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Effects of crude extracts of *Agaricus blazei* on DNA damage and on rat liver carcinogenesis induced by diethylnitrosamine

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ABSTRACT. The effects of crude extracts of the mushroom *Agaricus blazei* Murrill (Agaricaceae) on both DNA damage and placental form glutathione S-transferase (GST-P)-positive liver foci induced by diethylnitrosamine (DEN) were investigated. Six groups of adult male Wistar rats were used. For two weeks, animals of groups 3 to 6 were treated with three aqueous solutions of *A. blazei* (mean dry weight of solids being 1.2, 5.6, 11.5 and 11.5 mg/ml, respectively). After this period, groups 2 to 5 were given a single *ip* injection 200 mg/kg DEN and groups 1 and 6 were treated with 0.9% NaCl. All animals were subjected to 70% partial hepatectomy at week five and sacrificed 4, 24 and 48 h or 8 weeks after DEN or 0.9% NaCl treatments (10th week after the beginning of the experiment). The alkaline comet assay and GST-P-positive liver foci development were used to evaluate the influence of the mushroom extracts on liver cell DNA damage and on the initiation of

liver carcinogenesis, respectively. Previous treatment with the highest concentration of *A. blazei* (11.5 mg/ml) significantly reduced DNA damage, indicating a protective effect against DEN-induced liver cytotoxicity/genotoxicity. However, the same dose of mushroom extract significantly increased the number of GST-P-positive liver foci.

Key words: *Agaricus blazei*, DNA damage, GST-P-positive liver foci, Hepatocarcinogenesis

INTRODUCTION

Among the mushroom species of higher *Basidiomicetes*, *Agaricus blazei* Murrill, a species native to Brazil, where it is popularly known as "sun mushroom", has recently received attention in folk medicine due to its use in the treatment of ailments. Since 1965, strains have been exported from Brazil to Japan, where this mushroom has become popularly known as "Himematsutake" or "Agarikusutake". This edible mushroom is often consumed as food and tea in different parts of the world, especially because of its reported medicinal properties.

In Brazil, infusion of the dried fruiting bodies of the mushroom *A. blazei* has been popularly consumed both as a stimulant and for auxiliary treatment of various diseases, including cancer. Nevertheless, no epidemiological and few *in vivo* experimental data exist on the beneficial effects of the crude aqueous extract of this mushroom. Recently, we demonstrated that this extract provides significant protection against mutagenicity induced by cyclophosphamide and methyl methanesulfonate, both *in vivo* and *in vitro* (Delmanto et al., 2001; Menoli et al., 2001; Martins de Oliveira et al., 2002). Various polysaccharides and protein-bound polysaccharides isolated from mycelia and fruiting bodies of *A. blazei* have shown anti-tumor activity in tumor-bearing mice by host immune response activation (Kawagishi et al., 1989; Itoh et al., 1994, 1997; Fujimiya et al., 1998; Mizuno et al., 1998).

Dielthylnitrosamine (DEN) is a potent genotoxic carcinogen that has been used as initiating agent in some two-stage (initiation-promotion) alternative protocols for hepatocarcinogenesis (Ito et al., 1988; Dragan et al., 1991). It has been reported that after its metabolic biotransformation, DEN produces the promutagenic adducts O^6-ethyldeoxyguanosine and O^4- and O^6-ethyldeoxythymidine that may initiate liver carcinogenesis (Dragan et al., 1994; Verna et al., 1996). Consequently, the analysis of DNA damage may be relevant to evaluate the modifying influences of chemopreventive agents on the initiation stage of cancer (Moore et al., 1999).

The comet assay or single cell gel electrophoresis assay is a rapid and sensitive procedure for quantifying DNA lesions in individualized cells, both *in vitro* and *in vivo* (Tice et al., 1991; Fairbairn et al., 1995; Gontijo et al., 2001). The alkaline comet assay version was specially developed for detection of the DNA single-strand breaks and alkali-labile sites (Singh et al., 1988) and is also indicated to evaluate *in vivo* genotoxicity induced by carcinogen exposure (Anderson et al., 1998; Tsuda et al., 2000).

The DEN-partial hepatectomy (PH) model has proven to be a consistent bioassay for the detection of chemical hepatocarcinogens and for the assessment of the beneficial potential of chemopreventive agents (Ito et al., 1988, 1996; Moore et al., 1999). This 8-week-long medium-term rat liver assay uses as endpoint marker the development of putative preneoplastic foci

of altered hepatocytes that express the placental form of the enzyme glutathione S-transferase (GST-P) (Ito et al., 1988). It has been indicated as a practical approach for the assessment of the potential hazard or benefit of chemicals, when associated with other surrogate end-points (Moore et al., 1999).

The standard DEN-PH assay protocol uses 200 mg/kg DEN to establish the initiation of the hepatocarcinogenesis process (Ito et al., 1988). In order to determine the protective influence of the aqueous solutions of the mushroom *A. blazei* against liver damage and preneoplasia development induced by the DEN dose level used in the DEN-PH assay, we evaluated DNA damage and foci of altered hepatocyte development by the comet assay and by the immunohistochemical expression of the placental enzyme, glutathione S-transferase, respectively.

