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Oxidative stress and cell damage in a model of precancerous lesions and advanced hepatocellular carcinoma in rats

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ABSTRACT

Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer deaths throughout the world. This study was aimed to analyze oxidative stress and cell damage in a multistage model of liver carcinogenesis induced by diethylnitrosamine (DEN) in rats. Male Wistar rats weighing 145–150 g were divided into three groups: control, precancerous lesions (PL) (which received 100 mg DEN once a week every 6 weeks up to 28 weeks), and advanced HCC (50 mg DEN once/twice per week up to 19 weeks). Lipid peroxidation (TBARS), superoxide dismutase (SOD) activity, and expression of transforming growth factor-1 beta (TGF)-1β, endothelial and inducible nitric oxide syntahese (eNOS, iNOS), NADPH quinone oxireductase (NQO)-1, nuclear factor erythroid 2-related factor (NrF)2, kelch-like ECH-associated protein (Keap)1 and heat shock protein (HSP)70 were measured. TBARS concentration was augmented in the PL and advanced HCC groups. SOD activity, TGF-1β and Nrf2 expression were higher in animals with precancerous lesions. In advanced HCC, expression of NQO1 and iNOS increased while there was a decrease in HPS70 expression. Data obtained provide evidence for the differential activation of proteins involved in oxidative stress and cell damage during progression of carcinogenesis in an animal model of HCC.

Abbreviations: 2-AAF, 2-acetylaminofluorene; ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; DEN, diethylnitrosamine; EDTA, ethylenediamine tetraacetic acid; eNOS, endothelial nitric oxide synthase; GGT, gamma-glutamyl transferase; HCC, hepatocellular carcinoma; HSC, hepatic stellate cells; HSP70, heat shock 70-kDa protein; iNOS, inducible nitric oxide synthase; Keap1, kelch-like ECH-associated protein 1; MDA, malonaldehyde; NQO1, NADPH quinone oxireductase-1; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; PVDF, polyvinylidene fluoride; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactant substances; TGF-1β, transforming growth fator-1 beta; TTBS, Tris-buffered containing 0.05% Tween 20; UV, ultra violet.

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1. Introduction

The most common histological type of primary liver cancer is hepatocellular carcinoma (HCC). In 2008, there were approximately 694,000 deaths from HCC, making it the third most common cause of cancer death worldwide [1]. Chronic liver diseases are risk factors that predispose to HCC, as any agent or factor that chronically and slowly damages the hepatocytes induces mitosis and makes the DNA of these cells more susceptible to genetic alterations [2]. Such diseases include alcoholic cirrhosis, hepatitis B or C virus infection, α1-antitrypsin deficiency, hemochromatosis and tyrosinemia. In HCV-positive patients, for example, HCC appears on average 30 years after infection, almost exclusively in those with cirrhosis [3]. The development of HCC is a complex process, involving accumulation of genetic and epigenetic alterations, which passes through stages of initiation, promotion and progression, and numerous experimental observations have shown that viral products may contribute to the malignant transformation of hepatocytes [4].

Curative therapeutic approaches for HCC involve liver transplantation, or surgical and radiofrequency ablation, but these treatments are not yet effective [5]. Although surgical resection can sometimes be curative, few patients have resectable tumors because of the presence of cirrhosis or distant metastases; moreover, even after resection, preexisting liver cirrhosis persists and may cause other tumors in the remaining tissue. Orthotopic liver transplantation is the only truly curative therapy, although issues of recurrence and development of metastases remain. In case of unresectable tumor, treatment is limited, as HCC does not respond to chemotherapy and the liver does not tolerate high doses of radiotherapy [6].

HCC carries a high mortality rate and patients with chronic liver diseases usually take a long time before HCC occurs. Therefore, early diagnosis of HCC in precancerous lesions may improve the outcome of treatment, and it is necessary to encourage basic research to better understand the pathogenesis of this disease. Many experimental animal models of hepatocarcinogenesis have been described over the last decades. The most widely accepted, proposed by Farber et al. [7], combines chemical induction by diethylnitrosamine (DEN) with partial hepatectomy. Since then, DEN has been used to initiate the liver cancer either alone or in combination with other carcinogens [8–11]. However, fewer studies have characterized in detail the temporal evolution of oxidative stress and cell damage implicated in hepatocarcinogenesis. Understanding changes from pre-neoplastic to carcinoma lesions in oxidative stress, inflammation and liver fibrosis could be important to improve the knowledge on the transition of chronic inflammatory liver diseases to HCC. In the current study, we used a multistage model of chronic and intermittent exposure to DEN without partial hepatectomy to get insight into changes in markers of cell damage during progression of the disease. Two different protocols of drug exposure (designed to induce advanced HCC and precancerous lesions) allowed us to study effects of time on tumor onset, liver pathology, blood chemistry, and markers of oxidative stress and cell damage in the liver.

2. Materials and methods

2.1. Animals and procedures

Male Wistar rats weighing 145–150 g were used for this study and were obtained from the Central Animal Laboratory of the Federal University of Pelotas, Rio Grande do Sul (Brazil). The rats were caged at 24 °C, under a 12-h light–dark cycle and with free access to food and water until the time of the experiments at the Animal Experimentation Division of Hospital de Clínicas de Porto Alegre (Brazil). All experiments were performed in accordance with the *Guiding Principles for Research Involving Animals* (NAS) under protocol number 120355.

The animals were divided into three groups: CO: control, precancerous lesions (PL) and advanced HCC. Animals in the PL group were given diethylnitrosamine (DEN, Sigma Aldrich, St. Louis, MO) at a dose of 100 mg/kg body weight i.p. once a week every 6 weeks up to 28 weeks. Animals in the advanced HCC group received DEN at a dose of 50 mg/kg body weight i.p. twice a week for the first three weeks and once a week from weeks 4 to 6 and 11 to 13 up to 19 weeks. A single dose of 2-acetylaminofluorene (2-AAF, 100 mg/kg, Sigma–Aldrich, St. Louis, MO) was administered in week 4 to both DEN groups.

Following a 12-h fast, the animals were anesthetized with ketamine hydrochloride (Ketalar®, 100 mg/kg – PubChem CID: 15851) and xylazine (50 mg/kg – PubChem CID: 5707) and subjected to blood collection for measurement of biochemical parameters.

Samples of livers for histology, biochemical and molecular analyzes were taken from the same lobe (right medial lobe). The collected sample was withdrawn from the area where the nodules were visible. The animals were killed at the end of the experiment by exsanguination under deep anesthesia, as described in the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia [12].

2.2. Biochemical analysis

Serum levels of alanine aminotransferase (ALT) (U/L), aspartate aminotransferase (AST) (U/L) were determined by kinetic UV test. Gamma-glutamyl transferase (gamma-GT) (U/L), and alkaline phosphatase (AP) (U/L) were quantified by colorimetric kinetic test. They were measured using routine laboratory methods of the Hospital de Clínicas de Porto Alegre by enzymatic method (automated – Siemens Advia 1800 Chemistry system).

2.3. Histology

For histological examination, a specimen of liver was trimmed and fixed by immersion in 10% buffered formalin for 24 h. The blocks were dehydrated in a graded ethanol series and embedded in paraffin wax. Serial 3-μm sections were stained with hematoxylin and eosin and picrosirius red.

The percentage of fibrosis (%) in the liver tissue was determined by morphometric measurements. Ten images from each slide were captured from randomly selected high-power fields (200× magnification) containing the