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Dual effect of silymarin on experimental non-alcoholic steatohepatitis induced by irinotecan

Eudmar Marcolino Assis-Júnior [a], Anielle Torres Melo [a], Venúcia Bruna Magalhães Pereira [a], Deysi Viviana Tenazoa Wong [b], Nathalia Ribeiro Pinho Sousa [a], Christiane Mendes Gonçalves Oliveira [a], Lara Raissa Cavalcante Malveira [a], Leonardo Silva Moreira [a], Marcellus Henrique Loiola Ponte Souza [c], Paulo Roberto Carvalho Almeida [b], Roberto César Pereira Lima-Júnior [a,*]

[a] Department of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, Brazil
[b] Department of Pathology and Forensic Medicine, Faculty of Medicine, Federal University of Ceará, Brazil
[c] Department of Clinical Medicine, Faculty of Medicine, Federal University of Ceará, Brazil

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ABSTRACT

Irinotecan-based regimens are commonly used for treatment of colorectal cancer, which is limited by mucositis and non-alcoholic steatohepatitis (NASH). Silymarin (SIL) prevents fatty liver disease in the clinical setting and in models of liver damage induced chemically. This study investigated the possible effect of SIL on irinotecan (IRI)-induced NASH. Swiss female mice were injected with saline (SAL 5 ml/kg i.p.), IRI (50 mg/kg i.p.), SIL (150 mg/kg p.o.) or IRI (50 mg/kg i.p.) + (SIL 1.5, 15 or 150 mg/kg p.o.) thrice/week/7 weeks. On the seventh week, blood samples were collected for transaminases assay and livers were collected for histopathology, measurement of the total lipids, malondyadehyde (MDA), non-protein sulfhydryl groups (NPSH), cytokines (IL-1β, IL 6 and IL-10), 3-nitrotyrosine (N-Tyr) and toll-like receptor 4 (TLR4) immunoexpression, quantification of NF-kB, α-smooth muscle actin (α-SMA), and *Escherichia coli* 16S rRNA gene (RRS) expression. IRI increased liver transaminases, neutrophil infiltration, lipid accumulation, MDA, IL-1β and IL-6 levels, N-Tyr and TLR4 immunostaining, NF-kB, α-SMA expression and RRS versus the SAL group ($p < 0.05$). Additionally, SIL (1.5 mg/kg) improved these parameters ($p < 0.05$), except neutrophil infiltration and RSS versus the IRI group. Furthermore, the SIL (15 mg/kg) only improved the inflammatory parameters, the expression of α-SMA and RRS versus the IRI group ($p < 0.05$). The higher dose of SIL (150 mg/kg) was even more deleterious than the intermediate dose. Therefore, silymarin showed a dual effect on liver damage induced by IRI. Hepatoprotection seems to involve the inhibition of oxidative stress and protein nitrosylation, preventing activation of hepatic fibrosis mechanisms.

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1. Introduction

Colorectal Cancer (CRC) is the third most common type of cancer among men (10% of total cases) and the second among women (9.2% of total cases). Although the majority of cases (55%) occur in developed regions, the highest mortality rates occur in less developed regions (52% of deaths) (WHO-World Health Organization, 2004).

The main site of CRC metastasis is the liver in about 30% of patients (Kuvshinov and Fong, 2007). Clinical management of metastatic CRC involves tumor resection (Costa et al., 2014). Non-resectable tumors can become resectable by the use of conversion therapy, which is mainly based on irinotecan or oxaliplatin combined with 5-Fluorouracil (Bismuth et al., 1996; Adam et al., 2004; Pozzo et al., 2004). However, irinotecan-based regimens are related to limiting side effects such as intestinal mucositis (IM) (Ikuno et al., 1995; Freitas, 2007). In addition, about 1 in 12 patients undergoing irinotecan treatment develop non-alcoholic steatohepatitis (NASH), representing a 3.45-fold increased risk for those under chemotherapy treatment (Robinson et al., 2012), which might limit metastasectomy. Histopathological damage criteria were established by Kleiner et al. (2005), who consider steatosis, lobular inflammation and vacuolization as important parameters for NASH diagnosis, while fibrosis and portal infiltration are considered indicators of severe disease (Kleiner et al., 2005). Costa and co-workers developed an experimental model of irinotecan-related NASH characterized by oxidative stress, inflammatory infiltrate, lipid accumulation, fibrosis and hepatic failure (Costa et al., 2014).