MATERIAL AND METHODS

Animals and treatment

Male 4-week-old 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 ± 2°C, 55 ± 10% humidity, and with a 12-h light-dark cycle. They were fed with commercial Purina chow (Labina, Paulínia, SP, Brazil) and water *ad libitum* during a 2-week acclimation period.

The animals were randomly allocated into six groups (Figure 1). For two weeks, animals of groups 3 to 6 were treated with aqueous solutions of the *A. blazei*, with a mean dry

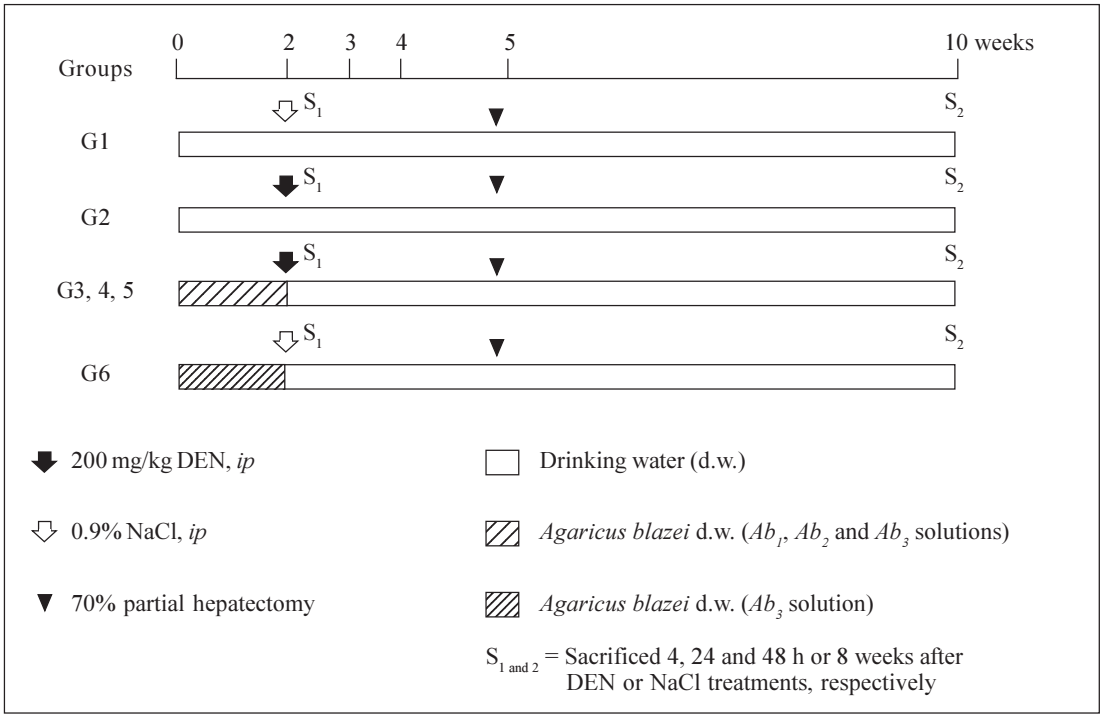

Figure 1. Experimental protocol.

weight of water-extractable solids of 1.2, 5.6, 11.5 and 11.5 mg/ml, respectively. After this period, groups 2 to 5 were given a single *ip* injection of 200 mg/kg DEN (Sigma, St. Louis, MO, USA), and groups 1 and 6 were treated with 0.9% NaCl only (DEN vehicle). All animals were subjected to 70% partial hepatectomy at week five and sacrificed 4, 24 and 48 h or 8 weeks after DEN or 0.9% NaCl treatments (10th week after the beginning of the experiment). The University Ethics Committee for Animal Research approved the protocols used in this study (Protocol No. 99/22).

Preparation and administration of aqueous solutions of *Agaricus blazei*

A sample of *A. blazei* Murrill (lineage 99/26) 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* mushroom was added to 1000 ml of deionized water (2.5% w/w) and left for 2 h at room temperature. This preparation corresponds to the popular form of use of *A. blazei* for beneficial health effects. This solution, referred from here on as "the crude aqueous extract", was then centrifuged (800 *g* for 10 min) and filtered (commercial non-sterile filter). The final solution was provided as 10% (Ab_1), 50% (Ab_2) and 100% (Ab_3) of the full 2.5 (w/w) aqueous extract of *A. blazei*. The mean amount of water-extractable solids in Ab_1, Ab_2, and Ab_3 solutions was 1.2, 5.6, and 11.5 mg/ml, respectively. The yields were 4.8, 22.4 and 46%, respectively. The solutions were prepared daily and offered to rats *ad libitum*, in aluminum foil-wrapped bottles to avoid light decomposition. They were the sole source of drinking fluid, starting two weeks before DEN initiation.

