Antigenotoxicity of Agaricus blazei mushroom organic and aqueous extracts in chromosomal aberration and cytokinesis block micronucleus assays in CHO-k1 and HTC cells

M.F. Bellini a, J.P.F. Angeli a, R. Matuo a, A.P. Terezan b, L.R. Ribeiro c, M.S. Mantovani a,*

a Departamento de Biologia Geral, Universidade Estadual de Londrina—Campus Universitário, Cx. Postal 6001, 86051 990 Londrina, PR, Brazil
b Departamento de Química, Universidade Federal de São Carlos, 13565 905 São Carlos, SP, Brazil
c Departamento de Patologia, UNESP, 18600 000 Botucatu, SP, Brazil

Received 17 May 2005; accepted 9 August 2005
Available online 22 September 2005

Abstract

Agaricus blazei (Ab) has become popularly known for its medicinal properties. Scientifically, it has been tested with regard to its capacity to protect genetic material against damage. We examined different organic extracts (methanolic extract—ME, hexanic extract—HE and n-butanoic extract—BE) and an aqueous extract (AE) of Ab, for their capacity to induce DNA damage as well as for their protective effect. Genetic damage was determined by the chromosomal aberration assay (CA) in CHO-k1 cells for all extracts and the cytokinesis block micronucleus assay (CBMN) in non drug-metabolizing (CHO-k1) and drug-metabolizing (HTC) cell lines for extract BE only. The extracts did not show clastogenicity but showed anticlastogenicity. The greatest percent reduction obtained were with BE (105%) and AE (126%) treatments in CA. BE treatment did not display genotoxicity in CHO-k1, but was genotoxic in HTC. However, BE was shown to be antigenotoxic causing decreased micronucleus frequency in HTC and CHO-k1 cells. These results suggest that all the extracts contained protective substances, but in some cases they could show a genotoxic effect with regard to metabolism. Therefore, these findings warrant caution in the use of this mushroom by the population.

Keywords: Antigenotoxicity; Agaricus blazei; Drug metabolism; HTC cells; CHO-k1 cells; Chromosomal aberration assay

1. Introduction

Modern life exposes humans constantly to a large number of chemical, physical and biological agents. These agents can therefore interact in many ways with the human organism, and as a consequence act in a beneficial, neutral or harmful manner. Health problems and their solutions as a result of the above-mentioned agents’ interactions have stirred interest among the scientific community.

Protective substances have been found in the diet, particularly in mushrooms including Agaricus blazei Murrill ss. Heinem. (Ab) (Lohman et al., 2001). A. blazei is a Brazilian mushroom popularly known as the sun mushroom, and it is frequently consumed as food or tea in different parts of the world, due to its medicinal effects. This mushroom is believed to fight physical and emotional stress, stimulate the immune system, improve the life quality in diabetics, reduce cholesterol, fight osteoporosis and ulcers, treat circulatory and digestive problems, antitumor activity (Mizuno, 1995), anticarcinogenic properties (Takeda et al., 2000), antimutagenic and anticlastogenic effects.

* Corresponding author. Fax: +55 43 3371 4527.
E-mail address: biomsm@uel.br (M.S. Mantovani).
(Delmanto et al., 2001; Menoli et al., 2001; Oliveira et al., 2002; Bellini et al., 2003; Luiz et al., 2003a,b).

Kawagishi et al. (1989) were the first to separate the active compounds found in the Ab fruiting body. The authors detected polysaccharides with antitumor activity, the major fraction among these being FIII-2-b, which comprised a protein complex consisted of 43.3% protein, with (1→6)-β-D-glucan, and 50.2% carbohydrate. Polysaccharides composed of α-glucose chains with β-1,3 and β-1,6 linkages associated with antitumor activity act by stimulating the immune system, especially NK and macrophage cells (Itoh et al., 1994).

According to Wasser and Weiss (1999), the polysaccharides found in this basidiomycete could have important nutritional properties and many effects against the cancer development. Due to the large consumption of this mushroom in popular medicine, more data is needed on action mechanisms of its components. DNA damage are correlated with cancer development, therefore, the aim of the study was to determine the DNA-damaging potential and the antigenotoxic effects of A. blazei organic and aqueous extracts in a eukaryotic system in vitro with non drug-metabolizing (CHO-k1) and metabolizing (HTC) cells.

