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Anticlastogenic effect of aqueous extracts of *Agaricus blazei* on CHO-k1 cells, studying different developmental phases of the mushroom

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Abstract

The *Agaricus blazei* Murill (ABM) mushroom, known as the sun mushroom, is native to Brazil and has become known for its medicinal properties. This study examined the anticlastogenic effect of *Agaricus blazei* in Chinese hamster ovary cells, CHO-k1, by means of a chromosome aberration test using methyl methanesulphonate (MMS, 10^{-4}M) as the DNA damage inducing agent. Two mushroom lines were used, ABM 99/26 and ABM 97/11, and the latter was used in the young (Y) and sporulating (S) developmental phases. The cells were treated for 12 h with MMS alone or combined with aqueous extracts of *A. blazei* at a final concentration of 0.15%, which were prepared at three different temperatures: (a) hot (60 °C), (b) room temperature (25 °C) and (c) chilled (4 °C). Mushroom extracts showed a marked anticlastogenic effect against DNA damage, as evidenced by a decrease in the number of cells with breaks, regardless of the line used, or the developmental stage or the temperature at which the extract was prepared. Generally, the extracts were more effective in reducing the isochromatid type breaks. The data obtained suggest that extracts of *A. blazei* mushroom are anticlastogenic under the conditions tested, mainly during the G1 and S stages of the cell cycle, where chromosome breaks of the isochromatid type are produced by the MMS agent.

Keywords: *Agaricus blazei*; CHO-k1 cells; Chromosome aberration; mushroom; Anticlastogenicity; Cell culture

1. Introduction

Interest in the medicinal properties of natural products has increased due to their popular use in traditional medicine. Many protective substances have been found in the diet, particularly in mushrooms, including *Agaricus blazei* Murill (ABM) (Lohman et al., 2001). The basidiomycete mushroom *A. blazei* (sun mushroom, Himematusutake, Kawariharatake), which is native to Brazil, is frequently consumed as a food or in a tea in different parts of the world, due to its medicinal effects (e.g. aphrodisiac, anti-stress, diabetes, gastric disturbances, osteoporosis), including possible anticarcinogenic and antimutagenic effects.

The fresh basidiocarp consists of 85–87% water. When dehydrated, it is rich in proteins (40–45%) and carbohydrates (3–4%) and also contains dietary fibers (6–8%), lipids (3–4%) and vitamins, especially B1, B2 and niacin. Ergosterol (0.1–0.2% dry weight) and linoleic acid (70–80% of the total lipids) are the predominate lipids (Osaki et al., 1994; Mizuno, 1995b). Chang et al. (2001) showed that *A. blazei* provides an appreciable quantity of non-volatile aromatic components plus soluble sugars, the most common being arabitol, glucose and trialose.

Takeda et al. (2000) described the spontaneous regression of a hepatocellular carcinoma in a 69 year-old man related to the use of *A. blazei*. Osaki et al. (1994) showed an antimutagenic effect against benzopyrene in the AMES/Salmonella test with hexane and chloroform/methanol extracts of *A. blazei*. Aqueous extracts of a mixture of *A. blazei* lines were shown to

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have an in vivo protective effect against clastogenicity induced by cyclophosphamide in mice. Animals pretreated with three different mushroom extracts, prepared at 4, 21 and 60 °C, had a significantly reduced micronucleus frequency in polychromatic erythrocytes and reticulocytes (Delmanto et al., 2001). Menoli et al. (2001) used the micronucleus test in binucleated cells and the comet assay (single microgel electrophoresis) in V79 cells treated with MMS and also found aqueous extracts from a mixture of *A. blazei* lines to have a protective effect.

In the present study, the anticlastogenic potential of *A. blazei* lines ABM 99/26 and ABM 97/11 (young — Y and sporulating — S) was tested in vitro using the chromosome aberration test in MMS-treated CHO-k1 cells, in which aqueous extracts were prepared at different temperatures.