Silymarin is a naturally-occuring, polyphenol-rich antioxidant, which acts as a free radical scavenger (Fraschini et al., 2002). It is reported that silymarin reversibly inhibits P-450 cytochrome enzymes

* Corresponding author at: Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Rua Cel Nunes de Melo, 1127, Rodolfo Teófilo, 60430-270 Fortaleza, Ceará, Brazil.

E-mail address: robertocesar@ufc.br (R.C.P. Lima-Júnior).

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(Fraschini et al., 2002), stabilizes mitochondrial (Bindoli et al., 1997) and microsomal membranes (Trouillas et al., 2008). Polyak et al. (2007) and Kim et al. (2013) reported anti-inflammatory and immunomodulatory effects of silymarin with the involvement of NF-κB inhibition (Lee et al., 2013) and over-expression of immunomodulators such as IL-10 and IFN-γ (Wilasrusmee et al., 2002). In addition, Täger et al. (2001) reported that silymarin treatment over-expressed the endogenous IL-1β inhibitor (IL-1ra). However, the effects of silymarin vary according to the dose and route of administration.

Silymarin hepatic protection has been widely demonstrated in animal models and in the clinical setting (Cacciapuoti et al., 2013; Zhang et al., 2013; Trouillas et al., 2008; Chen et al., 2012). It is effective in the treatment of obesity-related NASH (Cacciapuoti et al., 2013), prevents the establishment of alcoholic steatohepatitis (Zhang et al., 2013), and protects against chemically-induced liver damage and fibrosis by carbon tetrachloride (Trouillas et al., 2008) and thioacetamide (Chen et al., 2012).

In the present study we aimed to evaluate the effects of silymarin on irinotecan-induced non-alcoholic steatohepatitis.

2. Materials and methods

2.1. Chemicals and antibodies

Irinotecan hydrochloride (Evoterin®, Evolabis, São Paulo, Brazil, vial of 100 mg); Silymarin S0292 (Sigma-Aldrich, MO, USA); Monoclonal primary antibody rabbit anti-nitrotyrosine (Life Technology, NY, USA); Monoclonal primary antibody rabbit anti TLR-4 (Life Technology, NY, USA); Polyclonal primary antibody anti-NF-kB NLS rabbit (Santa Cruz Biotechnology, CA, USA); Polyclonal primary antibody Rabbit Anti-Actin β (Santa Cruz Biotechnology, CA, USA); Primary Antibody Monoclonal Goat Anti-α-SMA (Sigma-Aldrich, MO, USA); Secondary antibody linked to alkaline phosphatase anti-rabbit IgG (Sigma Aldrich, MO, USA); Secondary antibody linked to alkaline phosphatase anti-goat IgG (Sigma-Aldrich, MO, USA); ImunoCruz - Anti-rabbit ABC kit (Santa Cruz Biotechnology, CA, USA).

2.2. Animals

Swiss mice, weighing between 20 and 25 g, and obtained from the animal facility at Federal University of Ceará were used. The animals had free access to drinking water and food and were kept in a controlled temperature (22 ± 2 °C) environment with a light-dark cycle (12 h/12 h). All animal care and experimental procedures complied with the laboratory animal care and use principles outlined by the National Institutes of Health (NIH publication no. 85–23, revised 1985) and were approved by the local Ethics in Animal Research Committee (CEPA) of the Federal University of Ceará (Protocol number 21/12).