2. Material and methods

2.1. Preparation of Ab extracts

Ab extracts, strain 99/26, were kindly provided by Dr. Edson Rodrigues Filho and Ms. Ana Paula Terezan from the Chemistry Department of Universidade Federal de São Carlos. The fruiting body of the mushroom (896.90 g) was extracted with dichloromethane/hexane (50:50), dichloromethane (100), dichloromethane/methanol (50:50), methanol (100) and methanol/water (50:50). The hydroalcoholic extract obtained was first partitioned with dichloromethane, and the organic phase was concentrated and partitioned with methanol and hexane, yielding a hexanic fraction (HF/7.02 g) and methanolic fraction (MF/11.80 g). Subsequently, the hydroalcoholic extract was partitioned with ethyl acetate and n-butanol, respectively, yielding an aqueous fraction (AF/55.00 g) and an n-butanol fraction (BF/16.93 g). These were dissolved in DMSO (dimethyl sulfoxide, Mallinckrodt) at a concentration of 5 mg/mL. Aliquots were stored frozen until use time. The final concentration in cultures was 100 μg/mL. This fraction were denominated: methanolic extract—ME, hexanic extract—HE, n-butanolic extract—BE and aqueous extract—AE.

2.2. DNA damage-inducing agents

DNA damage was induced in CHO-k1 cells using ethyl methanesulfonate (EMS, Acros), a direct-acting alkylating agent. A stock solution was prepared in sterile phosphate-buffered saline (PBS), Ca²⁺ and Mg²⁺ free, pH 7.4, and used at a final concentration of 310 μg/mL in cultures. 2-Aminoanthracene (2-AA, Acros), an indirect-acting alkylating agent, was used to induce DNA damage in HTC. The stock solution was prepared in dimethyl sulfoxide (DMSO, Mallinckrodt) and used at a final concentration of 1 μg/mL in cultures.

2.3. Cell lines

The Chinese hamster ovarian cell line CHO-k1 (wild-type) used in this study was provided by Dr. Catarina S. Takahashi in the Mutagenesis Laboratory, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. HTC rat hepatoma cells derived from Rattus norvegicus were acquired from the Rio de Janeiro Cell Bank. Cells were grown in DMEM/F-12 medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco), as monolayer in 25 cm² flasks in a BOD type incubator at 37 °C. Under these conditions, the cell cycle time was approximately 12 h for CHO-k1 and 24 h for HTC.

2.4. Chromosomal aberrations assay—CA

CHO-k1 cells were grown for one complete cell cycle (12 h) before treatments. The treatments were as follows: (a) control (solvent control, DMSO, 2%); (b) EMS (100 μg/mL); (c) ME (100 μg/mL); (d) HE (100 μg/mL); (e) BE (100 μg/mL); (f) AE (100 μg/mL); (g) ME combined with EMS; (h) HE combined with EMS; (i) BE combined with EMS; and (j) AE combined with EMS. The treatments were simultaneous and were carried out for one cycle. Colcemid (Demecolcine, Gibco) (0.1 μg/mL) was added at the treatments ending an hour before fixing. At harvest, the cells were trypsinized (0.025%) and then hypotonized in 1% sodium citrate solution at 37 °C for 20 min. The cells were fixed in methanol/acetic acid (3:1) and the slides were stained with 5% Giemsa for 5 min.

Three separate experiments were carried out for each treatment. A total of 300 metaphases were analyzed treatment type (100 cells/treatment/repetition). Chromosomal aberrations were classified as isochromatid (i.e. isochromatid breaks—ic; dicentric—dic; and ring—r) or chromatid (chromatid breaks—ct; triradial—tr, quadri-radial—qr and complex rearrangement—cr) (Bez et al., 2001). The mitotic index (MI), corresponded to the number of cells in metaphase among 1000 cells analyzed per culture, was expressed as a mean percentage.

