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Protective effects of *Ginkgo biloba* against rat liver carcinogenesis

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Abstract

Ginkgo biloba (EGb) has been proposed as a promising candidate for cancer chemoprevention and has shown protective effects on the liver against chemically induced oxidative injury and fibrosis. The potential beneficial effects of EGb were investigated in two rat liver carcinogenesis bioassays induced by diethylnitrosamine (DEN). In a short-term study for anti-initiating screening, male Wistar rats were fed a basal diet or supplemented diet with 500 or 1000 ppm EGb and initiated 14 days later with a single dose of DEN (100 mg/kg i.p.). The respective groups were killed 24 h or 2 weeks after DEN-initiation. Liver samples were collected for the analysis of proliferating cell nuclear antigen (PCNA), transforming growth factor alpha (TGF-α), p53, apoptosis and induction of single hepatocytes and minifoci positive for the enzyme glutathione *S*-transferase P-form (GST-P). In a medium-term study for anti-promoting screening, the animals received a single dose of DEN (200 mg/kg i.p.) and, 2 weeks later, were fed a basal diet or supplemented diet with 500 or 1000 ppm EGb for 6 weeks. All animals underwent 70% partial hepatectomy (PH) at week 3 and killed at week 8. Liver samples were colleted to analyze development of preneoplastic foci of altered hepatocytes (FAH) expressing GST-P. In the short-term study, pretreatment of rats with 1000 ppm EGb significantly reduced the rates of cell proliferation, apoptosis and p53, TGF-α immunoreactivity and the number of GST-P-positive hepatocytes. In the medium-term study, EGb treatment during the post-initiation stage failed to reduce the development of DEN-induced GST-P-positive foci. Thus, EGb presented inhibitory actions during initiation but not promotion of rat liver carcinogenesis induced by DEN.

Keywords: Diethylnitrosamine; Putative initiated hepatocytes; GST-P-positive preneoplastic foci; Liver carcinogenesis; *Ginkgo biloba*; Chemoprevention

1. Introduction

Ginkgo biloba extract (EGb) is a commercial medicinal herb which comes from the green leaves of the ginkgo tree, one of the oldest living plant species [1]. EGb is a mixture, composed mainly of flavone glycosides and terpenoids (ginkgolides and bilobalide), that has presented various pharmacological activities [2–4]. This extract has shown several *in vivo* effects, including augmentation of blood flow and inhibition of platelet activating factor. It protects the cell membrane against damage induced by free radicals and presents protective effects against myocardial and brain ischemia/reperfusion injury [2–5]. EGb has been widely used in the clinical treatment of cardiovascular and neurological diseases like Alzheimer's, dementia, labyrintopathies and other cognitive dysfunctions [2,6].

EGb has shown protective effects on the liver against chemically induced oxidative injury and fibrosis [7–10].

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Also, EGb has been shown to modulate the hepatic phase I enzymes, including the induction or inhibition of specific cytochrome P450 (CYP) isozymes, and phase II enzymes, specifically the induction of glutathion *S*-transferases, DT-diaphorase and quinine reductase [11–14]. Thus, changes in metabolizing phase I and phase II enzymes by EGb can alter the balance between the activation of procarcinogens or detoxification of potential carcinogens.

There are few *in vivo* and *in vitro* studies on the potential anti-carcinogenic effects of EGb. Crude EGb or their specific compounds induced cellular death or anti-proliferative activities against the following cancer cell lines: HepG2, Hep 3B, and SMMC-7721 human hepatocellular [15,16], SCC 1483 human oral [17] and OVCA429, 433 and 420 human ovarian [18]. EGb, when fed orally to female Swiss mice, reduced forestomach tumor multiplicity induced by benzo(*a*)pyrene [19]. EGb and bilobalide, given orally to male Fischer 344 rats, inhibited the development of colonic aberrant crypt foci induced by azoxymethane [20].

Foci of altered hepatocytes (FAH) have been described as putative preneoplastic lesions detected in various rodent models of chemical liver carcinogenesis [21]. More recently, different types of FAH with similar morphological and biochemical alterations in the hepatocellular phenotype were identified in chronic human liver diseases associated with, or predisposing to hepatocellular carcinoma [22]. Glutathione *S*-transferase P-form (GST-P) expression is a useful marker for preneoplastic and neoplastic rat liver lesions [23]. Single hepatocytes and minifoci highly positive for GST-P develop very early in carcinogen-treated rat liver, and are considered precursors of large FAH and nodules [24,25]. The detection of GST-P-positive single cells and minifoci or large GST-P-positive FAH is an important tool for analyzing relevant carcinogenic or anti-carcinogenic responses during the initiation and promotion stages of rat liver carcinogenesis [26–28].

Using a short-term (anti-initiating screening) and a medium-term (anti-promoting screening) liver bioassay, the present study investigated the modifying influence of a *G. biloba* extract (EGb) on the initiation and promoting phases of rat liver carcinogenesis induced by diethylnitrosamine (DEN).

2. Material and methods

2.1. Animals and treatments

Four-week-old male Wistar rats were obtained from CEMIB (UNICAMP Campinas, SP, Brazil). The animals were kept in polypropylene cages (five animals/cage) covered with metallic grids in a room maintained at $22 \pm 2\,^{\circ}C$, $55 \pm 10\%$ humidity under a 12-h light:12-h dark cycle. They were fed commercial NUVILAB-CR-1 chow (NUVITAL, Curitiba, PR, Brazil) and water *ad libitum* for a 2-week acclimation period before beginning the experiment. Samples of lyophilized extract of *G. biloba* leaves (EGb), generously supplied by the CentroFlora Group (Botucatu, SP, Brazil), were obtained from hydroalcoholic extraction by a spray dryer system. The EGb (cod. 500821) used in this study contained known amounts of approximately 24% flavone glycosides (i.e., quercetin, kaempferol and isorhamnetin) and 6% terpene trilactones (i.e., ginkgolide A–C, bilobalide) as determined by the HPLC method. EGb was supplemented to basal diet at 500 and 1000 ppm, levels which correspond to 1 and 2 times the doses that were used in an *in vivo* feeding study for beneficial effects against rat colon carcinogenesis [20].

2.2. Experimental design

The protocols used were consistent with Ethical Principles for Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA). The animals were randomly allocated to two experimental protocols: a short- and a medium-term liver bioassay, respectively (Fig. 1).

2.2.1. Experiment 1 (short-term bioassay)

This study was performed to investigate the modifying effects of EGb intake on the first stage of rat liver carcinogenesis in an initiation bioassay model that uses the detection of initiated hepatocytes and minifoci positive for GST-P as the endpoint [24,25]. The animals were randomly allocated into four groups: groups G1A and G4A were fed basal diet and groups G2A and G3A were fed a diet supplemented with 500 or 1000 ppm EGb during 2 weeks. Then, groups G1A to G3A were given a single i.p. injection of 100 mg/kg b.w. of diethylnitrosamine and group 4A (control) received 0.9% NaCl (DEN vehicle). Twenty-four hours after DEN administration, groups G1A to G3A were fed basal diet *ad libitum*. The animals were sacrificed 24 h or 2 weeks after DEN treatment (end of week 4).

2.2.2. Experiment 2 (medium-term bioassay)

This study was performed to investigate the modifying effects of EGb intake on the second stage of rat liver carcinogenesis in a post-initiation bioassay model that uses preneoplasia detection (GST-P-positive foci) as the endpoint [26–29]. The animals were randomly allocated