

Journal of Ethnopharmacology 83 (2002) 25-32



www.elsevier.com/locate/jethpharm

# Influence of aqueous extract of *Agaricus blazei* on rat liver toxicity induced by different doses of diethylnitrosamine

L.F. Barbisan<sup>a</sup>, M. Miyamoto<sup>a</sup>, C. Scolastici<sup>a</sup>, D.M.F. Salvadori<sup>b</sup>, L.R. Ribeiro<sup>b</sup>, A.F. Eira<sup>c</sup>, João Lauro Viana de Camargo<sup>b,\*</sup>

<sup>a</sup> Departamento de Morfologia, Instituto de Biologia, UNESP, Botucatu 18618-000, SP, Brazil

<sup>b</sup> Departamento de Patologia-TOXICAN, Faculdade de Medicina, Universidade Estadual Paulista (UNESP), Botucatu 18618-000, SP, Brazil <sup>c</sup> Departamento de Produção Vegetal, Módulo de Cogumelos, Faculdade de Ciências Agronômicas, UNESP, Botucatu 18603-970, SP, Brazil

Received 1 June 2001; received in revised form 1 March 2002; accepted 20 June 2002

### Abstract

The modifying potential of prior administration of an aqueous extract of the mushroom *Agaricus blazei* Murrill (Agaricaceae) (*Ab*) on hepatotoxicity induced by different doses of diethylnitrosamine (DEN) in male Wistar rats was evaluated. During 2 weeks, animals of groups G3 (*Ab*+DEN<sub>50</sub>), G5 (*Ab*+DEN<sub>100</sub>), G7 (*Ab*+DEN<sub>200</sub>), and G8 (*Ab*-treated) were treated with the *A. blazei* through drinking water. After this period, groups G2 (DEN<sub>50</sub>), G3 (*Ab*+DEN<sub>50</sub>), G4 (DEN<sub>100</sub>) G5 (*Ab*+DEN<sub>100</sub>), G6 (DEN<sub>200</sub>), and G7 (*Ab*+DEN<sub>200</sub>) were given a single i.p. injection of 50, 100 and 200 mg/kg of DEN, respectively, while groups G1 (non-treated) and G8 (*Ab*-treated) were treated with 0.9% NaCl only. All animals were killed 48 h after DEN or NaCl treatments. The hepatocyte replication rate was estimated by the index of the proliferating cell nuclear antigen (PCNA) positive hepatocytes and the appearance of putative preneoplastic hepatocytes through expression of the enzyme glutathione S-transferase placental form (GST-P). After DEN-treatment, ALT levels, PCNA labeling index, and the number of GST-P positive hepatocytes were lower in rats that received *A. blazei* treatment and were exposed to 100 mg/kg of DEN. Our findings suggest that previous treatment with *A. blazei* exerts a hepatoprotective effect on both liver toxicity and hepatocarcinogenesis process induced by a moderately toxic dose of DEN. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Agaricus blazei; Murrill; Chemoprotection; Hepatoprotection; Liver toxicity; Diethylnitrosamine

### 1. Introduction

Mushrooms have been used as an important source of nutrition and/or therapy throughout the world since ancient times (Chang, 1996). However, some species of mushrooms are common sources of human poisoning (mainly species containing  $\alpha$ -amanitin) and have been associated with cancer development in experimental animals (mainly species containing carcinogenic compounds, such as hydrazine and diazonium ions) (Toth, 1995; Larrey and Pageaux, 1995; McPartland et al., 1997).

Immunological, hypocholesterolemic, antiviral, antibacterial and antiparasitic activities have been associated with some substances isolated from higher species of Basidiomycetes (Mizuno et al., 1995; Wasser and Weis, 1999; Ooi and Liu, 1999). Among these, *Agaricus blazei* Murrill (Agaricaceae), a species native to Brazil, where it is popularly known as 'cogumelo do sol' (or 'Himematsutake' in Japan), has received attention in folk medicine due to its possible medicinal values. In Brazil, infusion of the dried fruiting bodies of this mushroom has been popularly consumed both as a stimulant and as an auxiliary treatment of various diseases, including cancer (Fujimiya et al., 1998; Takaku et al., 2001; Delmanto et al., 2001; Menoli et al., 2001). Nevertheless, no epidemiological or experimental data exist on the beneficial effects of the crude aqueous extract of this species of mushroom.