Comet assay

Four hours after DEN or NaCl treatments, five animals of each experimental group were sacrificed and a small piece of the left hepatic lobe was collected and placed onto a small Petri disc with ice-cold mincing solution (Ca^{2+}- and Mg^{2+}-free HBSS containing 20 mM EDTA and 10% DMSO). The liver samples were cut into smaller pieces, using a disposable microtome razor blade, and the solution was aspirated. Then, a fresh mincing solution was added and the liver samples were minced again to finer pieces. Resulting cell suspensions were collected and filtered (100 µm nylon mesh). All samples were stocked on ice in appropriate conditions to avoid light until the comet assay procedures. The quantity of liver cells in the cell suspensions was determined in Giemsa-stained smears.

The viability of the liver cells was indirectly determined by analyzing the comet images after electrophoresis (Vaghef and Hellman, 1998) and by spontaneous DNA diffusion without DNA denaturation and electrophoresis (Vasques and Tice, 1997). The comet image was considered to be from a nonviable cell when it presented a "cloudy" appearance or a very small head and a tail like a balloon (necrotic or apoptotic cells). The viability of the cell suspension was considered acceptable when the frequency of such images was less than 2% (Vaghef and Hellman, 1998). Higher values were considered as indicative of cytotoxicity due to the carcinogen treatment. The extent of spontaneous DNA diffusion was evaluated by measuring the diameter of the liver cell "nucleoids", considering that cells containing extremely low molecular weight DNA associated with apoptosis or necrosis develop spontaneous DNA diffusion into the agarose gel and consequently have larger diameters (Vasques and Tice, 1997).

The comet assay was performed under alkaline conditions according to a previously described standard protocol (Speit and Hartmann, 1999). Briefly, an aliquot of 5 µl of each prepared hepatic cell suspension was mixed with 120 µl of 0.5% low melting point agarose at 37°C and layered onto conventional microscope slides, precoated with 1.5% normal melting point agarose. The slides were placed overnight in freshly prepared cold lysing solution (1% Triton X-100, 2.5 mM NaCl, 0.1 mM Na_2EDTA, 10 mM Tris with 10% DMSO, pH 10.0) and then in a horizontal electrophoresis cube with alkaline electrophoresis solution (0.3 M NaOH, 1 mM Na_2EDTA, pH >13) at 4°C for 20 min. The electrophoresis was performed at 25 V and 300 mA for 20 min. After electrophoresis, the slides were washed twice for 5 min in neutralizing buffer (0.4 M Tris-HCl, pH 7.5), fixed for 5 min in absolute alcohol, air-dried, and stored at room temperature. In order to evaluate extremely low molecular weight DNA diffusion, two slides from each animal were removed after lysis procedure, rinsed with neutralizing solution, fixed and air-dried, and stored until analysis.

Immediately before analysis, the DNA was stained with 50 µl of 20 µg/ml ethidium bromide. The slides were examined with a 40X objective lens with epi-illuminated fluorescence microscopy (Olympus-Bx60, excitation filter: 515-560 nm; barrier filter: 590 nm) attached to a color CCD video camera and connected to an image analysis system (Comet II, Perspective Instruments, UK). Coded slides were scored blindly and 100 hepatic cell images were randomly analyzed for each animal (50 images per slide).

The comets were analyzed by a visual scoring method and computerized image analysis. The comets were classified into five categories, defined as types 0, 1, 2, 3 and 4 - where 0 indicates no or very low damage, 1, 2 and 3 indicate low, medium and long DNA migration, respectively, and 4 indicates apoptotic or necrotic DNA migration (Speit and Hartmann, 1999). The metrics for comet analysis included spontaneous DNA migration (measure of diameters larger than 34 microns of the "nucleoids", expressed in microns), tail migration (distance from the end of the head to the end of the tail, expressed in microns), and tail moment (product of DNA density in the tail and the mean distance of DNA migration in the tail, expressed in arbitrary units).

Histology and GST-P-positive foci analysis

After sacrifice at the end of the 10th week, samples of each of the liver lobes were weighed and fixed in 10% phosphate-buffered formalin for hematoxylin-eosin (HE) staining and immunohistochemical demonstration of GST-P-positive foci, using the avidin-biotin complex method (Hsu et al., 1981).

Paraffin-embedded liver samples were cut into 5-µm thick sections, placed on poly/D/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. Nonspecific protein binding was minimized by the use of 1% nonfat dried milk in PBS for 60 min at 4°C. Slides were incubated 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) overnight at 4°C. Then, the slides were successively incubated with a biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories Inc., CA, USA) diluted at 1:200 in 1% BSA for 60 min, followed by an avidin-biotin-horseradish peroxidase complex kit (Vector Laboratories Inc.) diluted at 1:100 in PBS for 45 min. Subse-

quent chromagen color development was made using 0.038% 3-3'-diaminobenzidine tetrahydrochloride (Sigma) 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 a cover slip fixed with a xylene-based mounting medium.

GST-P-positive foci larger than 0.15 mm^2 in diameter were measured using a Nikon photomicroscope (Microphot-FXA) connected to a KS-300 apparatus (Kontron Elektronic, Germany). Data are reported as number and area (mm^2) of preneoplastic GST-P-positive foci per liver section (cm^2).

