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Protective effects of phycocyanin on ischemia/reperfusion liver injuries

Neyla Ben Gdara*,[1], Amel Belgacem[1], Ikram Khemiri, Safa Mannai, Lotfi Bitri

Department of Biology, University of Tunis El Manar, Faculty of Sciences of Tunis, University Campus 2092, El Manar, Tunis, Tunisia

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A B S T R A C T

In this study, phycocyanin (Pc) extracted from *Spirulina platensis* was used to evaluate its antioxidants effects after ischemia/reperfusion injury (IRI) using the ex-vivo model of isolated perfused rat liver. The rats were divided into eight groups : Control group, where livers were directly perfused after their removal; Cold Ischemia group (CI), livers were treated in the same way as the control group, except that after their collection, they were stored for 12 h and 24 h in the Krebs Henseleit (KH) preservation solution at 4 °C and Treated group (PHY), livers were preserved in the same way as the preceding group except that the KH solution was enriched with phycocyanin at two different concentrations. Pc, a powerful antioxidant, significantly reduced ischemia/reperfusion injury in the liver. In fact, the addition of phycocyanin to the preservation solution significantly decreased the activity of liver transaminases (AST) and (ALT), alkaline phosphatase (ALP), the rate of lipid peroxidation (MDA) and the activity of certain antioxidant enzymes, essentially glutathione-S-transferase (GST) and glutathione peroxidase (GPx). On the other hand, Pc increases the level of thiol groups in hepatic tissues. In conclusion, the results show the Pc-enriched KH conservation solution is effective in preserving the hepatic graft and protecting it against IRI by acting as a potent antioxidant against the products of oxidative stress.

1. Introduction

Organ transplantation is an effective therapy for most pathologies leading to irreversible loss of vital organs. Different metabolic changes take place during different stage of the liver transplantation procedure. After liver preservation and before transplantation, the hepatic graft undergoes diverse and complex molecular, cellular and biochemical alterations known as ischemia/reperfusion syndrome (IR) [1,2]. During transplantation, there are two types of ischemia, cold ischemia and warm ischemia followed by reperfusion [3]. Cold ischemia is associated with a reduction of cellular ATP levels, and the increased cytosolic calcium levels [4], while warm ischemia and reperfusion is characterized by production and release of Reactive Oxygen Species (ROS) which are the main mediators of hepatic ischemia reperfusion injury (IRI) [5,6], and later by an inflammatory disorder mediated by activation of Kupffer cells, endothelial and parenchymatous cells damage [7]. *Spirulina platensis*, a blue green microalgae belonging to Cyanobacteria family, is used in many countries as dietary supplement and known to exhibit broad spectrum biological, pharmacological and therapeutic activities [8]. The species known as "Spirulina of Gatrana" is widely distributed in the center (Sidi Bouzid) and in the north east of Tunisia (Hammamet). Phycocyanin (Pc), is one of the major blue pigment constituent of Spirulina. It was reported that Pc had an anti-inflammatory [9], antioxidant [10] and hepatoprotective [9] proprieties. In spite of the experimental advances supporting the therapeutic potential of Pc, little research has been done to evaluate the effect of Pc on IRI. Therefore, the present study aimed to investigate and evaluate the potential effect of Pc against IRI by using an ex-vivo model of isolated perfused rat liver.

2. Materials and methods

2.1. Culture conditions

Spirulina platensis was obtained from Medina Bio Spa Naturel in the north east of Tunisia (Hammamet region). This microalga is cultivated in special basins and in culture medium based on Zarrouk synthetic medium [11]. Then it was harvested and dried in solar gas or electric