Considerable efforts have been made to isolate from the *A. blazei* mushroom polysaccharides and proteinbound polysaccharides that have shown anti-tumor

<sup>\*</sup> Corresponding author. Tel.: +55-14-68026238; fax: +55-148-212348

E-mail address: decam@fmb.unesp.br (J.L.V. de Camargo).

activity in tumor-bearing mice (Kawagishi et al., 1989; Itoh et al., 1994; Ito et al., 1997; Mizuno et al., 1998; Fujimiya et al., 1998). Isolated protein-bound polysaccharides such as  $(1 \rightarrow 6)$ - $\beta$ -D-glucan-protein complex (designed as FIII-2-b fraction) (Kawagishi et al., 1989),  $(1 \rightarrow 4)$ - $\beta$ -D-glucan/ $(1 \rightarrow 6)$ - $\beta$ -D-glucan-protein complex (designed as acid-treated fraction, ATF; Fujimiya et al., 1998), and sole-isolated polysaccharides containing  $(1 \rightarrow 4)$ - $\beta$ -D-glucan and  $(1 \rightarrow 6)$ - $\beta$ -d-glucan complex (Mizuno et al., 1998) obtained from fruiting bodies of A. blazei have shown potential anti-tumor activity through specific and non-specific immune response activation. The enhanced immune response was also observed with the isolated polysaccharide-protein complex (designed as ATOM) obtained from the cultured mycelia of A. blazei (Ito et al., 1997). Direct tumor-specific cytocidal activity was observed solely with the  $(1 \rightarrow 4)$ - $\beta$ -D-glucan/  $(1 \rightarrow 6)$ - $\beta$ -D-glucan complex ATF crude (380 kDa) and LM-3 fraction (20 kDa) as demonstrated by selective cell cycle arrest and apoptosis of Meth A tumor cells in vitro (Fujimiya et al., 1998, 1999).

In contrast to the well-established anti-tumor activity of *A. blazei* observed in tumor-transplantable models, no reports exist on the modifying potential of this mushroom on chemical carcinogenesis models. Diethylnitrosamine (DEN), a potent genotoxic carcinogen, is used in chemical hepatocarcinogenesis models due to its initiating activity (i.e. induction of preneoplastic hepatocytes). Also, it induces acute necrosis when given at high doses and produces several types of promutagenic DNA adducts in hepatocytes of the rodents (Swenberg et al., 1991; Verna et al., 1996). Prolonged exposure to DEN is associated mainly with the development of liver and oesophageal tumors in rats (Peto et al., 1991; Verna et al., 1996).

Compensatory hepatocyte proliferation following cell necrosis is proposed to be necessary for initiation of the carcinogenesis process, presumably by conversion of promutagenic DNA adducts into mutation or other heritable genetic damage that leads to the establishment of initiated cells (Kato et al., 1993). The induction of putative preneoplastic glutathione S-transferase, placental form (GST-P) positive hepatocytes, is thought to represent the earliest recognizable stage of liver carcinogenesis following initiation by DEN and other carcinogens (Moore et al., 1987; Satoh et al., 1989; Kato et al., 1993). Therefore, GST-P expression is considered a useful marker for analyzing relevant factors of the initiation stage of hepatocarcinogenesis (Moore et al., 1987).

In the present study, we analyzed the modifying influence of prior administration of an aqueous extract of the mushroom *A. blazei* on necrosis, proliferation, and on the development of GST-P positive hepatocytes induced by different doses of DEN.

#### 2. Materials and methods

#### 2.1. Animals and treatment

Male 4-week-old Wistar rats were obtained from the CEMIB (UNICAMP Campinas, SP, Brazil). The animals were kept in polypropylene cages (five animals per cage) covered with metallic grids in a room maintained at  $22\pm2$  °C,  $55\pm10\%$  humidity with a 12-h light and 12-h dark cycle. They were fed with commercial Purina chow (LABINA, Paulinia, SP, Brazil) and water ad libitum for a 2-week acclimation period.

The animals were randomly allocated to eight groups of ten rats. For 2 weeks, animals of groups G3 (Ab+ DEN<sub>50</sub>), G5 (Ab+DEN<sub>100</sub>), G7 (Ab+DEN<sub>200</sub>), and G8 (Ab) were treated with an aqueous extract of the mushroom *A. blazei* (see below). After this period, groups G2 (DEN<sub>50</sub>), G3 (Ab+DEN<sub>50</sub>), G4 (DEN<sub>100</sub>), G5 (Ab+DEN<sub>100</sub>), G6 (DEN<sub>200</sub>) and G7 (Ab+DEN<sub>200</sub>) were given a single i.p. injection of 50, 100 or 200 mg/kg of DEN (Sigma Chemical Co., MO, USA), respectively, for initiation of liver carcinogenesis. Groups 1 (nontreated) and G8 (Ab-treated) were treated with 0.9% NaCl only (DEN vehicle). All animals were killed 48 h after DEN or 0.9% NaCl treatments. The University Ethical Committee for Animal Research approved the protocols used in this study (Protocol number 99/22).

# 2.2. Preparation and administration of aqueous extract of A. blazei

Sample of *A. blazei* was obtained from the Departamento de Produção Vegetal, Faculdade de Ciências Agronômicas, UNESP, Botucatu, SP, Brazil.

Twenty-five grams of powdered dry fruiting bodies of *A. blazei* (lineage AB 99/26) were added to 1000 ml of deionized water (2.5% w/w) and left for 2 h at room temperature. This solution corresponds to the popular form of the use of *A. blazei* for beneficial health effects. This solution, referred to from here on as 'the crude aqueous extract', was then centrifuged ( $800 \times g$  for 10 min) and filtered (commercial non-sterile filter). The final solution was diluted 50%. The mean amount of solids in the solution (i.e. the mean dry weight of waterextractable material) was 5.6 mg/ml, with a medium yield of 22.4%. It was prepared daily and offered ad libitum to rats in aluminium-foil-wrapped bottles to avoid light decomposition. It was the sole source of drinking fluid, starting 2 weeks before DEN treatment.