2. Material and methods

2.1. Preparation of aqueous extracts of the mushroom

The dehydrated powders of the fruiting body of the *A. blazei* lines ABM 99/26 and ABM 97/11 (Y and S) were kindly provided by Dr. A.F. da Eira of the Department of Agronomy at UNESP, Botucatu, São Paulo, Brazil.

The aqueous extracts were obtained by mixing 5 g of mushroom powder with 200 ml distilled water. Different processes were then carried out to obtain the three different extracts: (a) hot — the extract was heated to 60 °C for 5 min, and filtered after 15 min at ambient temperature; (b) ambient temperature—the extract was kept at 25 °C for 2 h before being filtered; (c) chilled—the extract was kept at 4 °C for one hour before filtering. The extracts were subsequently first filtered through ordinary filter paper, and then sterilized by ultrafiltration (0.2 μm). Aliquots of the extracts were frozen and thawed at ambient temperature just before use. Each extract was tested in cell culture at a final concentration of 0.15%.

2.2. Cell culture and treatment

Chinese hamster ovary cells, CHO-k1, obtained from Laboratory of Mutagenesis - USP - Ribeirão Preto, Brazil, were grown as a monolayer in plastic culture flasks (25 cm^2) in culture medium consisting of Ham-F10 (Gibco BRL) and D-MEM (Gibco BRL) (1:1), supplemented with 10% fetal bovine serum (Gibco BRL), at 37 °C in a BOD type chamber. The cell cycle was 12 h under these conditions. The cells were cultivated for a complete cycle before the treatments. Five treatments were carried out for experiments with ABM 99/26: (a) negative control; (b) MMS; (c) MMS + ABM 4 °C; (d) MMS + ABM 25 °C and (e) MMS + ABM 60 °C. Eight treatments were carried out for the tests with ABM 97/11: (a) negative control; (b) MMS; (c) MMS + ABM-Y 4 °C; (d) MMS + ABM-Y 25 °C; (e) MMS + ABM-Y 60 °C; (f) MMS + ABM-S 4 °C; (g) MMS + ABM-S 25 °C and (e) MMS + ABM-S 60 °C. Three independent replications were carried out for each treatment, on three different days. Cells were treated with MMS alone or combined with the extracts for 12 h.

2.3. DNA damage inducing agent

The alkylating agent methyl methanesulphonate (MMS) was used as the DNA damage inducing agent. It was prepared at a final concentration of 10^{-4} M in Ca^{++}- and Mg^{++}-free phosphate buffered saline (PBS), pH 7.4.

2.4. Chromosome aberration

Colcemid (Demecolcine, Sigma) (0.1 μg/ml) was added at the end of the treatments 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.

2.5. Slide analysis

One hundred metaphases were analyzed per culture for a total of 300 cells per treatment. Chromosome aberrations (Brusick et al., 1984; Bez et al., 2001) were classified as isochromatid (i.e. chromosome breaks — ic; dicentric — dic and ring —r) or chromatid (chromatid breaks — ct; triradial — tr, quadriradial — qr and complex rearrangements — rc). Metaphases with more than 10 aberrations were classified as multiple aberrations (ma). The mitotic index (MI) corresponded to the number of cells in metaphase among 1000 cells analyzed per culture and was expressed as a percentage. The MI data were expressed as the mean of replicates. The percentage of reduction in chromosome aberrations (Waters et al., 1990) was calculated by the number of cells with chromosome aberrations found after treatment with MMS less the number found with the anticlastogenic treatment (MMS + extract) divided by the number found with MMS less the number found in the negative control.

2.6. Statistical analysis

The data on the total number of cells with chromosome aberrations were analyzed by the χ^2 test (2×2 table) at $\alpha = 0.05$ level of significance.