Abbreviations: Pc, Phycocyanin; IRI, Ischemia/Reperfusion Injury; CI, Cold Ischemia; KH, Krebs Henseleit; AST, Aspartate Aminotransferase; ALT, Alanine Aminotransferase; ALP, Alkaline Phosphatase; MDA, Malondialdehyde; GST, Glutathione-S-Transferase; GPx, Glutathione peroxidase; IR, Ischemia/Reperfusion; ROS, Reactive Oxygen Species; TBARS, Thiobarbituric Acid Reaction; TBS, Tris-buffered Saline; GSH, Glutathion Solution; DTNB, 5,5 dithio-bis-2-nitrobenzoique; CDNB, 1-chloro-2,4-dinitrobenzene; TNB, 5-thiobis (2-nitrobenzoic acid)

* Corresponding author.
E-mail addresses: neyla.bengdara@fst.utm.tn (N.B. Gdara), amel.belgacem2@fst.utm.tn (A. Belgacem), ikram.khemiri@fst.utm.tn (I. Khemiri), safa.mannai@fst.utm.tn (S. Mannai), L.Bitri@fst.rnu.tn (L. Bitri).

[1] These authors contributed equally to this work.

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driers. The dried Spirulina was then grounded in the form of powder or in the form of flakes and stored in sachets protected from moisture and light.

2.2. Phycocyanin extraction

Spirulina platensis powder (4 g) was dissolved in distilled water (500 ml). Calcium chloride (0.5 g) was dissolved in distilled water (500 ml). The two solutions were mixed under magnetic stirring at room temperature for 8 h. The mixture was firstly filtered and then centrifugated at 10,000 rpm for 20 min at 4 °C. The supernatant was recovered and used to calculate the phycocyanin concentration (0.0953 mg ml^{-1}) [12], to verify the extraction yield (23.825 mg g^{-1}) [13] and then stored in the dark at 4 °C until further use.

2.3. Animals

Wistar male rats weighing 200g were used for experiments. Animals were provided by Pasteur Institute of Tunis and then stored in cages lined with wood chips in the animal house of the Faculty of Science of Tunis, under standardized environmental conditions. Rats had free access to water and rodent pellet diet provided by the company BADR (Utica, Tunisia). Animals were treated in accordance with its guidelines and according to Medical Ethics Committee for the Care and Use of Laboratory Animals of the Pasteur Institute of Tunis, Tunisia (approval number: FST/LNFP/Pro 152012). We have done our best to minimize the number of animals used and reduce animal's suffering.

2.4. Hypothermic conservation

Rats were anesthetized with 20% urethane solution (5 ml kg^{-1}). After midline laparotomy, and injection of 200 IU of heparin diluted in physiological liquid, the portal vein and the vena cava inferior were cannulated for the influent and effluent perfusate. After hepatectomy, livers were immersed in a cylindrical plastic vial containing 40 ml Krebs Henseleit bicarbonate buffer supplemented with mannitol and glutathione (reduced form), pH = 7.30 at 4 °C. Then the plastic vial is placed in the fridge for 24h. This step concerns all treatment groups except for control group.

2.5. Liver perfusion experiments

Livers were perfused according to the technique described previously [14,15]. Perfusions were performed in a closed circuit, in a humid thermoregulated chamber (37 °C) during 120 min. The perfusion medium, an isotonic Krebs Henseleit (KH) solution (120 mM NaCl, 4.7 mM Kcl, 1.2 mM KH_2PO_4, 1.2 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, pH = 7.30, 37 °C) was saturated with 95% O_2 / 5% CO_2 [16]. The perfusion fluid circulation was provided by a peristaltic pump at constant pressure and calibrated for a basal hepatic flow rate about 3 ml/min/g liver [17,18]. Before starting the experimental protocol, livers were perfused in single-pass mode for 15 min to ensure the rinsing of liver and their stabilization. Once the peristaltic pump is running, the perfusion fluid returns to the livers through the portal vein and exits through the lower vena cava before returning to the pump. Livers have been perfused in a recirculation mode. Samples of liver effluents were collected every 10 min to determine liver toxicity parameters and tissue samples were taken from the left lateral hepatic lobe at the end of reperfusion and subsequently frozen at −20 °C until MDA and antioxidant enzymes assay.