# 2.3. Sampling of the blood and tissue, histology and immunohistochemical procedures

Immediately before killing, whole blood was collected from the abdominal aorta for alanine aminotransferase (ALT) determination, which was carried out spectrometrically with the use of a commercial kit (Boehinger Mannheim, Germany). After killing, samples of the left, median and right liver lobes were fixed in 10% phosphate-buffered formalin for hematoxylin-eosin (HE) staining and immunohistochemical demonstration of proliferating cellular nuclear antigen (PCNA) and glutathione S-transferase, placental form (GST-P) positive cells, using the avidin-biotin complex (ABC) method (Hsu et al., 1981).

Paraffin-embedded liver samples were cut into 5-µm thick sections, placed on poly/<sub>D</sub>/lysine-coated slides, deparaffinized in xylene and rehydrated with graded alcohol to water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 5 min. Non-specific protein binding was minimized by the use of 1% non-fat dried milk in PBS for 60 min at 4 °C. The slides were incubated overnight at 4 °C with rabbit anti-rat GST-P primary antibody (Medical and Biological Laboratories Co., Tokyo, Japan) diluted at 1:1000 in 1% bovine serum albumin (BSA, Sigma Chemical Co., MO, USA) or anti-mouse PCNA primary antibody (Dako A/S, Denmark) diluted at 1:200 in 1% BSA. Then the slides were successively incubated with a biotinylated goat anti-rabbit or horse anti-mouse IgG secondary antibody (Vector Laboratories Inc., CA, USA) diluted at 1:200 in 1% BSA for 60 min followed by an avidin-biotinhorseradish peroxidase complex kit (Vector Laboratories Inc., CA, USA) diluted at 1:100 in PBS for 45 min. Subsequent chromagen color development was made using 0.038% 3-3'-diaminobenzidine tetrahydrocloride (DAB, Sigma Chemical Co.) and 0.025% hydrogen peroxide in 0.1 M Tris-HCl, pH 7.4 for 4 min. The sections were counterstained with Harris's hematoxylin, dehydrated, cleared in xylene and coverslipped with a xylene-based mounting medium.

# 2.4. Quantitative data on necrosis, hepatocyte proliferation, and GST-P expression

Histological liver sections stained by HE were examined for the extension of necrosis induced by the different doses of DEN. The intensity of liver necrosis was evaluated as 0 (absent), 1 (light, limited to zone 3 of the liver acinus, with a few necrotic hepatocytes near the central vein with or without inflammation), 2 (mild, limited to zone 3 of the liver acinus, with necrotic hepatocytes around the central vein as well as inflammation), and 3 (extensive, reaching zone 2 of the liver acinus, numerous necrotic hepatocytes and conspicuous inflammation).

Detection of proliferating cellular nuclear antigen (PCNA) (Foley et al., 1993) was estimated in order to evaluate the liver compensatory cell proliferation induced by the different doses of DEN. PCNA labeling index were determined by dividing the number of the PCNA (G1+S+G2+M phases) labeled hepatocytes by the total number of scored hepatocytes ( $\sim 20.000$  hepatocytes) under a 40  $\times$  objective in 160 semisuccessive random microscopic fields of predetermined size taken along the left, median, and right liver-lobe sections.

The total number of GST-P positive hepatocytes in the median hepatic lobe, isolated or grouped in minifoci, was calculated and expressed as the number per liver section  $(cm^2)$ . The areas of the liver sections were measured with the aid of a KS-300 image analysis system (Kontron Elektronik, Germany).

#### 2.5. Statistical analysis

Comparisons among groups regarding the body and liver weights were carried out by analysis of variance (ANOVA) and Student's *t*-tests. The ALT levels, PCNA labeling index, and the number of GST-P positive hepatocytes were analyzed by the Manny–Whitney and Kruskal–Wallis tests (Zar, 1984). The contrast between groups was analyzed by the Tukey test. A significant difference between the groups was assumed when P < 0.05.

### 3. Results

# 3.1. Consumption of food and liquid and body and liver weights

At the end of the second week, the average ingestion of the aqueous extract of *A. blazei* was significantly increased in relation to water ingestion (Table 1, P <0.001). Food consumption and mean body-weight gain of the different groups during *A. blazei* treatment were similar (Tables 1 and 2). The average solid ingestion of *A. blazei* (i.e. water-extractable material) is indicated in Table 1.

After DEN administration, final mean body weight as well as food and water consumption (data not shown) of the groups initiated with 100 and 200 mg/kg of DEN [groups G4 (DEN<sub>100</sub>), G5 (Ab + DEN<sub>100</sub>), G6 (DEN<sub>200</sub>), and G7 (Ab + DEN<sub>200</sub>)] were significantly reduced when compared with the non-initiated groups [Groups 1 (non-treated) and G8 (Ab-treated)] (Table 3, P < 0.0001). Significant decreases in the relative liver weights were found in rats which received the intermediate and high doses of DEN [groups G4 (DEN<sub>100</sub>), G5 (Ab + DEN<sub>100</sub>), G6 (DEN<sub>200</sub>), and G7 (Ab + DEN<sub>200</sub>)] (Table 3, P < 0.0001).