2.3. Experimental design

The animals were divided into six experimental groups (n = 8–10): group 1 - SAL: Saline 5 ml/kg, p.o. 1 h before saline 5 ml/kg i.p; group 2 - SIL: Silymarin 150 mg/kg, p.o. 1 h before saline 5 ml/kg i.p.; group 3 - IRI: Saline 5 ml/kg, p.o. 1 h before irinotecan 50 mg/kg, i.p.; group 4 - S1.5: Silymarin 1.5 mg/kg p.o. 1 h before irinotecan 50 mg/kg i.p.; group 5 - S15: Silymarin 15 mg/kg p.o. 1 h before irinotecan 50 mg/kg i.p.; group 6 - S150: Silymarin 150 mg/kg p.o. 1 h before irinotecan 50 mg/kg i.p. The animals were injected three times a week on alternate days. On the sixth day of each week the animals received silymarin or saline reinforcement. This schedule of treatment was continued for seven consecutive weeks. The survival rate (%) of the animals was recorded.

2.4. Serum concentration of liver transaminases

The mice were anaesthetised using 2.5% tribromo-ethanol solution (10 ml/kg, i.p.) for blood sample collection at the retro orbital plexus. After centrifugation (at 100 ×g for 10 min), the plasma was obtained for alanine and aspartate aminotransferase (ALT and AST) biochemical analysis following the manufacture's guidebook (Labtest Diagnostica commercial kits, Minas Gerais, Brazil).

2.5. Histopathological analysis

Liver samples were fixed in 10% formalin buffered solution, dehydrated and embedded in paraffin. Microtome sections (5 mm) were stained using hematoxylin-eosin (H&E) and mounted on 24 mm^2 coverslips, to be examined by optical microscopy (×100). The slides were analysed by an experienced pathologist who was unaware of treatment and group sorting. NASH diagnosis and graduation were made using NASH Activity Score (NAS), as proposed by Kleiner et al. (2005). The concomitant presence of the following three histological parameters is necessary to diagnose NASH: steatosis (<5% score 0; 5–33% score 1; 33–36% score 2; >66% score 3); lobular inflammation (no infiltrated by field score 0, mild infiltration score 1; moderate infiltration score 2; intense infiltration score 3) and hepatocyte ballooning (Score 0 = none, score 1 = mild ballooning, score 2 = severe ballooning).

In addition, neutrophil infiltration sites in all histopathologic slides were counted blind by two independent collaborators (10 fields). The first field choice was random; the following fields were contiguous to the previous field.

2.6. Liver lipid contents

Lipid content was assayed using the Bligh and Dyer (1959) method. Briefly, samples were homogenized in chloroform:methanol (1:2), 1.9 ml per 100 mg tissue sample, before adding more chloroform (0.63 ml) and stirring. Distilled water (0.63 ml) was added to reach a final proportion of 2:2:1 chloroform:methanol:water. The mixture was centrifuged at room temperature, 200 ×g for 2 min, obtaining a two-phase system (polar above, nonpolar below). The lower phase was placed into a tare tube and evaporated at 100 °C. Total lipid was expressed in mg of lipids/g of liver.

2.7. Malondialdehyde assay (MDA)

Liver samples were homogenized in 1.15% KCl solution (10% homogenate). A homogenate aliquot (500 μl), placed in a test tube, was mixed with 3 ml 1% H3PO4 and 0.6 ml 1% thiobarbituric acid (TBA). This mixture was placed in a water bath (45 min) and cooled in ice, n-butanol (4 ml) was added, and the mixture was vortexed (1 min) and centrifuged at 1200*g* for 15 min. The butanolic fraction was placed in a cuvette and read in spectrophotometer at 520 and 535 mm and the difference between the two readings was used to calculate the MDA, based on the molar extinction coefficient (13,700 M/cm) (Mihara and Uchiyama, 1978).

2.8. Non-protein sulfhydryl groups (NP-SH)

400 ul aliquots of 10% homogenate liver samples in cold sodium EDTA (0.02 M) were added to 320 μl of distilled water and 80 μl of trichloroacetic acid (TCA - 50%). The samples were then centrifuged at 1200 g for 15 min, and 200 ul from the supernatant was placed in a test tube with 0.4 M Tris buffer pH 8.9 (400 uL) and 0.01 M DTNB (10 ul) and incubated at room temperature for 5 min. The samples were then read in spectrophotometer (412 nm). A calibration curve was produced using reduced glutathione. NP-SH concentration was calculated

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