Statistical analysis

Comparisons of body and liver weights among groups were carried out using analysis of variance and Student *t*-test. The DNA migration data and the number and area of the GST-P-positive foci were analyzed by Mann-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 $P < 0.05$.

RESULTS

Food and liquid consumption and body and liver weights

The two-week A. blazei treatment had no influence on the mean body weight gain (Table 1), on food consumption (~25 g/rat/day for all the experimental groups) or on liver morphology, evaluated by HE (data not shown). Significantly increased ingestion of the aqueous extract of *A. blazei* was observed for 50 and 100% of the full aqueous extract (2.5 w/w) of *A. blazei* (Ab_2 and Ab_3 solutions) (group G1 *vs* group G6, $P < 0.001$; group G2 *vs* groups G4 and G5, $P < 0.001$) (Table 1). The average ingestion of *A. blazei* (i.e., water-extractable material) calculated from the mean ingestion of the aqueous solutions of *A. blazei* was approximately 0.18, 0.90 and 1.97 g of solids/kg/day (Table 1).

No differences were found in the mean body and liver weights between non-initiated (groups G1 and G2) and DEN-initiated animals (group G2 to group G5) at the end of the experimental period (data not shown).

DNA damage evaluation

In untreated animals (group G1), there was a low frequency of necrotic hepatocytes, indicated by type 4 comet images (Figure 2). DEN treatment induced a higher frequency of type 4 comet images (necrosis/apoptosis process) and increased DNA migration in the alkaline comet assay, when compared to the non-initiated animals (group G1 v*s* group G2) (Figures 2 and 3). Necrosis/apoptosis of liver cells in DEN-treated animals was also indicated by increased spontaneous DNA diffusion (Table 2) and by extensive centrilobular liver necrosis observed 48 h after DEN treatment (Figure 4).

Agaricus blazei treatment alone did not change the basal level of DNA damage in liver cells, evaluated by DNA migration in the comet assay (Table 2 and Figure 2). Significantly smaller values of the DNA migration parameters (tail migration and tail moment) and of sponta-

Table 1. Mean body weight gain, liquid consumption and ingestion of *Agaricus blazei* solids for two weeks before DEN or 0.9% NaCl treatments[1].

Groups/Treatment[2]		Effective number of rats	Body weight gain (g)	Liquid consumption (g/rat/day)	Ingestion of solids[3] (g/kg/day)
Non-initiated					
G1	Control	15	72.5 ± 6.0	34.3 ± 2.7	-
G6	Ab_3	15	73.4 ± 5.0	39.3 ± 2.9*	2.03 ± 0.16
Initiated					
G2	DEN	20	76.5 ± 6.2	34.1 ± 3.9	-
G3	Ab_1 + DEN	20	79.1 ± 9.0	34.6 ± 2.2	0.18 ± 0.02
G4	Ab_2 + DEN	20	71.1 ± 5.5	38.0 ± 3.2**	0.90 ± 0.12
G5	Ab_3 + DEN	20	72.0 ± 9.5	38.3 ± 3.2**	1.94 ± 0.21

[1]Values are reported as mean ± SD; [2]DEN (diethylnitrosamine, 200 mg/kg, *ip*) and Ab_1, Ab_2, Ab_3 = aqueous extract of *A. blazei* with mean dry weight of water-extractable solids of 1.2, 5.6 and 11.5 mg/ml, respectively; [3]Mean ingestion of water-extractable material based on mean dry weight of the aqueous extract of *A. blazei*. *Significantly different from control animals (group G6 *vs* group G1, $P < 0.001$); **Significantly different from DEN-only initiated animals (groups G4 and G5 *vs* group G2, $P < 0.001$).

