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Rosmarinic acid attenuates hepatic fibrogenesis via suppression of hepatic stellate cell activation/proliferation and induction of apoptosis

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ABSTRACT

Objective: To investigate the antifibrotic role of rosmarinic acid (RA), a natural polyphenolic compound, on HSCs activation/proliferation and apoptosis *in vitro* and *in vivo*.

Methods: The impact of RA on stellate cell line (HSC-T6) proliferation, activation and apoptosis was assessed along with its safety on primary hepatocytes. *In vivo*, rats were divided into: (i) normal; (ii) thioacetamide (TAA)-intoxicated rats for 12 weeks; (iii) TAA + silymarin or (iv) TAA + RA. At the end of experiment, liver functions, oxidative stress, inflammatory and profibrogenic markers, tissue inhibitor metalloproteinases type-1 (TIMP-1) and hydroxyproline (HP) levels were evaluated. Additionally, liver histopathology and immunohistochemical examinations of alpha-smooth muscle actin (α-SMA), caspase-3 and proliferation cellular nuclear antigen (PCNA) were determined.

Results: RA exhibited anti-proliferative effects on cultured HSCs in a time and concentration dependent manner showing an IC_{50} of 276 μg/mL and 171 μg/mL for 24 h and 48 h, respectively, with morphological reversion of activated stellate cell morphology to quiescent form. It significantly improved ALT, AST, oxidative stress markers and reduced TIMP-1, HP levels, inflammatory markers and fibrosis score (S1 *vs* S4). Furthermore, reduction in α-SMA plus elevation in caspase-3 expressions of HSCs *in vitro* and *in vivo* associated with an inhibition in proliferation of damaged hepatocytes were recorded.

Conclusions: RA impeded the progression of liver fibrosis through inhibition of HSCs activation/proliferation and induction of apoptosis with preservation of hepatic architecture.

1. Introduction

Liver fibrosis and its end-stage cirrhosis are major causes of morbidity and mortality worldwide. Liver fibrosis is not an independent disease but rather a sequel of chronic liver diseases including viral hepatitis, alcoholic or nonalcoholic fatty liver disease, autoimmune hepatitis and parasitic infection [1]. Preventing or inhibiting the progression of fibrosis may reduce the economic burden and, more importantly, the decline in health-related quality of life [2].

Hepatic stellate cells (HSCs) play a crucial role in the progression and resolution of hepatic fibrosis [3]. In normal liver, HSCs store retinoids and vitamin A [4], whereas upon injury they become activated and trans-differentiated into myofibroblast-like cells. Such trans-differentiation is provoked by cytokines release including transforming growth factor (TGF)-β1,

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platelet derived growth factor (PDGF) and tumor necrosis factor-α (TNF-α) complemented with boosted proliferation and expression of α-smooth muscle actin (α-SMA), loss of vitamin A stores plus overproduction of extracellular matrix (ECM) [3] thereby accelerating the progression of liver fibrosis [5].

Despite the extensive knowledge of the mechanisms underlying hepatic fibrosis, so far the potent and effective anti-fibrotic drug remains indefinable [6]. Currently, developing drugs from natural products have driven worldwide attention [7] for being safe and cost-effective making them potential anti-fibrotic agents. However, for many natural products, the mechanism of action is still unknown thus impeding drug development [8].

Rosmarinic acid (a-O-caffeoyl-3,4-dihydroxyphenyllactic acid; RA), a naturally occurring hydroxylated compound, is commonly found in various plants from *Lamiaceae* family including *Rosmarinus officinalis* (*R. officinalis*) (rosemary) [9]. RA has a variety of salutary biological activities including anti-inflammatory [10], antioxidant [11], antiangiogenic [12] and anticancer [13] activities. Consequently, this study provides insights on the antifibrotic effects of RA through focusing mainly on HSCs both in *vitro* and in a rat model of thioacetamide (TAA)-induced fibrosis.

