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Non-mutagenic or Genotoxic Effects of Medicinal Aqueous Extracts from the *Agaricus blazei* Mushroom in V79 Cells

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Summary *Agaricus blazei* Murill is a mushroom largely consumed due to its medicinal properties. Effects of aqueous extract from its lineage AB97/11 in 2 fruiting body development stages (closed and opened pileus) were evaluated on chinese hamster V79 cells using cytokinesis blocking micronucleus (CBMN) and comet assays. The cells were treated at 0.15% concentration of aqueous extract prepared at different temperatures: ice-cold (4°C), room temperature (21°C) and warm (60°C). The extracts were applied in mutagenicity and antimutagenicity protocols (simultaneous, pre-incubation and continuous). The results showed that the aqueous extracts of *Agaricus blazei* lineage AB97/11 obtained at the 3 temperatures and both development stages did not present mutagenic or antimutagenic effect in V79 cells either in CBMN or comet assay.

Key words *Agaricus blazei* Murill, Antimutagenesis, Cytokinesis block micronucleus assay, Comet assay, V79 cells, Methyl methanesulphonate.

It has been suggested that daily use of antimutagens and anticarcinogens would be an effective way to prevent cancer, a procedure known as chemical prevention (Gomes *et al.* 1996, Surh *et al.* 1996). Many protective substances have been found in human diet, especially in mushrooms, including *Agaricus blazei* (Lohman *et al.* 2001). *A. blazei* is an edible mushroom native to southern Brazil, known in Japan as *himematsutake* or *kawariharatake.* Popularly, it is used as an aphrodisiac and to treat stress, diabetes, gastric troubles, osteoporosis and even cancer.

In the chemical composition of this mushroom have been found antimicrobial (*i.e. trans*-11-octadienoic, 13-hydroxi-*cis*-9 acid), antimutagenic (*i.e.* linoleic acid) (Osaki *et al.* 1994) and antitumoral substance (Itoh *et al.* 1994, Fujimiya *et al.* 1998, 1999). Polysaccharides composed of D-glucose chains with β-1,3 and β-1,6 linkages associated with antitumoral activity act by stimulating the immune system, especially NK and macrophage cells.

A. blazei aqueous extracts have already shown antimutagenic effect against cyclophosphamide in the micronucleus assay in mice bone marrow cells (Delmanto *et al.* 2001) and in micronucleus and comet assays against methyl methanesulphonate (MMS) in cultured V79 cells (Menoli *et al.* 2001).

The present study assessed effects of aqueous extracts from *A. blazei* fruiting body (lineage AB97/11) at 2 development phases (closed and opened pileus) using the cytokinesis block micronucleus (CBMN) and the comet assays in V79 cells on the mutagenic activity of MMS.

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Materials and methods

Sun mushroom aqueous extracts

Dehydrated fruiting body of the *A. blazei* lineage AB97/11 in 2 development stages (closed and opened pileus) was produced and supplied by the Faculty of Agronomic Sciences-UNESP, Botucatu, São Paulo, Brazil. After agitating, the aqueous solutions obtained by diluting 5 g of the fruiting body pound in 200 ml distilled water for 10 min, were incubated at 3 different temperatures: (a) ice-cold (4°C) for 1 h; (b) room temperature (21°C) for 2 h and warm (60°C) for 5 min and filtered after cooling for 15 min at room temperature. The solutions were initially filtered in common filter paper and later sterilized in 0.2 mm bacteriological filter membrane. Aliquots were frozen for later use at room temperature. Each extract was applied to cell cultures at the final dilution of 0.15% (300 μl in 5 ml culture).

Mutagenic agent

The alkylating agent methyl methanesulphonate (MMS), used as a DNA damage inducer, was freshly dissolved in Ca^{++} and Mg^{++} free phosphate buffer saline (PBS) pH 7.4. Different MMS concentrations were used depending on the cell treatment protocol and each concentration was chosen from tests carried out in our laboratory (4×10^{-4} M or 10^{-4} M in CBMN and 1.2×10^{-4} M in comet assay) using the DNA damage induction that no promoted cytotoxicity as selection criteria.