#### 3.2. Necrosis and ALT levels

After 48 h, DEN-induced necrosis was restricted to zones 3 and 2 of the liver acinus. In the animals treated

Food and liquid consumption and ingestion of solids during 2 weeks before DEN or 0.9% NaCl treatments							
Groups	Treatments <sup>a</sup>	Number of rats	Food consumption (g per rat per day)	Liquid consumption (g per rat per day)	Ingestion of solids (g/kg per day) <sup>b</sup>		
Non-initiate	ed						
G1	Non-treated	10	$24.93 \pm 0.11$	$36.77 \pm 2.32$	_		
G8	Ab	10	$25.50 \pm 0.49$	$44.18 \pm 1.94^{\circ}$	1.18 + 0.12		
Initiated							
G2	DEN <sub>50</sub>	10	$24.93 \pm 1.52$	$36.32 \pm 2.35$	_		
G3	$Ab + DEN_{50}$	10	$24.70 \pm 0.21$	$44.15 \pm 2.09^{d}$	$1.13 \pm 0.11$		
G4	DEN <sub>100</sub>	10	$24.60 \pm 0.42$	$38.85 \pm 2.55$	_		
G5	$Ab + DEN_{100}$	10	$24.20 \pm 0.78$	$43.32 \pm 2.43^{d}$	$1.14 \pm 0.12$		
G6	DEN <sub>200</sub>	10	$24.70 \pm 0.71$	$38.30 \pm 2.93$	_		
G7	$Ab + DEN_{200}$	10	$25.98 \pm 0.32$	$45.85 \pm 1.44^{\rm d}$	$1.15\pm0.08$		

Table 1 Food and liquid consumption and ingestion of solids during 2 weeks before DEN or 0.9% NaCl treatment:

Values are mean  $\pm$  S.D.

<sup>a</sup> DEN<sub>50,100,200</sub> = diethylnitrosamine 50, 100 and 200 mg/kg, respectively; Ab, Previous A. blazei treatment.

<sup>b</sup> Mean ingestion of water-extractable material based on mean dry weight of the aqueous solutions A. blazei.

<sup>c</sup> Significantly different from non-treated animals (group G1 vs. group G8 P < 0.001).

<sup>d</sup> Significantly different from the only DEN-initiated animals (groups G2, G4 and G6 vs. groups G3, G5 and G7, P < 0.001).

with the low and intermediate doses of DEN (50 and 100 mg/kg), light and moderate liver necrosis in zone 3 of the liver acinus was observed with or without associated inflammatory process. In the animals treated with the highest dose of DEN (200 mg/kg), extensive liver necrosis in zones 3 and 2 of the liver acinus was observed with associated inflammatory process (Fig. 1B). By semiquantitative analysis, the intensity of liver necrosis was DEN dose-dependent and apparently was not altered by prior treatment with the mushroom *A. blazei*.

ALT evaluation was adopted as a marker of liver injury. Fig. 2A shows the levels of serum enzyme activity 48 h after the administration of each dose of DEN or 0.9% NaCl. DEN-induced acute elevation in the ALT levels occurred in a dose-dependent manner and was consistent with the cytotoxicity observed histologically in the liver. However, ALT levels were lower in rats that had received previous *A. blazei* treatment and were exposed to 100 mg/kg of DEN than in rats only initiated with that dose of the carcinogen [groups G4 (DEN<sub>100</sub>) vs. G5 (Ab+DEN<sub>100</sub>)] (Fig. 2A, P < 0.0001). Also, serum ALT levels were lower in animals that received *A. blazei* treatment only when compared with animals of the non-treated group [groups G8 (Ab-treated) vs. G1 (non-treated)] (Fig. 2A, P < 0.0001).

# 3.3. PCNA labeling and induction of GST-P positive single cells and mini-foci in the rat liver

In DEN-initiated animals, the mean number of PCNA positive hepatocytes was significantly increased in relation to control and not directly dependent on the dose of DEN (Fig. 2B, Fig. 3). A linear increase from 50 to 100 mg/kg was observed, but no further increment was observed at the highest dose of 200 mg/kg of DEN (Fig. 2B). A lower PCNA labeling index was observed in rats that received *A. blazei* treatment previously to 100 mg/kg of DEN when compared with rats solely initiated

