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Antimutagenic effects of the mushroom *Agaricus blazei* Murrill extracts on V79 cells

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Abstract

Agaricus blazei Murrill, a native mushroom in Brazil, has been widely consumed in different parts of the world due to its medicinal power. Its anticarcinogenic activity has been shown in experimental animals, and antimutagenic activity has been demonstrated only in *Salmonella*. In this work, the mutagenic and antimutagenic activities of mushroom teas of strains AB96/07, AB96/09 and AB97/11 were evaluated in Chinese hamster V79 cells, using the comet assay and the micronucleus test. The cells were treated with three different concentrations (0.05, 0.1 and 0.15) of teas prepared from a 2.5% aqueous solution, under three different temperatures: (1) room (20–25°C); (2) ice-cold (2–8°C); and (3) warm (60°C). The teas were applied in co-, pre- and post-treatments in combination with the mutagen methyl methanesulfonate (MMS; 1.6×10^{-4} and 4×10^{-4} M). The duration of the treatment was 1 h in the comet assay and 2 h in the micronucleus test. The results showed that the mushroom was not mutagenic itself. Nevertheless, the mushroom is an efficient antimutagen against the induction of micronuclei by MMS in all concentrations and preparations tested. The observed reductions in the frequencies of micronuclei ranged from 61.5 (room temperature 0.1% tea in post-treatment) to 110.3% (co-treatment with warm and ice-cold 0.15% tea). In the comet assay, the antimutagenic activity was detected only when the cells were pre-treated with the following teas: warm 0.1 and 0.15%, room temperature 0.05% and ice-cold 0.1%. The results indicate that the mushroom *A. blazei* extracts are antimutagenic when tested in V79 cells. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Agaricus blazei Murrill; Antimutagenesis; Micronucleus test; Comet assay; V79 cells; Methyl methanesulfonate

1. Introduction

The research and the discovery of natural factors or human diet compounds that may prevent the genetic effects of mutagens and carcinogens have assumed great importance [1], mainly due to the perceived risk of oncogenesis.

Agaricus blazei Murrill, an edible mushroom, is native to southern Brazil. Since 1965, the strains have been exported from Brazil to Japan, where this mushroom has become popularly known as “Himematsutake” or “Kawariharatake”. It has been widely cultivated and studied in Japan. At present, *Agaricus blazei* is often consumed as food and tea in

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different parts of the world, especially because of its imputed medicinal properties.

Popularly, this mushroom is used to combat physical and emotional stress, stimulate immunity, improve life quality of diabetics, reduce cholesterol, combat diseases such as osteoporosis and gastric ulcer, alleviate the bloodstream and digestive problems, and it is used as an effective antioxidant and anticarcinogen. Nevertheless, scientific knowledge about its biological properties is still insufficient.

The fruiting body of the Basidiomycete *A. blazei* was the source of linoleic acid (an unsaturated fatty acid that represents 70–78% of lipids total of its fruiting body), obtained in hexanic extracts, and *trans*-11-octadecadienoic, 13-hydroxy-*cis*-9 acid. This was isolated in chloroform:methanol (2:1) extracts. Linoleic acid was identified as the main substance with antimutagenic activity against the mutagen benzopyrene in *Salmonella* in the Ames assay. The second identified component has exhibited antibacterial activity but not antimutagenic capacity [2].

Various anticarcinogenic polysaccharides extracted from Basidiomycete fungus are known, the majority composed of chains of D-glucose in β-1,3 linkages. However, the main feature of the *A. blazei* is a unique D-glucan content consisting solely of unsubstituted β-1,6 linked residues associated with antitumor activity [3]. This sugar was part of the most active antitumor fraction isolated from its fruiting body. The antitumor fraction is obtained in the form of a protein–polysaccharide complex, named FIII-2-b, constituted as 50.2% carbohydrate and 43.3% protein. The antitumoral activity of this complex was confirmed later [4–6].

A protective effect of *A. blazei* teas was demonstrated in vivo against the clastogenicity induced by cyclophosphamide. Mice pre-treated with three different teas of the mushroom, obtained at 4, 21 and 60°C, showed a significant reduction in the frequencies of micronuclei in polychromatic erythrocytes and in reticulocytes, after 25 and 50 mg/kg of cyclophosphamide [7].

