-

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tion of Gal N. Phycocyanin (50, 100 and 200 mg kg^{-1}) was dissolved in a physiological saline solution that was administered intraperitoneally (i.p.) in a single injection with a volume of 1 mL/kg, 1 h before administering Gal N. This was also dissolved in saline, adjusted to pH 7.0 with 1N Na0H and given at doses of 600 mg/kg of body weight i.p. in a single injection with a volume of 1 mL/kg. Rats treated with the vehicle and a group of rats that were not treated were also included in these experiments. Twenty-four hours later, blood was withdrawn and serum was obtained for determinations of ALT, AST and malondialdehyde (MDA). Shortly afterwards, the animals were killed by decapitation and small pieces of the left lobule of the liver were taken for histological and ultrastructural studies.

Enzyme assays in rat serum.

Kits of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) from Boehringer-Mannheim (Germany) were used according to the methods described by Bergmeyer [8].

Determination of lipid peroxides in rat serum

Lipid peroxidation was estimated by measuring MDA using the thiobarbituric acid method [9], as follows: to 0.5 mL of the serum, 1 mL of 20% TCA was added and the test tube was left to stand for 10 min at room temperature. After centrifuging at 4000 g for 10 min, the supernatant was decanted and the precipitate was washed twice with 1.25 mL of 5 mM sulphuric acid. Then 1.5 mL of 0.2% TBA in 2 M sodium sulphate pH 3.5 were added to this precipitate and the color was developed by heating in a boiling water bath for 2 h. After cooling, the resulting chromogen was extracted with 1.75 mL of N-butyl alcohol by vigorous shaking. The separation of the organic phase was made through centrifugation at 4000 g for 10 min and its absorbance was determined at the wavelength of 530 nm. The results are expressed in mmol/L of serum using a calibration curve made with 1.1.3.3 tetraetoxypropane.

Histological techniques

Liver specimens were taken and fixed in 10% neutral buffered formalin (pH 7.4), processed and embedded in paraffin. The histological sections were stained with haematoxylin and eosin. A histological grading scale was used to determine liver damage: slight (I), moderate (II) and severe (III). The parameters evaluated were focal cell necrosis and infiltration of inflammatory cells. An expert pathologist scored the results in a blinded manner.

Transmission electron microscopy

Small liver pieces were quickly washed in sodium cacodylate buffer 0.1 M, pH 7.4. They were first fixed in 3.2% glutaraldehyde and then fixed in 2% osmium tetroxide buffered in sodium cacodylate, for 1 h each. Samples were dehydrated in ethanol and embedded in Spurr resin. Ultrathin sections were stained with saturated uranyl acetate and lead citrate [10] and observed in a JEOL 100 S Transmission Electron Microscope.

Statistical Analysis

Groups were compared using a one-way analysis of variance (ANOVA) with a completely randomized design and DUNCAN'S multiple range test. Values of p below 0.05 were considered significant.

Results

Effects of phycocyanin on ALT and AST activities as well as MDA content in rat serum

Phycocyanin at doses of 50, 100 and 200 mg kg^{-1}, i.p. administered 1 h before Gal N significantly reduced ($p<0.05$) the activities of ALT and AST, which were substantially increased by Gal N. Also, MDA concentrations in rat serum were lower than those of animals treated only with Gal N (Table 1).

Histopathology.

No macroscopic findings of peritonitis and liver damage were observed in rats pre treated only with phycocyanin 200 mg kg^{-1} i.p (data not shown). However, twenty four hours after the i.p. administration of Gal N (600 mg kg^{-1}) the liver showed various areas of focal cell necrosis accompanied by an inflammatory cell infiltration (Table 2).

When the animals were treated with Gal N plus phycocyanin (50 mg kg^{-1}), the inflammatory reaction was slightly reduced compared to the Gal N group. However, the treatment with doses of 100 and 200 mg kg^{-1} of phycocyanin induced a remarkable decrease in necrosis and leukocyte infiltration (Table 2).

Ultrastructural pathology.

The normal structure of hepatocytes was observed in non-treated control rats (Fig. 1), as well as in phycocyanin and vehicle-treated rats.

In the hepatocytes of rats treated with GalN, a proliferation of smooth endoplasmic reticulum vacuoles was found in the cytoplasm, as well as the dilation of the rough endoplasmic reticulum, nuclear envelope and

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Table 1: Effects of phycocyanin on ALT and AST activities and MDA content in rat serum.