Table 1
Anticlastogenic effect of aqueous extracts of *Agaricus blazei,* ABM 99/26, in CHO-k1 cells in vitro

Treatments	MI	Number of cells with aberrations		% Reduction
	Mean±S.D.	Total[b]	%	
Control	5.8±0.23	15	5	–
MMS	4.4±0.04	55* ($P<0.0001$)	18.3	–
ABM 4 °C[a]	4.7±0.65	27* ($P=0.0013$)	9	70
ABM 25 °C[a]	4.5±0.74	26* ($P=0.0008$)	8.6	72.5
ABM 60 °C[a]	4.7±0.53	16* ($P<0.0001$)	5.3	97.5

*Statistically significant difference.
[a] MMS combined with aqueous extracts of *A blazei* Murill prepared at different temperatures, at a final concentration of 0.15%.
[b] Total number of cells with aberrations out of 300 cells examined; MMS-methyl methanesulphonate, 10^{-4} M; MI, mitotic index; S.D., standard deviation.

Table 2
Anticlastogenic effect of aqueous extracts of *Agaricus blazei*, ABM 99/26 in the young (Y) and sporulating (S) forms, in CHO-k1 cells in vitro

Treatments	MI	Number of cells with aberration		% Reduction
	Mean±S.D.	Total[b]	%	
Control	9.4±1.27	19	6.3	–
MMS	7.8±1.57	48* ($P=0.0003$)	16	–
ABM-Y 4 °C[a]	8.8±0.09	24* ($P=0.0039$)	8	82.7
ABM-Y 25 °C[a]	8.9±1.03	24* ($P=0.0039$)	8	82.8
ABM-Y 60 °C[a]	9.3±1.06	21* ($P=0.0009$)	7	93.1
ABM-S 4 °C[a]	7.8±1.20	27* ($P=0.0136$)	9	72.4
ABM-S 25 °C[a]	8.0±0.88	26* ($P=0.0091$)	8.6	75.9
ABM-S 60 °C[a]	7.5±0.41	28* ($P=0.0197$)	9.3	69

MMS—methyl methanesulphonate at 10^{-4} M concentration; MI—mitotic index; DP—standard deviation.
* Significant statistical difference compared to MMS.
[a] MMS combined with aqueous extracts of *A. blazei* Murill prepared at different temperatures, at a final concentration of 0.15%.
[b] Total number of cells with aberrations out of 300 cells examined.

3. Results

Table 1 shows the anticlastogenic effect of *A. blazei* extract for ABM 99/26, while the data for ABM 97/11 are shown in Table 2. There was a significant reduction in both cases in the total number of cells with breaks, when cells were incubated with MMS plus *A. blazei* extract compared with MMS alone, regardless of the temperature at which the extract was prepared (4, 25 and 60 °C) or the developmental stage (Y or S). These tables also show the mean mitotic index (MI) obtained for each treatment, with variations from 4.4 to 5.8 for the ABM 99/26 line and 7.5 to 9.4 for the ABM 97/11 line.

The percentage of reduction of chromosome aberrations in cells varied from 70.0 to 97.5% for ABM 99/26 extracts (Table 1) and from 69.0 to 93.1% for ABM 97/11 extracts (Table 2). With the ABM 99/26 extracts, the hot preparation (60 °C) produced a greater protective effect (Table 1) as did ABM 99/26 in the young developmental stage (Table 2). However, for the sporulating stage the most efficient extract was that prepared at ambient temperature (25 °C) (Table 2). Extracts of ABM 97/11 in the young developmental phase (Table 2) produced an overall greater reduction in percentage of chromosomal damage than those prepared with the mushroom in the sporulating developmental phase.

The types of chromosome aberrations detected are shown in Tables 3 and 4, respectively, for ABM 99/26 and ABM 97/11. In summing the total number of

Table 3
Types of chromosome aberrations found in CHO-k1 treated with MMS combined with aqueous extracts of *Agaricus blazei* (ABM 99/26)

Treatments	Number of chromosome aberrations									
	Type of break									Other
	ct	tr	qr	rc	Total[b]	ic	r	dic	Total[c]	ma
Control	0	3	0	1	4	3	6	1	10	1
MMS	18	8	4	16	46	13	4	3	20	0
ABM 4 °C[a]	15	5	3	8	31	6	0	2	8	0
ABM 25 °C[a]	9	9	1	6	25	7	1	1	9	0
ABM 60 °C[a]	2	3	3	1	9	3	3	2	8	0

ma, multiple aberrations.
[a] MMS combined with aqueous extracts of *A. blazei* Murill prepared at different temperatures, at a final concentration of 0.15%.
[b] Chromatid breaks (i.e. chromatid breaks — ct; triradial — tr, quadriradial — qr and complex rearrangements — rc).
[c] Isochromatid breaks (i.e. chromosome breaks — ic; dicentric — dic and ring —r).