2.6. Treatment groups

Livers were divided into eight groups (n = 4, for each one) as follows (the grouping is described in detail in scheme1). Control group where perfusions were directly performed after livers removal. Two Control groups were performed for each cold ischemia group. Cold Ischemia groups (CI 12 h; CI 24 h), livers were treated in the same way as the control group, except that after their collection, they were stored for 12 h and 24 h in the KH preservation solution at 4 °C and Groups treated with Pc, livers were preserved in the same way as the preceding group except that the KH solution was enriched with phycocyanin at two doses (0.1; 0.2 mg ml^{-1} $g^{-1}$of liver).

2.7. Liver toxicity assay

Aspartase aminotransferase (AST) and alanine aminotransferase (ALT) levels in the liver effluents were estimated using commercial kits provided by Biomaghreb (Ariana Tunis, Tunisia) and with JENWAY 6300 analyzer (JENWAY, Ukraine, UK).

2.8. Oxidative stress parameters

Oxidative stress was evaluated by measuring the concentration of Malondialdehyde (MDA) and the level of thiol groups in liver tissue, as well as the activity of antioxidant enzymes (GPx and GST). Sample tissues (1 g) were homogenized in 10 ml TBS (50mM Tris, 150 mM NaCl, pH = 7.4) in a Dounce homogenizer and centrifugated at 9000 rpm for 20 min at 4 °C. The supernatant was recovered and stored at −20 °C for subsequent analysis.

The concentration of (MDA) in the tissue allows estimating lipid peroxide quantity. The assay is performed by the TBARS (thiobarbituric acid reaction) method [19]. The absorbance was measured at 530 nm. In brief, 500 μl of the supernatant were mixed with 200 μl of TBS buffer and 500 μl of trichloroacetic acid-BHT (20% TCA-1% BHT) and then vortexed and centrifugated at 3000 rpm for 10 min. 800 μl of the supernatant was recovered and mixed with 160 μl HCL (0.6 M) and 640 μl of Tris-TBA (25 Mm–120 mM TBA) and then vortexed and incubated at 80 °C for 10 min. The MDA concentration was expressed as nmol/ mg protein.

Gpx activity was estimated according to the method developed by Flohe and Günzler [20], 200 μl of the supernatant were mixed with 200 μl of glutathion solution (GSH) (4 mM) and 100 μl of KH_2PO_4 buffer (0.1 M, pH 7.4). After 10 min of incubation at 37 °C, the reactions were started by the addition of 500μl of H_2O_2 solution (5 mM). At the end of the reaction, 500 μl of 5.5 dithio-bis-2-nitrobenzoïque (DTNB) were added and the absorbance was measured at 412nm. GPx activity was expressed as nmol of GSH oxidized/ min/ mg protein.

GST activity was determined in liver tissue according to the method of Habig et al. [21], 500 μl of the supernatant were mixed with 100 μl of glutathion solution (GSH) and 100 μl of PBS buffer (0.1 M, pH 7.8). The reactions were initiated by the addition of 100 μl of 1-chloro-2,4-dinitrobenzene (CDNB) (100 mM) and the absorbance was measured at 340nm. GST activity was expressed as nmol/min/mg protein.

Alkaline phosphatase (ALP) activity in liver homogenate was detected according to the method recommended by the German company for clinical chemistry DGKG using a commercial kits provided by Biomaghreb (Ariana Tunis, Tunisia).

The level of thiol groups was estimated in liver tissue using DTNB in an oxydo-reduction reaction. In fact, at basic pH, the free thiols of proteins reduce the disulfide DTNB to yellow TNB (5-thiobis (2-nitrobenzoic acid)) and measured at 412nm. Results were expressed as mM [22].

Total protein concentrations in liver homogenate samples were determined with Lowry method [23].

2.9. Statistical analysis

Data analysis was performed using Prism 6.0 program (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Results were expressed as mean ± standard error of the mean. Statistical comparisons with the control were made by the T tests-one per row applicable to

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