Type of treatment	ALT activity (U/L)	AST activity (U/L)	MDA (μmol/l)
Non treated controls	18,3 ± 6,1	70,9 ± 13,4	3,0 ± 0,3
Phycocyanin 200 mg kg^{-1}	20.3 ± 6.2	67.2 ± 10.6	2.2 ± 0.4
Gal N 600mg kg^{-1}	267.0 ± 59.8	357.8 ± 64.9	15.1 ± 1.7
Phycocyanin 50 mg kg^{-1} plus GalN	169.0 ± 28.7*	251.8 ± 12.1*	12.2 ± 1.1*
Phycocyanin 100 mg kg^{-1} plus GalN	59.3 ± 37.7**	111.5 ± 19.2**	10.5 ± 0.5*
Phycocyanin 200 mg kg^{-1} plus GalN	41.5 ± 19.2**	108.9 ± 18.9**	10.5 ± 1.6*

Phycocyanin was administered i.p., one hour before GalN, Values are the means from groups of six rats ± SD. Significantly different from Gal N group *p<0.05, **p<0.01

Table 2: Histological results

Type of treatment	# of animals	Degree of damage			Normal
		I	II	III	
Non treated controls	6	0	0	0	6
Phycocyanin 200 mg/kg	6	0	0	0	6
GalN 600 mg/kg	6	0	2	4	0
Phycocyanin 50 mg/kg plus GalN	6	2	2	0	2
Phycocyanin 100 mg/kg plus GalN	6	2	0	0	4
Phycocyanin 200 mg/kg plus GalN	6	2	0	0	4

A histological score was used to determine liver damage: slight (I), moderate (II) and severe (III). The parameters evaluated were focal cell necrosis and infiltration of inflammatory cells. The results were evaluated in a blinded manner.

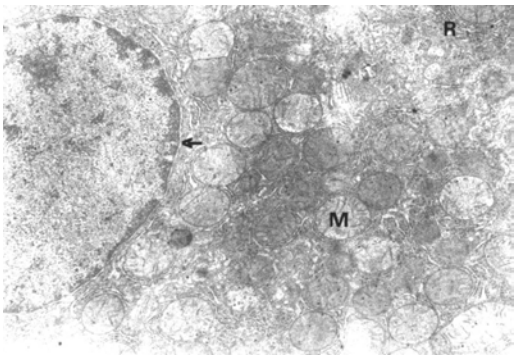

Fig. 1. Control rat hepatocyte. Observe the normal aspect of cell structures, such as rough endoplasmic reticulum (R), mitochondria (M) and nuclear envelope (arrow). X 11200.

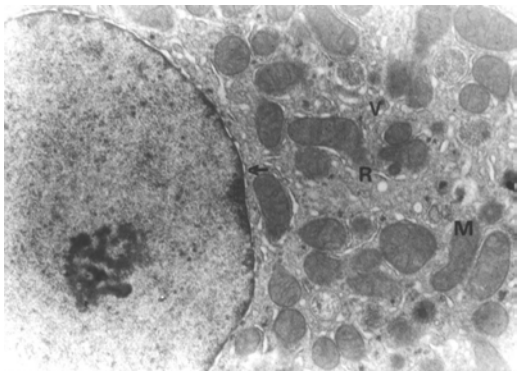

Fig. 3. Hepatocyte of a rat treated with GalN (600 mg/kg; i.p.) plus phycocyanin (50 mg/kg, i.p.). Note the slight dilation of the nuclear envelope (arrow) and the rough endoplasmic reticulum (R). A great deal of smooth endoplasmic reticulum vacuoles (V) is also found in the cytoplasm. Mitochondria (M) have a normal aspect. X 10400.

Golgi complex, while the mitochondria structure was normal. On the other hand, an extensive inflammatory infiltrate with the presence of neutrophils in the tissue was observed in these samples (Fig. 2).

In rats treated with GalN plus phycocyanin (50 mg/kg, i.p.), a moderate reduction of vacuoles of the smooth endoplasmic reticulum was noted in the cytoplasm of hepatocytes and there was less dilation of the nuclear envelope and the rough endoplasmic reticulum than in the hepatocytes of rats treated only with GalN (Fig. 3).

When the animals were treated with GalN plus phycocyanin (100 and 200 mg/kg, i.p.), hepatocyte morphology was normal. Thus, hepatocytes had a normal nuclear envelope, endoplasmic reticulum and Golgi complex, well preserved mitochondria and there were no signs of inflammation (Fig. 4).

Discussion

The present data show clearly that phycocyanin protects rats from Gal N-induced hepatitis, which was demonstrated by the reduction of ALT and AST activities and MDA content in rat serum (Table 1), as well as in histopathological and ultrastructural studies (Table 2, Figs. 1-4).

The mechanisms underlying the GalN-induced hepatitis in rats have been extensively investigated. One of them is the inhibition of protein synthesis by Gal N itself in the hepatocytes. Gal N induces biochemical alterations in the liver by trapping and depleting cellular uracyl nucleotides, followed by the inhibition of RNA and protein synthesis [11]. These events induce cellular damage in the hepatocyte and the subsequent development of acute hepatitis with disseminated hepatocellular necrosis and the infiltration of polymorphonuclear leucocytes (PMNL). However, other important mechanisms to explain the induction and development of Gal N hepatitis have also been well documented.