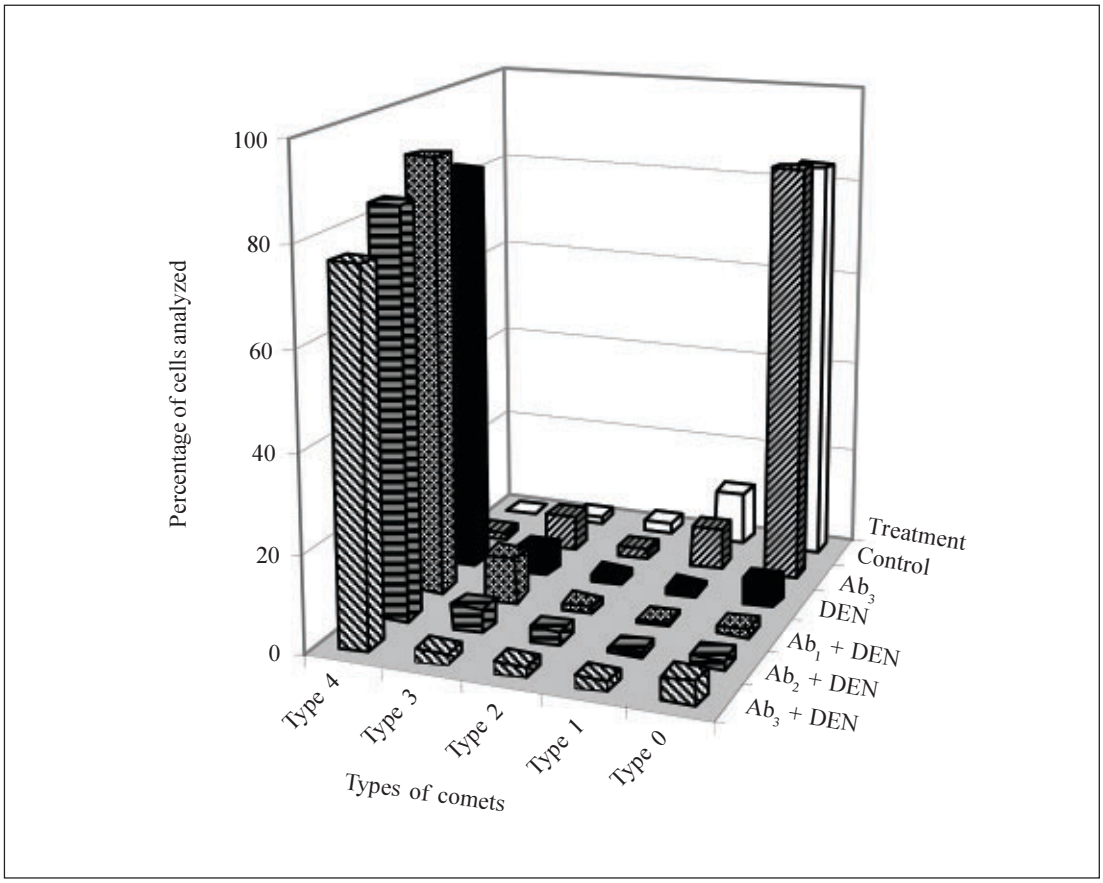

Figure 2. Frequency of comets 4 h after DEN or 0.9% NaCl treatments. DEN = diethylnitrosamine (200 mg/kg); Ab_1, Ab_2, and Ab_3 = aqueous extract of the mushroom *Agaricus blazei* with mean dry weight of water-extractable solids of 1.2, 5.6 and 11.5 mg/ml, respectively.

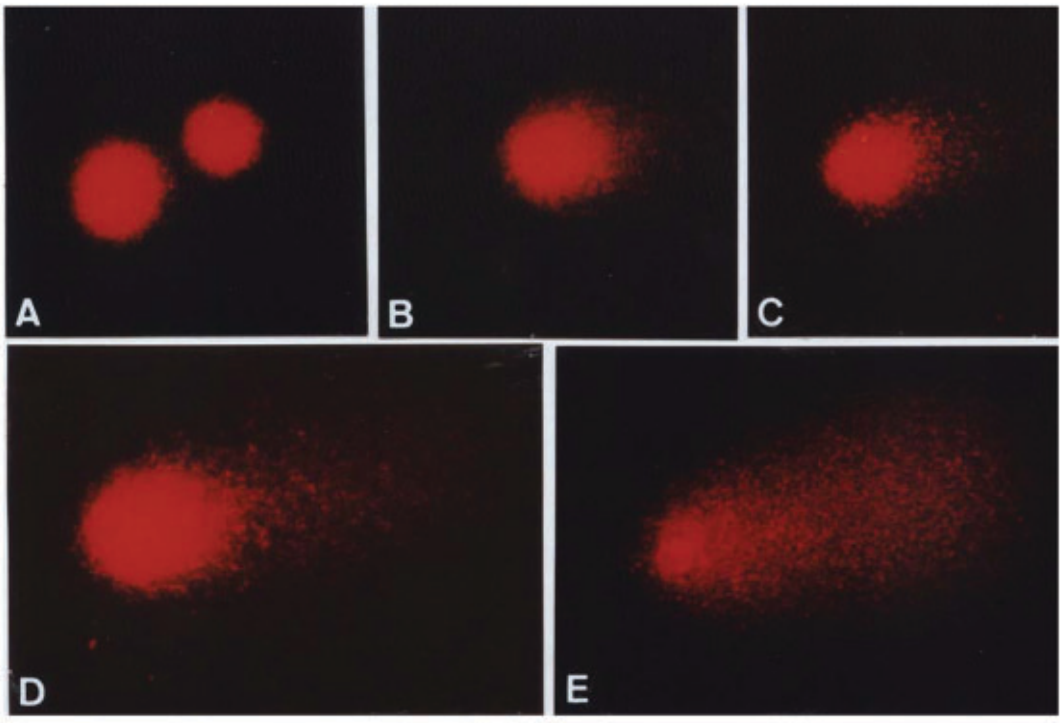

Figure 3. Comet images of liver cells 4 h after DEN treatment. There are type 0 (A), 1 (B), 2 (C), 3 (D) and 4 (E) comets (for details see Materials and Methods) (400X).

neous DNA diffusion analysis without electrophoresis were observed in rats that received the highest concentration of the mushroom *A. blazei* (Ab_3 solution) before 200 mg/kg DEN, than in rats solely treated with this dose of carcinogen (group G2 *vs* group G5, $P < 0.001$, Table 2).