2. Materials and methods

2.1. Drug and reagents

Tissue inhibitor metalloproteinases, type 1 (TIMP-1), platelet derived growth factor-BB (PDGF-BB) and tumor growth factor-β1 (TGF-β1) ELISA kits were purchased from Quantikine R&D systems (Minneapolis, USA). Serum alanine and aspartate aminotransferases kits were purchased from Spectrum (Cairo, Egypt). Dimethyl sulphoxide (DMSO), collagenase Type IV, thiazolyl blue tetrazolium bromide (MTT), sulforhodamine base (SRB), thioacetamide, 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent), chloramine T, 4-dimethylaminobenzaldehyde (Ehrlich reagent), standard hydroxyproline (HP), standard reduced glutathione were purchased from Sigma–Aldrich Chemical Co. (MO, USA). Anti-Interleukin (IL)-6, anti-lymphocyte common antigen (CD45), anti-caspase-3, anti-alpha smooth muscle actin (α-SMA) and anti-proliferating cell nuclear antigen (PCNA) monoclonal antibodies, mouse and rabbit specific HRP/DAB detection IHC kits were purchased from Santa Cruz Biotechnology (CA, USA). Silymarin (Legalon®) was purchased from Chemical Industries Development, CID (Cairo, Egypt). All other chemicals and solvents were of the highest grade commercially available.

2.2. Plant materials

The aerial parts of *R. officinalis* L. were obtained from the experimental station of medicinal, aromatic and poisonous plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt in spring 2014 and was kindly authenticated by Prof. Dr. Wafaa Amer, Botany Department, Faculty of Science, Cairo University, Giza, Egypt. A voucher specimen (2014067) was kept in the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

2.3. Extraction and isolation of RA

One kilogram of the powdered air-dried aerial parts of *R. officinalis* was extracted with methanol in a soxhlet until exhaustion. The methanolic extract was concentrated under reduced pressure to yield 200 g of the dry residue, which was suspended in distilled water (700 mL) and defatted by partition with methylene chloride (3 × 500 mL). The mother liquor was then fractionated over Diaion HP-20 using water, 50% methanol in water and 100% methanol. The solvent of each fraction was evaporated under reduced pressure at 40 °C and monitored using TLC, where a major spot was detected in the 100% methanol fraction. This fraction was purified by elution on sephadex LH-20 column using methanol-water (1:1 v/v) yielding pure white powder compound, Rf value (0.54 in ethyl acetate-methanol-water 100: 16.5: 13.5 v/v/v).

2.4. Animals

Adult male Sprague Dawley rats with (280 ± 30) g were purchased from the animal house of Theodore Bilharz Research Institute (TBRI), Giza, Egypt. The rats were kept in an environmentally controlled room at (20–22) °C, 12 h light/dark cycle and 50–60% humidity throughout the acclimatization and experimental periods with free access to water and *ad libitum* with rodent food pellet (El-Kahira Company for Oils and Soap, Tanta, Egypt). All experiments conducted followed the international guidelines for animal ethics and were approved by the Institutional Review Board of TBRI.

2.5. Cell culture and treatment

HSC-T6, an immortalized rat hepatic stellate cell line, was a generous gift from Prof. Scott L. Friedman (Division of Liver Diseases, Icahn School of Medicine at Mount Sinai University, New York). Primary isolated hepatocytes were freshly isolated from rats by a two-step portal collagenase perfusion of the liver as previously described [14]. HSCs and hepatocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL streptomycin under conditions of 95% air/5% CO_2 at 37 °C. RA was dissolved in a small volume of DMSO (equivalent to <1% of the final volume).

2.5.1. HSCs proliferation/morphology and hepatocyte cytotoxicity assays

Rat HSCs-T6 (5×10^3) were cultured in 96-wells tissue culture plates and treated with various concentrations of RA (0, 25, 50, 100, 200, 400 μg/mL) or vehicle (DMSO, 0.05% final concentration) for 24 and 48 h. The anti-proliferative activity of RA was determined using SRB staining as previously described [15]. Furthermore, HSC morphology was observed under phase-contrast microscope [EVOS® xl core cell culture microscope (Advanced Microscopy Group, USA)].

Cytotoxicity of RA on hepatocytes was determined using MTT assay on freshly rat hepatocytes isolated as described by Mosmann [16], where primary hepatocytes (5×10^3) were cultured in 96-wells tissue culture plates and treated with the same concentrations of RA for 24 h and 48 h.

2.5.2. HSCs activation and apoptosis assays

HSCs activation was assessed via determination of concentrations of TGF-β1 in culture media using the commercial ELISA kits according to the manufacturer's instructions and immunocytostaining of HSCs with α-SMA; moreover, HSCs apoptosis was assessed using caspase-3 immunocytostaining.

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