Cell culture and treatment

V79 cells (chinese hamster lung fibroblast) supplied by the Laboratory of Mutagenesis of USP (Riberão Preto, São Paulo, Brazil) were grown in culture flasks, in Ham's F10 culture medium plus Dulbecco's modified Eagle minimum essential medium (1 : 1) with 0.1% antibiotic-antimicotic solution, supplemented with 10% fetal bovine serum in a BOD type incubator at 37°C. The average cell cycle time of V79 was 12–14 h under these conditions. For both assays the cells were cultivated for 2 cell cycles (25 h) in complete culture medium before being treated according to one of the following treatment protocols (1) treatment with each extract at the 0.15% final concentration for 1 h (mutagenicity); (2) co-treatment with each extract and MMS for 1 h (simultaneous); (3) treatment with the mixture of each extract with MMS that after resting for 1 h was applied in culture for 1 h (pre-incubation); (4) co-treatment with each extract with MMS for 18 h (continuous). For each protocol there were (a) one culture treated with phosphate-buffered saline (PBS, pH 7.4) (negative control) and (b) a culture treated with alkylating agent MMS at the final concentrations of 4×10^{-4} M for mutagenicity, simultaneous and pre-incubation, 10^{-4} M for 3 simultaneous continuous (CBMN) and 1.2×10^{-4} M for the comet assay. The continuous mutagenicity protocol was not performed in the comet assay. All treatments described here were repeated independently 3 times for both assays.

Cytokinesis block micronucleus assay

The agents were added to 5 ml of complete culture medium 25 h after iniciating the cultive of cells which had been previously seeded in 25 cm^3 flasks. At the end of the treatments of mutagenicity, simultaneous and pre-incubation, the cells were washed twice with 5 ml of PBS (pH 7.4) at room temperature. After, this flasks received 5 ml of new complete culture medium with cytochalasin-B (3 μg/ml). The cells were cultivated for further 17 h to block cytokinesis and to yield binucleated cells. In the continuous treatment protocol the cells were not washed and cytochalasin-B was add 1 h after beginning of the treatment.

For harvesting the cells were trypsinized (0.025% trypsin at room temperature), hypotonized (1% sodium citrate) and fixed with methanol/acetic acid (3 : 1). The slides were stained with 5% Giemsa dissolved in phosphate buffer (0.06 M Na_2HPO_4, 0.039 M KH_2PO_4, pH 7.0) for 5 min and analyzed.

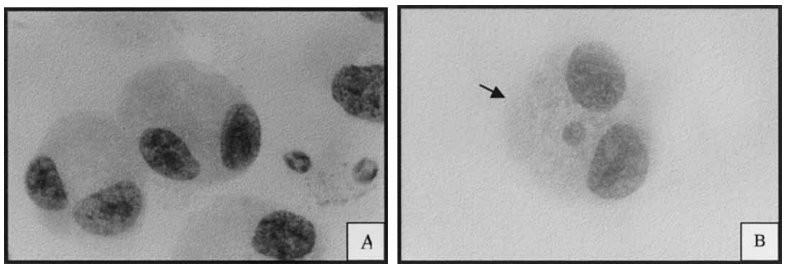

Fig. 1. Binucleated cells in V79 without micronuclei (A) or with micronuclei (B).

One thousand binucleated cells per treatment were analyzed for each experimental repetition. Only cells with conserved cytoplasm were scored. The following criteria were used to identify the micronuclei (MN) (Fig. 1): (1) MN should be smaller than 1/3 of the nucleus; (2) both MN and the nucleus should be round; (3) MN could not present connection with the nucleus; and (4) MN should not be refractive and should have coloring similar to the nucleus (Schmid 1976, Titenko-Holland *et al.* 1997).

Single cell gel electrophoresis assay

General procedures according to Speit and Hartmann (1999), were used for the comet assay with modifications.