GST-P quantitative data

Non-DEN-treated control and *A. blazei*-treated animals (groups G1 and G6) did not develop preneoplastic GST-P-positive liver foci (Table 3, Figure 5). Higher values of the number of but not the area of GST-P-positive liver foci per cm^2 of liver sections were observed in rats that received the highest concentration of *A. blazei* (Ab_3 solution) previously to 200 mg/kg DEN than in rats solely treated with this dose of carcinogen (group G2 *vs* group G5, $P < 0.001$, Table 3).

DISCUSSION

The potential of aqueous extracts of the mushroom *A. blazei* to ameliorate DNA damage and GST-P foci development induced by DEN was investigated in male Wistar rats.

Natural products have been traditionally accepted as remedies due to the popular belief that they produce few adverse side effects. Therefore, understanding the potential beneficial or adverse influence of natural products extensively used by human population is very important to implement public health safety measures. We did not observe adverse effects of treatment with relatively high doses of a crude aqueous solution of the mushroom *A. blazei*, determined from

Table 2. Tail migration, tail moment and DNA diffusion of liver cells from male Wistar rats 4 h after DEN or NaCl treatments[1].

Groups/Treatment[2]		Number of analyzed cells	DNA migration		Spontaneous DNA diffusion (μm)
			Tail migration (μm)	Tail moment	
Non-initiated					
G1	Control	500 (5)[3]	4.4 ± 0.5	0.8 ± 0.2	43.7 ± 0.3
G6	Ab_3	500 (5)	5.4 ± 0.7	1.1 ± 0.2	44.4 ± 0.3
Initiated					
G2	DEN	500 (5)	80.2 ± 1.2*	30.2 ± 0.8*	54.8 ± 0.4*
G3	Ab_1 + DEN	500 (5)	80.2 ± 0.9*	31.0 ± 0.6*	53.1 ± 0.4*
G4	Ab_2 + DEN	500 (5)	82.5 ± 1.1*	31.6 ± 0.8*	52.9 ± 0.4*
G5	Ab_3 + DEN	500 (5)	76.3 ± 0.8*.**	27.7 ± 0.6*.**	51.2 ± 0.4*.**

[1]Values are reported as mean ± SEM; [2]DEN (diethylnitrosamine, 200 mg/kg, *ip*) and Ab_1, Ab_2, Ab_3 = aqueous extract of *A. blazei* with mean dry weight of water-extractable solids of 1.2, 5.6, and 11.5 mg/ml, respectively; [3]Number of animals analyzed; *Significantly different from control animals (0.9% NaCl) (group G6 *vs* group G1, $P < 0.0001$); **Significantly different from DEN-only initiated animals (group G2 *vs* group G5, $P < 0.001$).

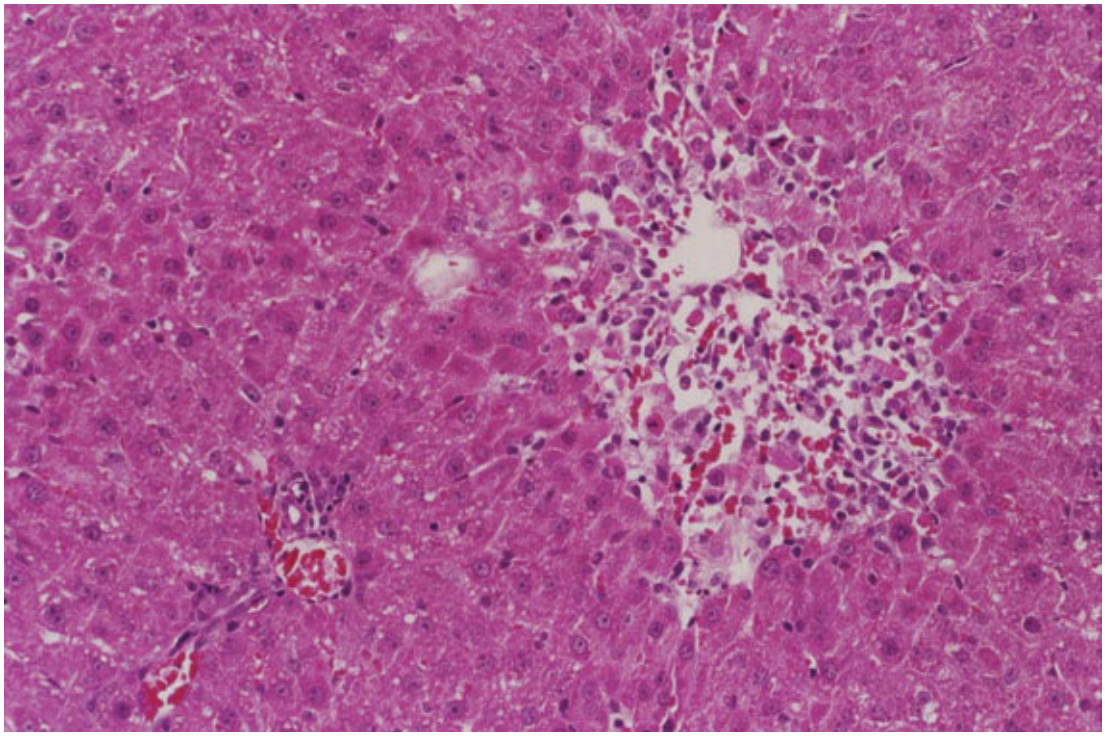

Figure 4. Severe necrosis and inflammatory reaction around the liver centrilobular vein 48 h after DEN treatment (250X).

body weight, liver morphology, DNA damage and GST-P foci data.