2.5. Cytokinesis block micronucleus assay—CBMN

Cells were grown for a complete cell cycle (12 h for CHO-k1 and 24 h for HTC) before treatments. The treatments were carried out as follows: (a) control (sol-
vent control, DMSO, 2%); (b) DNA damage-inducing agents (EMS-310 μg/mL or 2-AA-1 μg/mL); (c) BE (100 μg/mL); (d) BE combined with EMS or 2-AA; (e) BE pre-incubated with EMS or 2-AA (1 h). The culture treatments were simultaneous for 3 h, in serum-free culture medium. After treatments, the cells were washed with PBS (pH 7.4) and 5 mL of complete culture medium with 3 μg/mL cytochalasin-B (Cyt-B, Acros) were added. The cells were incubated subsequently for 18 h for CHO-k1 and 30 h for HTC. The procedures for harvesting and fixing cells were based on Menoli et al. (2001). Three independent repetitions were carried out for each treatment. The criteria utilized for the micronuclei determination in binucleated cells were established by Fenech (2000). A total of 6000 binucleated cells were analyzed per culture, which is 2000/treatment/repetition, in accordance with Garriot et al. (2002) and Phelps et al. (2002).

2.6. Reduction percentage and statistical analysis

The reduction percentage in chromosomal aberrations and micronuclei was calculated by dividing the number of cells with aberrations/micronuclei observed with EMS or 2-AA minus the number of cells with aberrations/micronuclei found with antigenotoxic treatments, by the number of cells with aberrations/micronuclei found with antigenotoxic treatments, by the number of cells with aberrations/micronuclei observed with EMS or 2-AA minus the number of cells with aberrations/micronuclei found in the control (Manhoharan and Banerjee, 1985; Waters et al., 1990).

The data were submitted to statistical analysis using Student’s t-test.

3. Results

The number of cells with chromosomal aberrations obtained in the clastogenicity and anticlastogenicity tests of Ab extracts is shown in Table 1. This table also presents the mitotic index (MI) obtained for each treatment, which varied 2.8–5.4%. The reduction percentage in cells, that showed chromosomal aberrations, was 63.15% with ME, 94.74% with HE, 105.26% with BE and 126.32% with AE.

Statistical analysis of the anticlastogenicity data indicated a significant difference with all the extracts, when compared with EMS, where BE was the more effective among the organic extracts and AE the more effective of all the extracts. None of the extracts tested was clastogenic in CA assay. The chromosomal aberrations types found are shown in Table 2. Table 3 presents the reduction percentage in total breaks types, where the breaks types more frequently reduced were the isochromatid type with BE (100%) and AE (144.4%) and the chromatid type with AE (100%) and HE (126.6%).

Table 4 indicates the mean and total micronuclei frequencies with the different treatments, along with the reduction percentage obtained in antigenotoxicity tests BE extract, respectively in cell lines CHO-k1 and HTC. It can be observed that the micronuclei frequency is relatively greater in HTC than in CHO-k1 with BE treatment indicating significant difference of control only in HTC cells (genotoxic effect). The antigenotoxic effect was observed in HTC and CHO-k1 cells with BE treatment. The pre-incubation treatment in HTC cells was more effective with reduction percentage of the 83.8%.