The V79 cells were seeded in flasks with 2.5 ml of complete culture medium, cultivated for 25 h and treated for 1 h applying genotoxicity, simultaneous and pre-incubation protocols. When the treatment had finished, the cells were trypsinized (200 μl 0.025% trypsin), centrifuged and re-suspended in culture medium (500 μl) after centrifugation. The cell suspension (10 μl) was mixed with 120 μl LMP (low melting point) agarose (0.5%) at 37°C and spread onto microscope slides pre-coated with 1.5% normal agarose. Coverslips were added to the slides and allowed to gel at 4°C for 10 min. After removing the coverslips, the slides were immersed in recently prepared lysing solution, composed of 89 ml solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 corrected with solid NaOH, 890 ml distilled water, 1% sodium Na-lauroyl sarcosine), 1ml Triton X-100 and 10 ml DMSO. Cellular lyse occurred in refrigerator 4°C, protected from the light, for 1 h. The slides were taken to the electrophoresis cube with pH >13.0 buffer (300 mM NaOH, 1 mM EDTA, prepared from a stock solution of NaOH 1ON, EDTA 200 mM, pH 10.0) at 4°C for 20 min for DNA denaturation. Electrophoresis was performed at 4°C for 20 min, 25 V and 300 mA (1.6 V/cm). Later the slides were neutralized with pH 7.5 buffer (0.4 M Tris–HCl) for 15 min (3 cycles of 5 min), dried and fixed in 100% ethyl alcohol for 10 min and kept up to reading. The slides were covered with 50 μl ethidium bromide 200 μg/ml and coverslip for staining. The material was assessed under a fluorescence microscope at 400× magnification, with 420–490 nm excitation filter and 520 nm barrier filter.

One hundred cells per treatment were analyzed visually (Kobayashi *et al.* 1995), for each experimental repetition, classifying the comets as: (class 0) cells with undetectable DNA damage that did not present tail; (class 1) cells with tail smaller than the diameter of the nucleus; (class 2) cells with tail length between once and twice the nucleus diameter; (class 3) cells with tail longer than twice the nucleus diameter (Fig. 2). Apoptotic cells that presented nucleus totally fragmented were not scored (Speit and Hartmann 1999).

Statistical analysis

The results of the 3 independent repetitions for both assays were summed and the statistical differences between treatments and controls were determined by the χ^2 test (Pereira 1991).

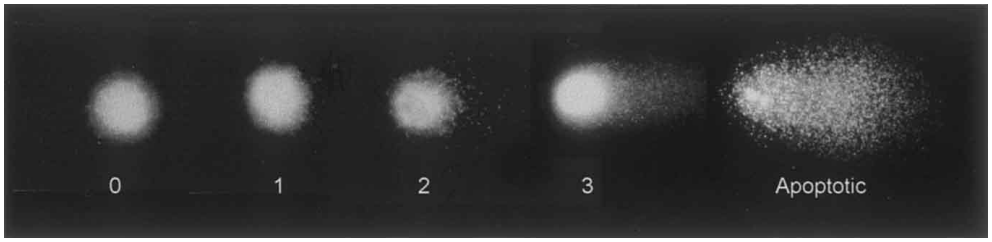

Fig. 2. Class of comets (0–3) and apoptotic nuclei in V79 cells.

Table 1. Asssessment of mutagenicity and genotoxicity of *A. blazei* aqueous extracts by cytokinesis block micronucleus and comet assays in V79 cells

Treatments	MN		Comet Class				
	Total	mean±S.D.	Total[1]	0	1	2	3
Negative control	39	13.0±1.0	99	203	94	4	1
MMS	103*	34.3±1.5	275*	25	210	62	3
Closed pileus[2]							
4°C	38	12.7±0.6	102	198	100	2	0
21°C	38	12.7±1.5	98	202	94	4	0
60°C	41	13.7±1.5	99	202	94	3	1
Opened pileus[2]							
4°C	40	13.3±1.5	102	198	101	1	0
21°C	41	13.3±0.6	98	202	97	1	0
60°C	41	13.7±1.2	100	200	99	1	0

MMS – methyl methanesulfonate 4×10^{-4} M in CBMN and 1.2×10^{-4} M in comet assay, SD – standard deviation, * Significant difference compared to negative control ($p<0.05$), [1] Total of cells with damage (class 1+2+3), [2] Treatments with aqueous extracts *A. blazei* at 0.15%.

Results and discussion

Biological data on the medicinal properties associated with mushrooms are rare, especially regarding antimutagenic and anticarcinogenic activities (Grüter *et al.* 1990). As the use of *A. blazei* is very popular and it has been prepared in diverse temperature conditions, 3 aqueous extraction temperatures were tested (4, 21, 60°C) in the present study. Our results showed that the aqueous extracts of the *A. blazei* lineage AB97/11 did not have mutagenic or genotoxic effect at the 0.15% concentration regardless of the temperature of extraction. The results of the mutagenicity or genotoxicity with *A. blazei* extracts are shown in Table 1. The fruiting body development phase also did not interfere in this biological response, suggesting that there is no significant presence of water soluble mutagenic substances either in this closed and opened pileus phases of development.

