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Prevention of rat liver fibrosis and carcinogenesis by coffee and caffeine

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ABSTRACT

Coffee has been inversely related to the incidence of human liver disease; however, whether caffeine is the component responsible for the beneficial effects of coffee remains controversial. This study evaluated the beneficial effects of coffee or caffeine in a medium-term bioassay for rat liver fibrosis/carcinogenesis induced by diethylnitrosamine (DEN) and carbon tetrachloride (CCl_4). One week after the DEN injection, the groups started to receive conventional coffee, instant coffee or 0.1% caffeine *ad libitum* for 24 weeks. The groups receiving conventional coffee or caffeine presented a significant reduction in collagen content and mRNA expression of collagen I. The groups receiving instant coffee or caffeine had a significant reduction in the size and area of pre-neoplastic lesions and in the mean number of neoplastic lesions. A significant increase in liver bax protein levels was observed in the groups receiving instant coffee or caffeine as compared to the control group. These data indicate that the most pronounced hepatoprotective effect against fibrosis was observed in the groups receiving conventional coffee and 0.1% caffeine, and the greatest effects against liver carcinogenesis were detected in the groups receiving instant coffee and 0.1% caffeine.

1. Introduction

The majority of human chronic liver diseases follow a common pathway that is initiated by inflammation leading to fibrosis and cirrhosis, which are associated with high indexes of morbidity and mortality worldwide (Wanless, 2004; Lim and Kim, 2008; Hernandez-Gea and Friedman, 2011). Liver fibrosis and cirrhosis are consequences of a continuous regenerative process in response to sustained noxious stimuli, such as chronic hepatitis B and C infections or chronic alcohol abuse (Wanless, 2004; Lim and Kim, 2008; Hernandez-Gea and Friedman, 2011). Cirrhosis is an irreversible and terminal step in the fibrotic process, characterised by impairment of hepatic architecture and function (Wanless, 2004; Lim and Kim, 2008; Fallowfield et al., 2006; Kisseleva and Brenner, 2007; Hernandez-Gea and Friedman, 2011).

The fibrotic/cirrhotic process leads to the formation of hepatic nodules and the accumulation of extracellular matrix proteins, mainly collagens I and III, proteoglycans and glycoproteins (Wanless, 2004; Kisseleva and Brenner, 2007; Hernandez-Gea and Friedman, 2011). This process starts with a cascade of events leading to recruitment of inflammatory cells and activation of collagen-producing cells, including hepatic stellate cells (Kisseleva and Brenner, 2007; Hernandez-Gea and Friedman, 2011). Because chronic fibrosis/cirrhosis can lead to the development of hepatocellular carcinoma, there have been increased efforts to develop preventive strategies to inhibit the progression of fibrosis/cirrhosis and consequently, tumour development (Fallowfield et al., 2006; Kisseleva and Brenner, 2007; Hoshida et al., 2012).

Coffee is one of the most popular and highly consumed beverages worldwide, and its moderate intake has been considered to be safe and beneficial for human health (Heckman et al., 2010; Butt and Sultan, 2011). Consumption of more than three cups of coffee per day has been inversely related to the incidence of non-alcoholic fatty liver disease, fibrosis/cirrhosis and hepatocellular carcinoma development in subjects with or without hepatitis B and/or C infection (Klatsky et al., 2006; Bravi et al., 2007; Catalano et al., 2010; Modi et al., 2010; Costentin et al., 2011; Leung et al., 2011). Mechanistic studies have suggested that coffee, caffeine and/or diterpenes cafestol and kahweol intake are beneficial due to antioxidant properties including increase in glutathione levels and modifying effects on phase I-activating and phase II-detoxifying enzymes, leading to protection against the development of liver diseases (Cadden et al., 2007; Lee et al., 2007; Huber et al., 2008; Cavin et al., 2008; Tao et al., 2008; Boettler et al., 2011). However, whether this protection is exerted by coffee *per se* or by specific components, such as caffeine, chlorogenic acid and the diterpenes cafestol and kahweol, remains controversial (Cadden et al., 2007; Huber et al., 2008; Cavin et al., 2008; Tao et al., 2008; Boettler et al., 2011). Experimental studies have suggested that the intake of instant coffee, conventional coffee, caffeine or the diterpenes cafestol and kahweol can reduce hepatotoxicant-induced liver fibrosis in male rats and mice

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(Ozercan et al., 2006; Lee et al., 2007; Shi et al., 2010; Shin et al., 2010; Moreno et al., 2011; Furtado et al., 2012).

A few *in vivo* studies attempted to elucidate the modifying effects of coffee or caffeine intake on the process of chemically-induced liver carcinogenesis (Hasegawa et al., 1995; Hosaka et al., 2001; Silva-Oliveira et al., 2010; Fujise et al., 2012). In addition, the available investigations are difficult to compare due to the different doses and types of coffee preparations adopted. Thus, the present study aimed to elucidate the beneficial effects of different coffee beverages (conventional and instant) and caffeine alone against chemically induced fibrosis and carcinogenesis in the liver of male Wistar rats.