Table 2

Body weights and weight gain during 2 weeks before DEN or 0.9% NaCl treatments

Groups	Treatments <sup>a</sup>	Number of rats	Body weight (g)		
			Initial	Final week 2	Gain
Non-initiate	ed				
G1	Non-treated	10	$203.6 \pm 16.1$	$279.3 \pm 14.4$	$70.7 \pm 15.8$
G8	Ab	10	$184.5 \pm 19.7$	$262.5 \pm 20.4$	$78.0\pm6.6$
Initiated					
G2	DEN <sub>50</sub>	10	$193.1 \pm 19.1$	$264.5 \pm 25.8$	$71.4 \pm 11.2$
G3	Ab + DEN50	10	$203.0 \pm 28.8$	$265.5 \pm 15.7$	$72.3 \pm 6.4$
G4	DEN100	10	$197.6 \pm 16.7$	$269.7 \pm 16.9$	72.1+11.7
G5	$Ab + DEN_{100}$	10	$194.0 \pm 13.0$	267.7 + 15.8	$73.7 \pm 5.4$
G6	DEN <sub>200</sub>	10	$206.7 \pm 20.4$	$285.6 \pm 21.4$	$78.9 \pm 9.5$
G7	$Ab + DEN_{200}$	10	$198.1 \pm 19.2$	$271.6 \pm 25.2$	$73.5 \pm 9.4$

Values are mean  $\pm$  S.D.

<sup>a</sup> DEN<sub>50,100,200</sub> = diethylnitrosamine 50, 100 and 200 mg/kg, respectively; Ab, Previous A. blazei treatment.

Final body weight, relative need weight and number of OSTF-F positive nepatocytes per cm 48 in aner DEN of 0.976 NaCi treatments						
Groups	Treatments <sup>a</sup>	Number of rats	Final body weight (g)	Relative liver weight (%)	GST-P <sup>+</sup> hepatocytes (cm <sup>2</sup> )	
Non-initi	ated					
G1	Non-treated	10	$283.7 \pm 25.4$	$4.4 \pm 0.2$	$0.6 \pm 1.8$	
G8	Ab	10	$275.8 \pm 24.5$	$4.5 \pm 0.2$	$0.2 \pm 0.7$	
Initiated						
G2	DEN <sub>50</sub>	10	$269.4 \pm 23.7$	$4.2 \pm 0.4$	$12.5 \pm 6.9^{b}$	
G3	$Ab + DEN_{50}$	10	$266.4 \pm 14.4$	$4.1 \pm 0.3$	$8.60 \pm 5.5^{b}$	
G4	$DEN_{100}$	10	$252.6 \pm 18.3^{b}$	$3.7 \pm 0.4^{b,c}$	$20.7 \pm 9.9^{b,c}$	
G5	$Ab + DEN_{100}$	10	$254.1 \pm 15.6^{b}$	$3.6 \pm 0.2^{b,c}$	$10.2 \pm 7.3^{b,e}$	
G6	DEN <sub>200</sub>	10	$246.8 \pm 22.9^{b,c}$	$3.2 \pm 0.4^{b,c,d}$	$22.3 \pm 12.9^{b,c}$	
G7	$Ab + DEN_{200}$	10	$238.9 \pm 17.8^{b}$	$3.1 \pm 0.4^{b,c,d}$	$23.5 \pm 7.9^{b,c}$	

Table 3 Final body weight, relative liver weight and number of GSTP-P positive hepatocytes per cm<sup>2</sup> 48 h after DEN or 0.9% NaCl treatments

Values are mean  $\pm$  S.D.

<sup>a</sup> DEN<sub>50,100,200</sub> = diethylnitrosamine 50, 100 and 200 mg/kg, respectively; Ab, Previous A. blazei treatment.

<sup>b</sup> Significantly different from control animals (groups G1 and G8), P < 0.0001).

<sup>c</sup> Significantly different from groups G2 and G3, P < 0.0001.

<sup>d</sup> Significantly different from groups G4 and G5, P < 0.0001.

<sup>e</sup> Significantly different from groups G4, P < 0.001.



Fig. 1. Liver sections stained with HE. (A) Normal liver from a control rat. (B) Severe necrosis and inflammatory reaction around the centrilobular vein in the liver 48 h after DEN administration (200 mg/kg). CR, Centrilobular region; PR, Periportal region; Bar, 100  $\mu$ m.

with this dose of carcinogen [groups G4 (DEN<sub>100</sub>) vs. G5 (Ab+DEN<sub>100</sub>)] (Fig. 2B, P < 0.0001]. Although not significantly different, a lower mean value of PCNA labeling index was observed in rats that received A. *blazei* treatment previously to 50 mg/kg of DEN, when



Fig. 2. (A) ALT serum levels 48 h after DEN or NaCl (vehicle) administration in the different experimental groups. (B) PCNA labeling index 48 h after DEN or NaCl (vehicle) administration in the different experimental groups. Different letters indicate significant differences among the groups at P < 0.0001. Values are mean  $\pm$ S.D.  $\Box$ , Non-treated group;  $\blacksquare$ , Previous *A. blazei* treated group;  $\blacksquare$ , DEN-initiated groups;  $\blacksquare$ , Previous *A. blazei* treatment and DEN-initiated groups.

compared with rats solely initiated with this dose of carcinogen [groups G3  $(Ab+DEN_{50})$  vs. G2  $(DEN_{50})$ ] (Fig. 2B).