Thus, a growing body of evidence supports that the release of ROS and cytokines such as tumor necrosis factor (TNF a) and interleukin 1 (IL 1) by Kupffer cells (KC), in the liver contribute to hepatocyte damage in GalN hepatitis [12, 13].

It has also been demonstrated that selective depletion of KC by gadolinium chloride, substantially attenuates GalN hepatitis in rats [14]. In addition, it is well known that GalN-induced hepatitis is reduced if the animals are treated with ROS inhibitors or scavengers such as N-acetylcysteine, cystiamine, silymarin and others [15-18].

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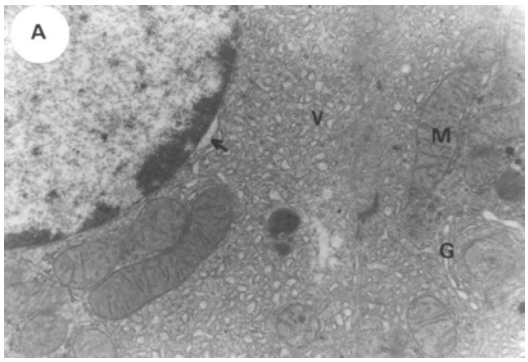

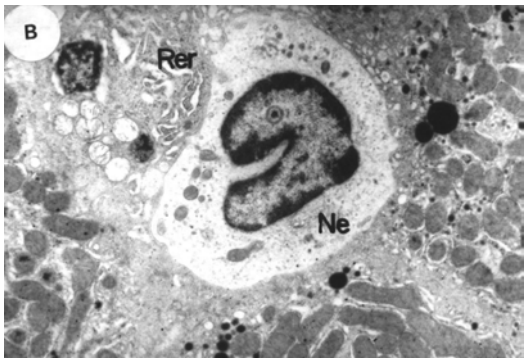

Fig. 2. Hepatic parenchyma of rats treated with GalN (600 mg/kg, i.p.). A) Hepatocyte. A great amount of smooth endoplasmic reticulum vacuoles (V) is observed in the cytoplasm, as well as the dilation of the nuclear envelope (arrow) and the Golgi complex (G). Mitochondria (M) show a normal structure. X 13000. B) Neutrophil (Ne) infiltrated in the tissue. In a neighboring hepatocyte, observe the dilation of the rough endoplasmic reticulum (Rer). X 6500.

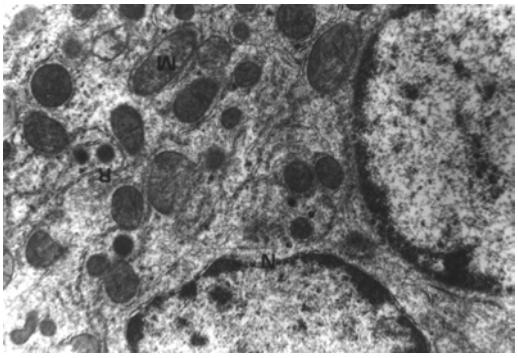

Fig. 4. A portion is shown of the rat hepatocyte after a treatment with GalN (600 mg/kg; i.p.) plus phycocyanin (200 mg/kg, i.p.). Normal nuclear envelope (N), mitochondria (M) and channels of rough endoplasmic reticulum (R) can be observed. X 10400.

In line with these results we have observed in isolated mouse liver perfusion studies, that KC functioning e.g. carbon phagocytosis and carbon-induced O_2 uptake as well as the sinusoidal release of LDH in the intact liver, are significantly reduced by low concentrations of phycocyanin (0.2 mg/mL). This hepatoprotective effect is consistent with the antioxidant behavior of the biliprotein [19].

Thus, it was demonstrated that phycocyanin is able to scavenge hydroxyl ($OH^{\bullet}$) and alkoxyl ($RO^{\bullet}$). It also inhibited *in vitro* the microsomal liver peroxidation and the chemiluminescent response of PMNL activated by zymosan, as well as the edema index in glucose oxidase-induced inflammation in the paw of mice, which is mediated by H_2O_2 and $OH^{\bullet}$ [1]. Furthermore, the biliprotein also reduced the levels of leucotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2) in arachidonic acid-induced mouse ear inflammation test [4, 20].

More recently, in our laboratory it has been found that phycocyanin inhibits in a dose-dependent way, the release of TNF-a in a murine model of endotoxic shock induced by LPS [21], which resembles GalN effects on the liver [13].

As a whole, our findings support the view that the hepatoprotective effects of phycocyanin in GalN-induced hepatitis in rats might be ascribed to its ROS scavenging properties and antilipoperoxidative effects as well as its inhibitory effects on cytokines (e.g. TNF-a) and on the metabolites of arachidonic acid. KC seems to be strongly involved in these protective effects of phycocyanin.

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Received in March, 2002. Accepted for publication in February, 2003.