We opted to investigate more into the study of BE

Table 1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>MI mean ± SD</th>
<th>( n^0 ) Cells with aberrations Mean ± SD</th>
<th>Total(^b)</th>
<th>Reduction %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Clastogenicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.1 ± 0.3</td>
<td>5.0 ± 1.0</td>
<td>15</td>
<td>5.0</td>
</tr>
<tr>
<td>ME(^b)</td>
<td>5.4 ± 0.5</td>
<td>5.0 ± 0.0</td>
<td>15</td>
<td>5.0</td>
</tr>
<tr>
<td>HE(^b)</td>
<td>4.6 ± 0.7</td>
<td>4.0 ± 0.0</td>
<td>12</td>
<td>4.0</td>
</tr>
<tr>
<td>BE(^b)</td>
<td>4.8 ± 1.0</td>
<td>4.6 ± 1.2</td>
<td>14</td>
<td>4.6</td>
</tr>
<tr>
<td>AE(^b)</td>
<td>5.1 ± 0.9</td>
<td>4.6 ± 1.2</td>
<td>14</td>
<td>4.6</td>
</tr>
<tr>
<td><strong>Anticlastogenicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMS</td>
<td>4.1 ± 0.3</td>
<td>11.3 ± 2.1(^*)</td>
<td>34</td>
<td>11.3</td>
</tr>
<tr>
<td>ME(^b) + EMS</td>
<td>3.4 ± 0.2</td>
<td>7.3 ± 1.5(^**)</td>
<td>22</td>
<td>7.3</td>
</tr>
<tr>
<td>HE(^b) + EMS</td>
<td>3.6 ± 0.7</td>
<td>4.3 ± 1.5(^**)</td>
<td>13</td>
<td>4.3</td>
</tr>
<tr>
<td>BE(^b) + EMS</td>
<td>2.8 ± 0.9</td>
<td>4.6 ± 2.1(^**)</td>
<td>14</td>
<td>4.6</td>
</tr>
<tr>
<td>AE(^b) + EMS</td>
<td>4.1 ± 1.2</td>
<td>3.3 ± 2.1(^*)</td>
<td>10</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Control—solvent control, DMSO, 2%; EMS—ethyl methanesulfonate, 310 μg/mL. Treatments followed by: \(^*\) differ statistically from control, \(^**\) differ statistically from EMS.  
\(^a\) Total number of cells with aberrations out of 300 cells examined. 
\(^b\) *Agaricus blazei* extracts, 100 μg/mL; ME—methanolic extract; HE—hexanic extract; BE—n-butanoic extract; AE—aqueous and MI—mitotic index; SD—standard deviation.
extract, which was found to be very effective organic extract of Ab in the chromosomal aberration assay.

4. Discussion

Natural products have been traditionally accepted as remedies due to popular belief that they present minor adverse effects. Therefore, understanding the natural products beneficial potential or adverse influence of used by human populations in implementing safety measures for public health.

In the present study, different responses were seen for the induction and chemoprevention of chromosomal aberrations and micronuclei by Ab extracts treatments. These extracts did not show clastogenicity but, demonstrated anticlastogenic effects in CA. However, BE treatment was genotoxic in HTC cells but not in CHO-k1 and antigenotoxic in both cells line when CBMN was utilized. It is possible that in CA assay each extract acts more effectively by a distinct anticlastogenic mechanism in chromosomal aberration formation, since BE (100%) and AE (144%) show a greater reduction percentage in isochromatid breaks and HE (126.6%) and ME (73.3%) in chromatid breaks. This could be correlated with the phase in the cell cycle in which the breaks occur; both

### Table 2
Types of chromosomal aberrations observed in CHO-k1 cells treated with different *Agaricus blazei* extracts with or without EMS

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Types of chromosomal aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>ME</td>
</tr>
<tr>
<td></td>
<td>HE</td>
</tr>
<tr>
<td></td>
<td>BE</td>
</tr>
<tr>
<td></td>
<td>AE</td>
</tr>
<tr>
<td></td>
<td>EMS</td>
</tr>
<tr>
<td></td>
<td>ME + EMS</td>
</tr>
<tr>
<td></td>
<td>HE + EMS</td>
</tr>
<tr>
<td></td>
<td>BE + EMS</td>
</tr>
<tr>
<td></td>
<td>AE + EMS</td>
</tr>
</tbody>
</table>

### Table 3
Total and reduction percentage in chromatid and isochromatid breaks in tests of *Agaricus blazei* n-butanol extract for anticlastogenic effects

### Table 4
Frequency of micronuclei in CHO-k1 and HTC cells in the tests of *Agaricus blazei* n-butanol extract for antigenotoxic effect using simultaneous treatment and simultaneous treatment with pre-incubation
chromatid and isochromatid alterations occur in S phase, isochromatid breaks occur during G1 and chromatid breaks occur in G2, suggesting that BE and AE are more effective in G1 and S phases and HE and ME in S and G2 phases.

The damage inducer agents inactivation (chemistry or enzymatically) by the antimutagenic agents interaction (desmutagenic action mechanism), supports the possibility that a compound can present genotoxic and antigenotoxic activities, because when the extract is alone, it would act in DNA causing damage after the cells metabolism and when it is associated to another damage inducer agent, the extract should inactive it and consequently would also reduce its molecules to cause damages in DNA. However, the dual action of the Ab organic extract having a protective effect and DNA damage-inducing effect needs to be better investigated with emphasis on the role of cell metabolism in this process.