As shown in Table 3, the induction of immunohistochemically detectable GST-P positive single cells/mini-



Fig. 3. Immunohistochemical demonstration of PCNA and GST-P expression in the rat liver, identified 48 h after DEN administration (200 mg/kg): (A) PCNA positive hepatocytes (dark nuclei). (B) Single GST-P positive hepatocytet. (C) Minifocus of GST-P positive hepatocytes. Bar =  $20 \mu m$ .

foci (Fig. 3B and C) 48 h after carcinogen exposure was not directly dependent on the dose of DEN. In DENinitiated animals, the mean number of GST-P positive hepatocytes was significantly increased in relation to those in non-initiated groups (Table 3, P < 0.0001). In the two control groups [group G1 (non-treated) and G8 (*Ab*-treated)], only one animal in each group presented GST-P positive hepatocytes, while all DEN-initiated animals presented altered hepatocytes. Furthermore, GSTP-positive mini-foci (two to five cells) were rarely observed 48 h after DEN administration (Fig. 3C). Only one animal of group 6 (200 mg/kg of DEN) presented a larger foci (>10 cells).

The induction of GST-P positive hepatocytes 48 h after carcinogen exposure in rats that had received previous *A. blazei* treatment and exposed to 100 mg/ kg of DEN was significantly lower than in rats solely initiated with this dose of carcinogen [groups G4 (DEN<sub>100</sub>) vs. G5 (Ab+DEN<sub>100</sub>)] (Table 3, P < 0.001). Although not significantly different, a lower mean value of induction of GST-P positive hepatocytes was observed in rats that had received *A. blazei* treatment previously to 50 mg/kg of DEN, when compared with rats solely initiated with this dose of carcinogen [groups G3 (Ab+DEN<sub>50</sub>) vs. G2 (DEN<sub>50</sub>)] (Table 3).

### 4. Discussion

Natural products have been traditionally accepted as remedies due to popular belief that they present minor adverse effects. Therefore, understanding the potential beneficial or adverse influence of natural products used by human populations is important in implementing safety measures for public health. In the present study, no adverse effects of the aqueous extract of the mushroom *A. blazei* treatment were observed, indicating that this treatment was not toxic to the animals used. The increased ingestion of the aqueous extract of *A. blazei* was not associated with changes in animal diuresis.

Lower increases of serum ALT levels and PCNA labeling index were more evident in rats that had received previous A. blazei treatment and were exposed to 100 mg/kg of DEN, but not in those exposed to 50 (subnecrogenic dose) or 200 mg/kg (frank necrogenic dose) of that hepatocarcinogen, indicating that previous A. blazei treatment may exert protection against a moderate, but not severe, chemical liver toxicity. Liver necrosis induced by the single doses of DEN occurred in a dose-dependent manner and corresponded to changes in serum ALT levels but not in compensatory hepatocyte proliferation data. The reduced PCNA labeling index observed in animals treated with 200 mg/kg of DEN may be attributed to the toxic environment induced by this dose of carcinogen, resulting in delayed entrance into critical phases of the cell cycle such as early S or M in presence of DNA lesions (Kaufmann et al., 1991; Dragan et al., 1994a), neutralizing any strategy to evaluate the probable chemopreventive action of the A. blazei treatment at this dose. In contrast to high toxicity induced by 200 mg/kg of DEN, the dose of 50 mg/kg of DEN exerted a weak liver toxicity as shown by histological analysis, ALT levels, and relative liver weight data. Similarly, no clear hepatoprotective effect from the previous A. blazei treatment was observed. Therefore, our findings indicate that elaboration of a DEN-induced hepatotoxicity model with a dose of 100

mg/kg of DEN is a better approach for screening hepatoprotective drugs.

In the present investigation, previous treatment with *A. blazei* at 100 mg/kg of DEN protected the induction of GST-P positive hepatocytes. This may indicate a hepatoprotective influence on the initiation step of liver carcinogenesis. In fact, GST-P expression has been used as a marker enzyme for preneoplastic lesions in rat hepatocarcinogenesis; single cells and minifoci positive for GST-P develop very early in carcinogen-treated rats and are considered precursors of GST-P positive liver foci, nodules and tumors (Sato, 1988; Moore et al., 1987; Satoh et al., 1989; Dragan et al., 1994b).

Both our findings and published data by our group demonstrate that aqueous extract of *A. blazei* provides significant protection against the mutagenicity induced by cyclophosphamide (CPA) and methyl methanesulfonate (MMS) in vivo and in vitro, respectively, (Delmanto et al., 2001; Menoli et al., 2001). Herein, we suggest that aqueous extract of *A. blazei* could provide significant protection against the in vivo mutagenicity and carcinogenicity induced by DEN, since reparative hepatocyte proliferation and DNA damage are required for mutagenesis and carcinogenesis processes (Kato et al., 1993; Verna et al., 1996).