In the comet assay, cells with damaged DNA displayed increased migration of DNA fragments (comet tail) from the nucleus (comet head), which may also be a feature of DNA fragmentation associated with the necrotic/apoptotic death process (Olive et al., 1993; Fairbairn

Table 3. Number and area of hepatic preneoplastic GST-P-positive foci in male Wistar rats at the end of the experiment (10th week)[1].

Groups/Treatment[2]		Effective number of rats	GST-P-positive foci	
			Number (foci/cm^2)	Area (mm^2/cm^2)
Non-initiated				
G1	Control	14	0	0
G6	Ab_3	14	0	0
Initiated				
G2	DEN	15	3.15 ± 1.10	0.27 ± 0.14
G3	Ab_1 + DEN	13	3.53 ± 1.18	0.24 ± 0.14
G4	Ab_2 + DEN	13	4.06 ± 1.68	0.26 ± 0.12
G5	Ab_3 + DEN	15	4.82 ± 1.88*	0.32 ± 0.16

[1]Values are reported as mean ± SD; [2]DEN (diethylnitrosamine, 200 mg/kg, *ip*) and Ab_1, Ab_2, Ab_3 = aqueous extract of *A. blazei* with mean dry weight of water-extractable solids of 1.2, 5.6 and 11.5 mg/ml, respectively; *Significantly different from DEN-only initiated animals (group G2 *vs* group G5, $P < 0.05$).

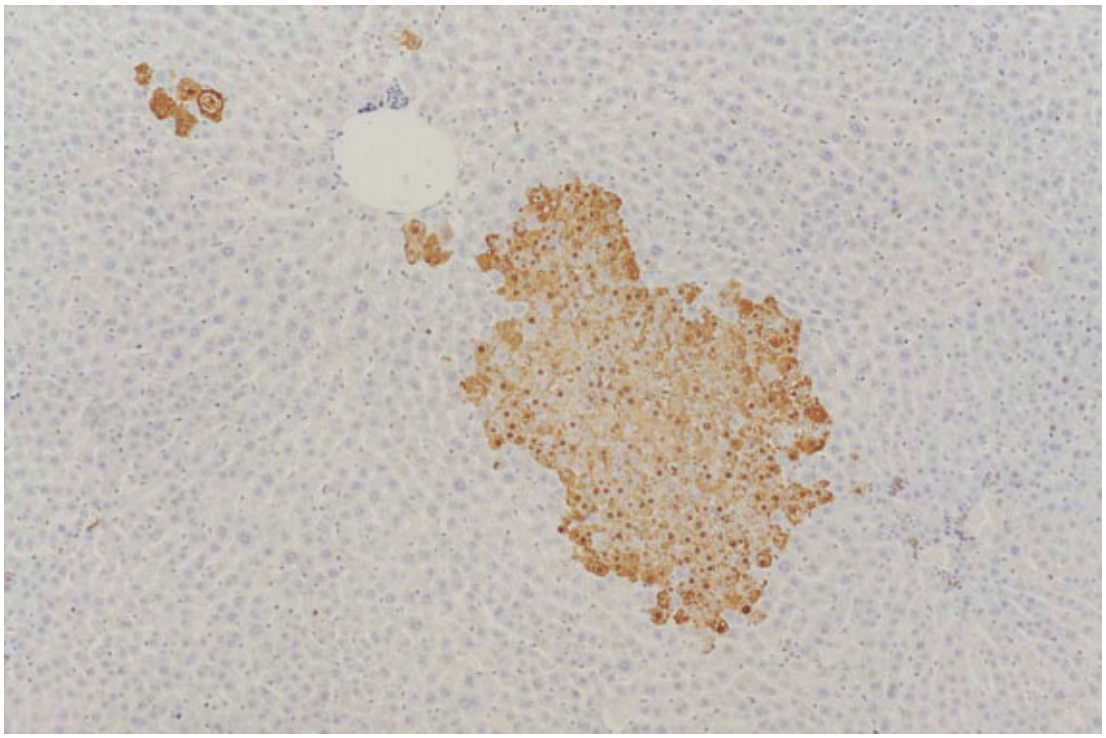

Figure 5. Immunohistochemically stained GST-P-positive liver foci (100X).

et al., 1996). In contrast with the conventional dye exclusion assay to evaluate cell viability (e.g., Trypan blue), we used two other approaches to evaluate concurrent liver cytotoxicity due to the DEN treatment. These alternative cytotoxicity assays included liver histological analysis for the detection of necrosis/apoptosis and the single-cell neutral diffusion assay to detect cells with low molecular weight DNA fragments, indicative of apoptosis or necrosis (Tice et al., 2000).