Table 2 show the results of the antimutagenicity in the CBMN assay. The extracts were not able to reduce significantly the cell number with MN in relation to the MMS in any of the cases.

Menoli *et al.* (2001) observed a protective effect on CBMN induction by MMS of aqueous extracts obtained from a mixture of *A. blazei* lineages as well as partial antigenotoxicity in comet assay. The results of the present investigation showed absence of antimutagenic effect of *A. blazei* extracts against MMS, what suggest that differences in lineages of *A. blazei*, could modify the mushroom effects.

Tables 3 exhibit the results of the simultaneous and pre-incubation protocols in the comet assay, where not significant reductions in the number of cells with DNA lesions were observed when compared to MMS. This is showing that *A. blazei* extracts did not have protective effect, ei-

Table 2. Assessment of antimutagenicity of *A. blazei* aqueous extracts in the simultaneous, pre-incubation and continuous treatments by cytokinesis block micronucleus in V79 cells

Treatments	Simultaneous		Pre-incubation		Continuous	
	Total	Mean±S.D.	Total	Mean±S.D.	Total	Mean±S.D.
Negative control	40	13.3±0.6	40	13.3±1.2	44	14.7±1.0
MMS	102	34.0±2.0	104	34.7±1.5	108	36.0±1.7
Closed pileus[1]						
4°C	101	33.7±0.6	104	34.7±1.2	96	32.0±0.6
21°C	99	33.0±1.0	102	34.0±2.0	89	29.7±1.5
60°C	98	32.7±1.8	100	33.3±1.5	98	32.7±1.2
Opened pileus[1]						
4°C	100	33.3±1.5	102	34.0±1.0	101	35.7±1.0
21°C	99	33.0±1.0	100	33.3±1.5	100	33.3±1.5
60°C	96	32.0±1.0	99	33.0±1.7	93	31.0±1.2

MMS – methyl methanesulfonate 4×10^{-4} M, S.D. – standard deviation, [1] Treatments with aqueous extracts *A. blazei* at 0.15%.

Table 3. Assessment of antigenotoxicity of *A. blazei* aqueous extracts in the simultaneous and pre-incubation treatments by comet assay in V79 cells

Treatments	Simultaneous					Pre-incubation				
	Total[1]	Comet class				Total[1]	Comet class			
		0	1	2	3		0	1	2	3
Negative control	98	202	96	2		97	204	87	8	1
MMS	279	21	206	69	4	275	27	201	67	5
Closed pileus[2]										
4°C	275	25	198	73	4	273	28	194	76	3
21°C	278	22	205	70	3	273	27	204	64	5
60°C	274	26	195	75	4	270	30	192	75	3
Opened pileus[2]										
4°C	277	23	204	71	2	274	26	199	73	2
21°C	276	24	198	74	4	271	29	196	71	4
60°C	278	22	206	69	3	274	26	203	67	4

MMS – methyl methanesulfonate 1.2×10^{-4} M, [1] Total of cells with damage (class 1+2+3), [2] Treatments with aqueous extracts *A. blazei* at 0.15%.

ther in relation to the number of damaged cells or to the level of damage observed (class types).

Geographic, climatic and intra specific variations may interfere in mushroom biological response (Chang 1996) and this type of interfering on its antimutagenic effect should be studied in *A. blazei*. Further to these factors, harvesting and conservation methods of this mushroom may promote loss or degradation of active compounds. The presence of antimutagenic potential in *A. blazei* cannot be excluded since may be that an incomplete extraction of the active components may occurs according to solvent used in the extraction. Thus, organic extracts should also be assessed.

However, Oliveira *et al.* (2002) demonstrated activity desmutagenic and bioantimutagenic to aqueous extracts by *A. blazei*. Desmutagenic are those compounds able to prevent mutagenic action mainly by trapping the mutagenic agents and acting outside the cell. Bioantimutagenic agents are those able to prevent the lesion or to stimulate the DNA repair mechanisms, acting inside the cell (Kada and Shimoi 1987). Different treatment protocols have been used in an attempt to elucidate antimutagenicity mechanisms (De Flora 1998). In the present study, 3 protocols that can demon-

strate desmutagenic and bioantimutagenic capability were used: simultaneous, pre-incubation and continuous treatments.

It is still premature to indicate or counter-indicate *Agaricus blazei* Murill in chemical-prevention. A greater knowledge of the chemical composition of this mushroom is needed to estimate the advantages and disadvantages of its use for human consumption.

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