The hepatoprotective effect of A. blazei aqueous extract could be due to a modifying influence on DEN metabolism, thus, reducing its liver toxicity, mutagenicity, and carcinogenicity. Changes in phase I or II enzyme can result in either bioactivation or detoxification, depending on the toxicity of the metabolites (Kedderis, 1996). It has been reported that after biotransformation, DEN produces the promutagenic adducts O<sup>6</sup>-ethyldeoxyguanosine and O<sup>4</sup> and O<sup>6</sup>-ethyldeoxythymidine that play a role in the initiation of liver carcinogenesis (Swenberg et al., 1991; Dragan et al., 1994a; Verna et al., 1996). Therefore, DNA adducts would have been formed in hepatocytes after DEN administration and enhanced post-necrotic compensatory cell replication, which is necessary for the conversion of DNA adducts to mutation in daughter cells, and could have contributed to the appearance of preneoplastic hepatocytes observed after DEN exposure.

The improvement in liver toxicity by crude extract of *A. blazei* may be due to the presence of some active principles which will influence DEN metabolism and consequent formation of DNA adducts and preneoplastic hepatocytes. In fact, crude extracts and polysaccharides isolated from mushrooms have shown protective activity against chemical liver injury. In this way, Ooi (1996) registered significant protection by crude extracts of *Lentinula edodes* (Tricholomataceae), *Tricholoma lobayence* (Tricholomataceae) and *Grifola frondosa* (Polyporaceae) against paracetamol-induced hepatotoxicity. Using this same experimental model, Yeung et al. (1994, 1995) proved that an isolated polysacchar-

ide peptide (COV-1) of *Coriolus versicolor* (Polyporaceae) increased the conjugation and excretion of reactive paracetamol metabolites and prevented the fall of the reduced glutathione (GSH) in the liver. Our findings suggest that the treatment with aqueous extract of *A*. *blazei* Murrill exerts a hepatoprotective effect on liver toxicity and on the initiation of hepatocarcinogenesis in an environment of moderate toxicity.

### Acknowledgements

We gratefully acknowledge the technical support provided by Paulo Roberto Cardoso and Mara Luíza Falagueira Ardanaz. The study was supported by Fundação de Amparo à Pesquisa do Estado de Sao Paulo (FAPESP, grant Number 98/07726-5) and Fundação para o Desenvolvimento da UNESP (FUNDU-NESP, grant Number 119/99-DFP). Scolastici C. (PBIC/UNESP) Salvadori D.M.F, Ribeiro L.R, and de Camargo, J.L.V. were recipients of fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

#### References

- Chang, R., 1996. Functional properties of edible mushrooms. Nutrition Reviews 54, S91–S93.
- Delmanto, R.D., de Lima, P.L.A., Sugui, M.M., da Eira, A.F., Salvadori, D.M.F., Speit, G., Ribeiro, L.R., 2001. Antimutagenic effects of *Agaricus blazei* Murill mushroom on the genotoxicity induced by cyclophosphamide. Mutation Research 496, 15–21.
- Dragan, Y.D., Hully, J.R., Nakamura, J., Mass, M.J., Swenberg, J.A., Pitot, H.C., 1994a. Biochemical events during initiation of rat hepatocarcinogenesis. Carcinogenesis 15, 1451–1458.
- Dragan, Y.D., Campbell, H.A., Baker, K., Vaughan, J., Mass, M., Pitot, H.C., 1994b. Focal and non-focal hepatic expression of placental glutathione S-transferase in carcinogen-treated rats. Carcinogenesis 15, 2587–2591.
- Fujimiya, Y., Suzuki, Y., Oshiman, K., Kobori, H., Moriguchi, K., Nakashima, H., Matumoto, Y., Takahara, S., Ebina, T., Katakura, R., 1998. Selective rumoricidal effect of soluble proteoglucan extracted from the basidiomycete, *Agaricus blazei* Murill, mediated via natural killer cell activation and apoptosis. Cancer Immunology and Immunotherapy 46, 147–159.
- Fujimiya, Y., Suzuki, Y., Takakura, R., Ebina, T., 1999. Tumorspecific cytocidal and immunopotentiating effects of relatively low molecular weight products derived from the basidiomycetes, *Agaricus blazei* Murril. Anticancer Research 19, 113–118.
- Foley, J., Ton, T., Maronpot, R., Butterworth, B., Goldsworthy, T.L., 1993. Comparison of proliferating cell nuclear antigen to tritiated thymidine as a marker of proliferating hepatocytes in rats. Environmental Health Perspectives 101 (S5), 199–206.
- Hsu, S.M., Raine, L., Fanger, N., 1981. Use of avidin-biotinperoxidase complex (ABC) and unlabeled antibody (PAP) procedures. Journal of Histochemistry and Cytochemistry 29, 557–580.
- Ito, H., Shimura, K., Itoh, H., Kawade, M., 1997. Antitumor effects of a new polysaccharide-protein complex (ATOM) prepared from *Agaricus blazei* (Iwade Strain 101) 'Himematsutake' and its

mechanisms in tumor-bearing mice. Anticancer Research 17, 277-284.