According to Bellini et al. (2003), Ab aqueous extracts, from strains 99/26 and 97/11 (this in the young and sporulating developmental phase, 0.15%), are anticlastogenic in relation to MMS in CHO-k1, when prepared at three different temperatures (4, 21 and 60 °C) and applied under continuous treatment conditions (12 h). The protective effect of Ab aqueous extracts have also been demonstrated in vivo against clastogenicity induced by cyclophosphamide (Delmanto et al., 2001). The protective effect for the aqueous extract against EMS, which is also an alkylating agent like cyclophosphamide and MMS, was found in this work, however, it may not suggest that this mushroom is effective against the DNA damage caused by alkylating agents, in general, because Luiz et al. (2003b) did not find any antimutagenic activity in Ab aqueous extracts against MMS in V79 cells, using the CBMN and comet assays.

Protective efficacy for the organic and aqueous extracts was observed, indicating that antimutagenic substances are present in different extracts types. These substances may be β-glucans, vitamins, essential amino acids, linoleic acid and others. The effectiveness demonstrated for the aqueous extract is an important aspect of the this mushroom protective activity, since humans consume Ab mainly in its natural form as a tea, not utilizing any type of organic solvent for the its compounds or active principles extraction.

Oliveira et al. (2002) studied Ab aqueous extracts, using simultaneous and simultaneous with pre-incubation conditions, and demonstrated a strong protective effect based on the CBMN assay under both conditions, suggesting a desmutagenic activity. However, under pre- and post-treatment conditions, a bio-antimutagenic activity was observed.

Menoli et al. (2001) observed a protective effect against CBMN induced by methyl methanesulfonate (MMS), when cells were treated with an aqueous extract of an Ab strains mixture, demonstrating desmutagenic activity. In the comet assay, the same authors observed antigenotoxic potential demonstrating the same mechanism. Our data show evidence of a substantial desmutagenic effect for BE extract, since with pre-incubation the micronuclei frequency observed was significantly higher than with simple simultaneous treatment in HTC cells but not in CHO-k1 cells.

In addition, not always Ab extracts have shown a protective effect. Delmanto et al. (2001) did not find a decrease in the micronuclei frequency after treating mice with a strain of the sun mushroom. Luiz et al. (2003b) did not find any antimutagenic activity in Ab aqueous extracts against MMS in V79 cells, using the CBMN and comet assays. Using the comet assay, Gutierrez et al. (2004) did not find a protective effect for Ab aqueous extracts in V79 cells, suggesting that differences in the cultivation, storage and extracts preparation could influence in its effectiveness as a nutritional supplement protecting against DNA damage as proposed by Chang (1996). In testing Ab aqueous extracts of three different origins (Botucatu-SP, Londrina-PR and Piedade-SP), Gutierrez et al. (2004) did not observe a genotoxic potential but an antigenotoxic activity only for Ab from Piedade-SP with pre-, post- and simultaneous treatments, and for Ab from Londrina-PR only with simultaneous treatment. These implicate that treatment type also influencing the protective effects of Ab.

Since ME, HE, AE and BE were found to be protective in the chromosomal aberration assay, but BE extract was both genotoxic and antigenotoxic in the CBMN assay, we can conclude that studies of the Ab biological effects are still in their infancy and that a better understanding is needed of the active principles regarding the action mechanism in the cell and interactions with cell physiology processes.

Acknowledgments

We are grateful to CAPES, CNPq and Fundação Araucária for financial support of the project. We also thank Dr. A. Leyva for his assistance in the preparation of the manuscript.

References


Lui, R.C., Jordão, B.Q., Eira, A.F., Ribeiro, L.R., Mantovani, M.S., 2003a. Mechanism of anticlastogenicity of Agaricus blazei Murrill mushroom organic extracts in wild type CHO(k1) and repair deficient (xrs5) cells by chromosome aberration and sister chromatid exchange assays. Mutation Research 528, 75–79.