Treatment with 200 mg/kg DEN induced liver cytotoxicity, evidenced by increased

spontaneous DNA diffusion and also by severe centrilobular liver necrosis. The increased DNA migration observed 4 h after DEN treatment was likely a sum of genotoxicity and cytotoxicity induced by the carcinogen (Hartmann and Speit, 1997). A protective effect of the treatment with the higher *A. blazei* aqueous extract concentrations against DEN cytotoxicity/genotoxicity can be postulated because there was a diminished tail extension and diminished DNA content in the tails of the hepatocyte comets in *A. blazei*-treated animals when compared to the animals treated only with the standard 200-mg/kg DEN dose. It was previously found that *A. blazei* extracts inhibit the mutagenicity of benzo(a)pyrene in the Ames Salmonella microsome assay (Osaki et al., 1994) as well as mutagenicity induced by cyclophosphamide in the mouse bone marrow micronucleus test (Delmanto et al., 2001). In general, crude extracts and polysaccharides isolated from mushrooms have protective activity against chemical liver injury (Yeung et al., 1995; Ooi, 1996). Treatment with crude extracts of *Lentinus edodes*, *Grifola frondosa*, *Tricholoma lobayence* and an isolated polysaccharide peptide of *Coriolus versicolor* provides significant protection against paracetamol-induced hepatotoxicity by preventing a decrease in hepatic reduced glutathione and by increasing the conjugation and excretion of the drug reactive metabolites (Yeung et al., 1995; Ooi, 1996). The protective effect of *A. blazei* against the cytotoxicity induced by DEN could result from a modification in DEN metabolism due to active principles in the *A. blazei* extracts.

Altered foci of hepatocytes are believed to be early markers of the rodent liver cancer development (Bannasch and Zerban, 1992). A variety of phenotypic abnormalities identify the altered foci of hepatocytes, including abnormal expression of enzymes such as GST-P (Sato, 1988). In the DEN-PH model, the necrogenic dose of 200 mg/kg DEN was adopted because it induces a large number of GST-P-positive foci, favoring the analysis of the anti-cancer promoting potential of chemicals (Hasegawa and Ito, 1992). In a parallel study on DEN-induced cancer initiation of Wistar rats, we found that a previous treatment with a moderate dose of *A. blazei* (5.6 mg/ml) can exert hepatoprotection against liver toxicity and against the development of single GST-P-positive hepatocytes induced by 100 mg/kg DEN (Barbisan et al., 2002). Therefore, it was assumed that higher doses of *A. blazei* would also exert a beneficial influence against the initiation step of liver carcinogenesis by the standard protocol for medium-term hepatocarcinogenesis assay, which uses 200 mg/kg DEN for initiation. However, an increased number of the GST-P-positive foci were observed in animals treated with the higher doses of *A. blazei* before DEN. This could be attributed to the toxic environment induced by the higher dose of the carcinogen, when compared to the previous study. Also, the protection against liver cytotoxicity by the crude mushroom extract in the animals treated with the necrogenic dose of DEN could increase the survival of initiated cells (putative preneoplastic cells) through the process of liver initiation, resulting in a significant enhancement of GST-P-positive foci.

Numerous efforts towards reducing the chemical initiation of the carcinogenesis process have been unfruitful (Schmitt et al., 1993; Tessitore et al., 1996, 1997; Hambly et al., 1997). Schmitt et al. (1993) and Tessitore et al. (1996, 1997) were unable to detect an influence of fasting on the initiation stage of rat liver and mammary carcinogenesis when relatively high doses of carcinogens were used. Hambly et al. (1997) showed that diets containing low risk factors for colorectal cancer (e.g., low in fat and high in fiber and calcium) reduced dimethylhydrazine-induced DNA damage, as assessed by the comet assay, but did not change the aberrant crypt multiplicity, a preneoplastic marker for colorectal cancer in carcinogen-induced rodent models. Based on these results it appears that DNA damage does not always correlate with the

development of histologically detected preneoplasia. We also showed that DNA damage does not correlate with GST-P-positive foci development. It is possible that chemically induced models using a relatively high dose of carcinogen are not well suited to study factors relevant to the initiation events (Tessitore et al., 1996, 1997; Rijken et al., 1999).

Apparently the protective influence of aqueous extracts of *A. blazei* against DEN genotoxicity, cytotoxicity and carcinogenicity is due to different mechanisms and is dependent on both the dose of the chemopreventive agent and of the carcinogen used. The highest concentration of *A. blazei* extract (11.5 mg/ml) demonstrated pro-carcinogenic properties by reducing the elimination of damaged cells (there was less apoptosis/necrosis of liver cells after DEN injection in rats receiving the mushroom extract) leading to the formation of an increased number of preneoplastic lesions.

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