- Itoh, H., Ito, H., Amano, H., Noda, H., 1994. Inhibitory action of a  $(1 \rightarrow 6)$ - $\beta$ -D-glucan-protein complex (FIII-2-b) isolated from *Agaricus blazei* Murill ('Himematsutake') on Meth A fibrosarcomabearing mice and its antitumor mechanism. Japanese Journal of Pharmacology 66, 265–271.
- Kato, M., Popp, J.A., Conolly, R.B., Cattley, R.C., 1993. Relationship between hepatocyte necrosis, proliferation, and initiation induced by diethylnitrosamine in the male F344 rat. Fundamental and Applied Toxicology 20, 155–162.
- Kaufmann, W.K., Rice, J.M., Mackenzie, S.A., Smith, G.J., Wenk, M.L., Devor, D., Qaqish, B.F., Kaufman, D.G., 1991. Proliferation of carcinogen-damaged hepatocytes during cell-cycle-dependent initiation of hepatocarcinogenesis in the rat. Carcinogenesis 12, 1587–1593.
- Kawagishi, H., Inagaki, R., Kanao, T., Mizuno, T., Shimura, K., Itos, H., Hagiwara, T., Nakamura, T., 1989. Fractionation an antitumor activity of the water-insoluble residue of *Agaricus blazei* fruiting bodies. Carbohydrate Research 186, 267–273.
- Kedderis, G.L., 1996. Biochemical basis of hepatocellular injury. Toxicology Pathology 24, 77–83.
- Larrey, D., Pageaux, G.P., 1995. Hepatotoxicity of herbal remedies and mushrooms. Seminars in Liver Disease 15, 183–188.
- McPartland, J.M., Vilgalys, R.J., Cubeta, M.A., 1997. Mushroom poisoning. American Family Physician 55, 1797–1809.
- Menoli, R.C.R., Mantovani, M.S., Ribeiro, L.R., Speit, G., Jordão, B.Q., 2001. Antimutagenic effects of the mushroom *Agaricus blazei* Murrill extracts on V79 cells. Mutation Research 496, 5–13.
- Mizuno, T., Saito, H., Nishitoba, T., Kawagishi, H., 1995. Antitumoractive substances from mushrooms. Food Reviews International 11, 23–61.
- Mizuno, M., Morimoto, M., Minato, K., Tsuchida, H., 1998. Polysaccharides from *Agaricus blazei* stimulate lymphocyte T-cell subsets in mice. Biosciences Biotechnology and Biochemistry 63, 434–437.
- Moore, M.A., Nakagawa, K., Satoh, K., Ishikawa, T., Sato, K., 1987. Single GST-P positive liver cells—putative initiated hepatocytes. Carcinogenesis 8, 483–486.
- Ooi, V.E.C., 1996. Hepatoptotective effect of some edible mushrooms. Phytotherapy Research 10, 536–538.

- Ooi, V.E.C., Liu, F., 1999. A review of pharmacological activities of mushroom polysaccharides. International Journal of Medicinal Mushrooms 1, 195–206.
- Peto, R., Gray, R., Brantom, P., Grasso, P., 1991. Dose and time relationship for tumor induction in liver and esophagus of 4080 inbred rat by chronic ingestion of *N*-nitrosodiethylamine or *N*nitrosodimethylamine. Cancer Research 51, 6415–6451.
- Sato, K., 1988. Glutathione S-transferase and hepatocarcinogenesis. Japanese Journal of Cancer Research 79, 556–572.
- Satoh, K., Hatayama, L., Tateoka, N., Tamai, K., Shimizu, T., Tatematsu, M., Ito, N., Sato, K., 1989. Transient induction of single GST-P positive hepatocytes by DEN. Carcinogenesis 10, 2107–2111.
- Swenberg, J.A., Hoel, D.G., Magee, P.N., 1991. Mechanistic and statistical insight to the large carcinogenesis bioassays on *N*nitrosodiethylamine and *N*-nitrosodimethylamine. Cancer Research 51, 6409–6414.
- Takaku, T., Kimura, Y., Okuda, H., 2001. Isolation of an antitumor compound from *Agaricus blazei* Murill and its mechanism of action. Journal of Nutrition 131, 1409–1413.
- Toth, B., 1995. Mushroom toxins and cancer. International Journal of Oncology 6, 137–145(Review).
- Verna, L., Whysner, J., Williams, G.M., 1996. N-Nitrodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor initiation. Pharmacology and Therapy 71, 57–81.
- Wasser, S.P., Weis, A.L., 1999. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. Critical Reviews in Immunology 19, 65–96.
- Yeung, J.H.K., Chiu, L.C.M., Ooi, V.E.C., 1994. Effects of polysaccharide peptide (PSP) on glutathione and protection against paracetamol-induced hepatotoxicity in the rat. Methods and Findings in Experimental and Clinical Pharmacology 16, 723–729.
- Yeung, J.H.K., Chiu, L.C.M., Ooi, V.E.C., 1995. Effects of polysaccharide peptide (PSP) on in vivo sulphation and glucuronidation of paracetamol in the rat. European Journal of Drug Metabolism and Pharmacokinetics 20, 287–292.
- Zar, J.H., 1984. Biostatististal analysis, second ed.. Pretince Hall, Englewwod Cliffs, p. 718.