Table 4
Types of chromosome aberrations found in CHO-k1 treated with MMS combined with aqueous extracts of *A. blazei* (ABM 97/11, in the young (Y) and sporulating (S) forms)

Treatments	Number of chromosome aberrations									
	Type of break									Other
	ct	tr	qr	rc	Total[b]	ic	r	dic	Total[c]	ma
Control	7	0	1	0	8	2	3	6	11	0
MMS	15	4	2	2	23	15	4	11	30	2
ABM-Y 4 °C[a]	5	3	1	0	9	4	6	5	15	0
ABM-Y 25 °C[a]	10	6	1	2	19	7	2	2	11	0
ABM-Y 60 °C[a]	12	1	1	0	14	5	1	2	8	0
ABM-S 4 °C[a]	4	2	7	1	13	4	3	3	10	0
ABM-S 25 °C[a]	7	2	3	0	12	5	3	9	17	0
ABM-S 60 °C[a]	9	4	6	0	19	6	2	3	11	0

ma, multiple aberrations.
[a] MMS combined with aqueous extracts of *A. blazei* Murill prepared at different temperatures, at a final concentration of 0.15%.
[b] Chromatid breaks (i.e. chromatid breaks — ct; triradial — tr, quadriradial — qr and complex rearrangements — rc).
[c] Isochromatid breaks (i.e. chromosome breaks — ic; dicentric — dic and ring —r).

breaks of the chromatid type (i.e. chromatid breaks — ct; triradial — tr, quadriradial — qr and complex rearrangements — rc) or isochromatid type (i.e. chromosome breaks — ic; dicentric — dic and ring —r), it is seen that *A. blazei* extract added to MMS reduced both types when compared to MMS treatment alone, regardless of the extract preparation temperature or the developmental phase (Y or S). Table 5 describes the percent reduction for the total number of the two types of breaks, and the greatest reduction in frequency occurred with the isochromatid type breaks.

4. Discussion

Edible and medicinal mushrooms have been studied for their potential therapeutic effects. *A. blazei* has a characteristic β-D-glucan polysaccharide that is associated with antitumor activity (Chang, 1996).

The data obtained in the present study showed that extracts of *A. blazei*, both ABM 99/26 and ABM 97/11 lines, were efficient protectors against DNA damage induced by MMS, suggesting the presence of a chemoprotective agent(s). However, Mizuno (1995a) warns that the basidiomycete properties should be considered together with the form of cultivation management, stock and processing. Menoli et al. (2001) also found an antimutagenic effect with a mixture of *A. blazei* lines in the micronuclei test in V79 cells treated with MMS.

In the present study, where the protective effect of extracts from different developmental stages were assessed, the young extracts were much more efficient and produced the greatest reduction in DNA damage. This indicates that riper mushrooms are less efficient probably due to a modification in their chemical composition during development.

Table 5
Reduction in the number of chromatid and isochromatid breaks found after treatment with MMS combined with aqueous extracts of *A. blazei* (ABM 99/26 and ABM 97/11)

Treatments[a]	% Reduction	
	Chromatid	Isochromatid
ABM 99/26 4 °C	35.7	120
ABM 99/26 25 °C	50	110
ABM 99/26 60 °C	88	120
ABM-Y 97/11 4 °C	94.7	78.9
ABM-Y 97/11 25 °C	42.1	100
ABM-Y 97/11 60 °C	68.4	115
ABM-S 97/11 4 °C	68.4	105.2
ABM-S 97/11 25 °C	78.9	68.4
ABM-S 97/11 60 °C	42.1	100

[a] MMS combined with aqueous extracts of *A. blazei* Murill, ABM 99/26 and ABM 97/11 (young and sporulating), prepared at different temperatures, at a final concentration of 0.15%.