2. Materials and methods

2.1. Animals and housing environment

Male Wistar rats, 4-week-old, were purchased from the Multidisciplinary Centre for Biological Investigation (CEMIB/UNICAMP, Campinas-SP, Brazil) and housed at the animal facility of the Department of Pathology-Botucatu School of Medicine/UNESP Sao Paulo State University. The animals were housed in polypropylene cages (four animals/cage) covered with metallic grids in a room maintained at 22 ± 2 °C with 55 ± 10% humidity, a 12-h light–dark cycle and continuous air exhaustion. The animals were clinically evaluated weekly, food and liquid consumption were measured daily throughout the experiment. The experimental protocol was approved by the local University Ethics Committee for Animal Research (protocol number 588).

2.2. Experimental design

After a two-week acclimation period, the animals were randomly allocated into four groups (12 rats per group). All groups were given a single intraperitoneal injection of 200 mg/kg body weight (b.w.) of diethylnitrosamine (DEN, Sigma–Aldrich Co., St. Louis Mo, USA). Carbon tetrachloride (CCl_4, Sigma–Aldrich Co., St. Louis Mo USA) was dissolved in corn oil and orally administered by gavage once a week (Mondays at 9.00 a.m.) at a dose of 0.5 ml/kg b.w. from weeks 2–10 and then at a dose of 1.0 ml/kg b.w. from weeks 11–24. One week after DEN administration, the groups received drinking water (G1), conventional coffee (G2), instant coffee (G3) or 0.1% caffeine (G4) *ad libitum* for five days/week from week 2–25. Freshly brewed conventional coffee (8 g of powder in 140 ml of hot water with filtration), instant coffee (2% w/v in hot water) or 0.1% caffeine (Sigma–Aldrich Co., St. Louis Mo, USA) were prepared daily (Hasegawa et al., 1995). All solutions were offered *ad libitum* to the rats in aluminium foil-wrapped bottles to avoid light decomposition. These solutions were the sole drinking fluid offered to the animals throughout the experiment. At week 25, the animals were fasted for 12 h and then euthanised by a lethal dose of sodium pentobarbital (45 mg/kg). Blood samples were collected by cardiac puncture for the analysis of the serum levels of alanine aminotransferase (ALT), triglycerides and total cholesterol.

Caffeine and chlorogenic acid and trigonelline content in the coffee beverages drunk by animals and caffeine in the serum of the groups receiving coffee beverages and caffeine alone were analysed by isocratic high performing liquid chromatography as previously described (Furtado et al., 2012; Silva et al., 2013). The system consisted of a Constametric Pump, model 3500 (Fremont, CA, USA), a Rheodyne 7161 (Contati, CA, USA) manual injector equipped with a 20 μL loop and a UN Lab Alliance Model 525 detector (San Jose, CA, USA). The signal produced was transmitted to a Data Jet Thermo Separation Products (San Jose, CA, USA) integrator. The separation was achieved on a 10 μm Phenomenex C18 column (Torrance, CA, USA). Mobile phase consisted of a 0.2% aqueous metaphosphoric acid solution:acetonitrile (80:20 v/v) with detection at 330 nm for chlorogenic acid, 275 nm for caffeine and 267 nm for trigonelline with a flow rate of 1.0 mL/min (Furtado et al., 2012; Silva et al., 2013). The concentrations of caffeine, chlorogenic acid and trigonelline were calculated from standard curves prepared with purified standards (Sigma–Aldrich, Co.) and data are expressed as mg/ml of the average from triplicate samples.

Immediately after euthanasia, the whole liver was excised, washed in 0.9% saline and weighed. Liver samples from all lobes were immersed in 10% buffered-formaldehyde for 24 h for histological and immunohistochemical analyses. Additional liver samples for molecular analyses were stored with RNA*later* (Qiagen, Gaithersburg, MD, USA) at −70 °C. Fixed liver samples were paraffin-embedded, and serial slices 3–4 μm thick were used for histopathological analysis by haematoxylin–eosin (HE) staining, collagen analysis by Picrosirius red staining and labelling glutathione S-transferase placental form (GST-P) and proliferating cell nuclear antigen (PCNA) by immunohistochemical analysis using a polymer system (Novo-Link Max Polymer Detection System, Leica/Novocastra). In HE-stained liver sections, preneoplastic and neoplastic lesions were identified and classified according to their predominant phenotype (Goodman et al., 1994).