Regarding the temperatures used to prepare the aqueous extracts, the ABM 99/26 line had a better protective effect when extracted at 60 °C, a difference not observed for the ABM 97/11 line. However, both lines and all types of extracts showed a reduction in DNA damage greater than 70%, which was similar to the findings reported by Oliveira et al. (2002) for the ABM 99/26 line in the micronuclei test under different treatment conditions. Oliveira et al. (2002) suggested that *A. blazei* may have protective effects both of the desmutagenic type, involving chemical inactivation of the DNA damage-inducing compound, and of the biomutagenic type, through modulation of the DNA replication and repair process.

MMS is an S-dependent alkylating agent and produces chromatid and isochromatid breaks, but the greatest reduction in the frequency of breaks detected was for the isochromatid type. This finding may indicate a greater protective efficiency of *A. blazei* extracts in the G1 and S phases of the cell cycle, where this type of aberration occurs. Other anticlastogenic substances also display this type of differential behavior. Bez et al. (2001) demonstrated that chlorophyll *a* and *b* also reduced isochromatid type breaks more efficiently than chromatid type breaks in human lymphocytes. This could be a common behavior of chemoprotective substances.

A. blazei is rich in polysaccharides which can stimulate the immune system, and β-1,6-D-glucan is the main polysaccharide with this activity. Ohno et al. (2001) when performing enzyme and functional studies on aqueous extracts of *A. blazei* (hot, NaOH and hot NaOH) demonstrated that the functional center of the β-1,6-glucan polysaccharide is a region rich in β-1,3 links.

Ethanol extracts of *A. blazei* basideocarp have been demonstrated to affect the immune system by stimulating macrophages and increasing the expression of cytokine mRNA (IL-8 and TNF) (Sorimachi et al., 2001a). Ethanol extracts of the mycelia were shown to reduce the cytopathic effects caused by the western equine encephalitis (WEE) virus (Sorimachi et al., 2001b). *A. blazei* also contains a substance with antimicrobial activity, 13-hydroxi-*cis*-9 acid, *trans*-11-octadienoic, obtained from the hexane extract of its basidiocarp (Osaki et al., 1994).

Extracts of *A. blazei* using chloroform/methanol (2:1) have been shown to have antitumor activity against solid tumors, in which ergosterol was found to be responsible for this activity. Although it is not cytotoxic to tumor cells, ergosterol was shown to be effective in blocking angiogenesis, causing the death of tumor cells by preventing neovascularization (Takaku et al., 2001). However, the anticlastogenic effect of *A. blazei* demonstrated in this study cannot be correlated, in terms of mechanism of action, directly to an antitumor effect. *A. blazei* extract acts mainly through the modulation of

the immune system, activating macrophages, neutrophils and lymphocytes, and does not have a direct effect on tumor tissue (Takusaburo and Yoshiaki, 1998). Among the substances identified to date, linoleic acid (Osaki et al., 1994) is the main candidate accounting for the antimutagenic activity described in various reported studies.

A. blazei steroids derived from cerevisterol and ergosterol have been shown to be cytotoxic. These steroids were isolated from the basidiocarp extracted with acetone and tested in HeLa S3 cells (epithelial carcinoma cells from human cervix) (Kawagishi et al., 1988). Mizushina et al. (1998) demonstrated the inhibitory effect of cerecisterol on DNA replication polymerases, especially DNA α polymerase. Other ergosterol derivatives, such as 4-hydroxy-17-methyl-incicterol (HMI), were also shown to inhibit DNA α polymerase in a dose-dependent manner. HMI inhibited the growth of cancer cells derived from leukemia and solid tumors and the same result was observed with its synthetic derivatives 17-methyl-incisterola and 4-acetyl-17-methyl-incisterol (Togashi et al., 1998).

Therefore, the use of *A. blazei* as a chemopreventive food is premature, and the medicinal potential and counter-indications of this mushroom need to be further explored.

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