2.3. Analysis of GST-P-positive PNL and cell proliferation

The number and size of GST-P-positive preneoplastic lesions were determined using a KS 300 imaging software (Kontron Elektronic, Munich, Germany). Data are expressed as number (lesions/cm^2), mean size (mm^2) and area (mm^2/cm^2) (Pinheiro et al., 2003; Furtado et al., 2012). GST-P-positive preneoplastic lesions were also categorised into three different classes according to size: class I: $\leqslant$1.0 mm, class II: 1.1–4.9 mm and class III: $\geqslant$5.0 mm. PCNA-S-phase-positive hepatocytes were counted among a total of 2000 hepatocytes within preneoplastic lesions under 40× microscope magnification (Pinheiro et al., 2003; Furtado et al., 2012).

2.4. Analysis of collagen content

Stereological-morphometric analysis of the area of collagen I and III fibres was performed using a Leica DMLB 80 microscope connected to a Leica DC300FX camera and images were analysed using the Leica Q-win software, Version 3 (Leica, Heidelberg, Germany). Fifteen fields from Picrosirius red-stained liver sections were randomly selected and the area of collagen fibres was determined (Furtado et al., 2012).

2.5. Quantitative Real-time PCR (RT-qPCR)

All RT-qPCR analyses were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Total RNA was extracted from the liver samples using the illustra RNAspin kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) in accordance with the manufacturer's instructions. Total RNA was resuspended in RNase-free water and then treated with TURBO DNase (Ambion, USA) to remove any possible DNA contamination. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL, USA) to measure optical density (OD) at 260 nm. cDNA was synthesised using High Capacity RNA-to-cDNA Master Mix (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Briefly, 1 μg of total RNA was added to 4 μl of Master Mix and brought to a total volume of 20 μl with RNase-free water. cDNA synthesis reaction conditions were conducted as follows: 5 min at 25 °C for primer annealing, 30 min at 42 °C for reverse transcription and 5 min at 85 °C for inactivation of the enzyme reverse transcriptase.

Amplification of the mRNA of interest was performed using TaqMan probes (Life Technologies, Carlsbad, CA, USA) with primers specific for collagens I and III (Applied Biosystems RN01463848_m1 and RN01437683_m1) and GAPDH (as an endogenous control, Applied Biosystems 4352338E). For each reaction, 10 μl of TaqMan® Universal PCR Master Mix with no AmpErase® UNG (2X) (Life Technologies, EUA) was mixed with 4 μl of the final product from the reverse transcription reaction and the final volume was adjusted to 20 μl with nuclease-free water. Each reaction followed the following design: 95 °C for 10 min, followed by 95 °C for 15 s and 60 °C for 1 min for 40 cycles. All reactions were performed using the Real-time PCR system 7900 HT (Life Technologies, USA). Quantification data (Cq) were calculated using SDS2.1 software with an automatic baseline configuration and threshold of 0.2. The Cq value refers to the point at which the corrected amplification curve (based on baseline) meets the threshold (in accordance with the RDML, http://rdml.org) (Lefever et al., 2009). Relative mRNA expression was achieved with the comparative Cp method (Livak and Schmittgen, 2001).

2.6. Western blot

Briefly, 300 mg of hepatic tissue was homogenised in 100 μl of extraction buffer (Tris–HCl 500 mM pH 7.6; NaCl 0.2 M; Triton X-100 1%; CaCl2 10 mM; protease inhibitor cocktail 2 μl/100 ml) and kept at 4 °C for 2 h. The solution was then centrifuged at 4000 rpm for 20 min at 4 °C. The supernatant was collected, and the total protein concentration was determined using the Branford's method at 495 nm in a microplate spectrophotometer (Spectra Max 190, Sunnyvale, CA, USA).

Aliquots of liver homogenates containing 70 μg of total protein were heated at 95 °C for 5 min in sample-loading buffer and then electrophoretically separated in a 12% SDS–PAGE gel under reducing conditions and transferred to nitrocellulose membranes (Sigma Chemical Co., St. Louis Mo, USA) (Aguiar e Silva et al., 2012). Membranes were blocked with non-fat milk in TBS-T (Tris 0.05 M, NaCl 0.15 M, pH 7.2, 1% Tween-20) for 1 h. Membranes were subsequently incubated with primary antibodies against bax (1:200, Santa Cruz Biotechnology, CA, USA), bcl-2 (1:200, Santa Cruz Biotechnology, CA, USA) and TGF-β1 (1:1,000; MAB240; R&D Systems, Minneapolis, USA) for 90 min, washed with TBS-T (3× 20 min. each) and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:1000 anti-mouse, Santa Cruz Biotechnology, CA, USA) for 1 h. Goat anti-β-actin antibody (1:1,000; Santa Cruz Biotechnology, CA, USA) served as a loading control. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies, the corresponding bands were detected using the 3,3′-diaminobenzidine (DAB) substrate. Bax, bcl-2, TGF-β1 and β-actin protein expression levels were quantified by measuring integrated optical density (IOD) of the bands by densitometric analysis. The bax, bcl-2 and TGF-β1 expression levels were normalised to the β-actin values and expressed as means ± S.E.