The present study assesses the mutagenic and/or antimutagenic potential of the *A. blazei* Murrill, in vitro, using both micronucleus (MN) and comet (single cell microgel electrophoresis) assays in V79 cells submitted to methyl methanesulfonate (MMS) treatments. The major interest is to provide evidence that the *A. blazei* may be a practical and effective protective agent against chemical mutagens and may be a natural source of antimutagenic substances.

2. Materials and methods

2.1. Extraction and preparation of teas

Ground dehydrated fruiting bodies of the cultured AB 96/97, AB 96/09 and AB 97/11 strains of *A. blazei* were kindly provided by Dr. A.F. da Eira from the Department of Agronomy of UNESP, Jaboticabal, SP (Brazil). The mushroom powder (5 g) was mixed with distilled water (200 ml) and after 10 min of agitation, this aqueous solution was incubated at different temperatures to isolate three different extracts: (a) ice-cold: the solution was maintained at 2–8°C for 1 h before filtering; (b) room temperature: the solution was maintained at 20–25°C, for 2 h before filtering; and (c) warm: the solution was heated at 60°C for 5 min, and filtered after cooling for 15 min at room temperature. These extracts were filtered first through common filter paper and, then, sterilized in 0.2 mm millipore filter paper. Aliquots of these extracts were frozen and later used at room temperature. Each tea was applied to cell cultures, in both assays, at three final dilutions: 100, 200, and 300 μl per 5 ml, named, respectively, as 0.05, 0.1 and 0.15% concentrations.

2.2. Culture and cell treatments

Chinese hamster lung V79 cells were grown as a monolayer in culture plastic flasks ($25\,cm^2$) in Ham's F10 nutrient mixture (GibcoBRL) plus Dulbecco's modified Eagle minimal essential medium (D-MEM–GibcoBRL) (1:1), supplemented with 10% fetal bovine serum (Cultilab) at 37°C in a BOD-type incubator. Under these conditions, the average cell cycle time was 14 h. The cells were cultivated for two complete cycles prior to the treatments with fresh complete medium containing the following test substances: (1) the alkylating agent MMS was used as the positive control treatment at final concentrations of 4×10^{-4} M in the micronucleus test and 1.6×10^{-4} M in the comet assay; (2) each mushroom extract, at each one of three final concentrations; (3) each mushroom extract co-treated with MMS (simultaneous

treatment); (4) each mushroom extract applied prior to MMS (pre-treatment); (5) each mushroom extract used immediately after MMS (post-treatment). One culture that was treated with the solvent PBS (pH 7.4) served as negative control in each experiment. The treatment duration with each test substance was 1 h in comet assay and 2 h in micronucleus test. The cells were washed twice with PBS (pH 7.4) after each treatment.

2.3. Chemicals

The alkylating agent methyl methanesulfonate (MMS, CAS: 66-27-3, Aldrich) was used as the DNA damaging agent. It was dissolved in sterilized Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS), pH 7.4. Cytochalasin-B (Cyt-B, CAS: 14930-96-2, Sigma) was diluted in dimethyl-sulfoxide (DMSO, CAS: 67-68-5, Merck) to get a stock solution of 300 μg/ml, which was kept at 4°C in the dark until use. The other main chemicals used were obtained from the following suppliers: normal melting point (NMP) agarose: Roth, Karlsruhe, Germany; low melting point (LMP) agarose (CAS: 9012-36-6): FMC, Rockland, MD, sodium *N*-lauroyl sarcosine (CAS: 7631-98-3) and EDTA (CAS: 60-00-4): Sigma.

2.4. Single cell gel electrophoresis test — the comet assay

The general procedure was carried out by the method described by Speit and Hartmann in 1999 [8], which is based on the original work of Singh et al. [9], and includes modifications introduced by Klaude et al. [10] as well as additional modifications.

V79 cells in early log-phase that had been seeded in 2.5 ml culture flasks (Nunc) were cultivated for 28 h (two complete cell cycles) in complete medium and treated for 1 h with each test substance, as described above. At the end of the treatments, cells were washed twice with 5 ml ice-cold PBS (pH 7.4) and trypsinized with 0.2 ml of a 0.025% solution. After about 2 min, this process was stopped by adding complete culture medium. The cells were collected by centrifugation at 900 rpm for 5 min and the pellet was gently resuspended in 1 ml of culture medium. An aliquot of 10 μl of the cell suspension was mixed with 120 μl of 0.5% LMP agarose at 37°C, and rapidly spread onto microscope slides pre-coated with 1.5% NMP agarose. Coverslips were added and the slides were allowed to gel at 4°C for 10 min. The coverslips were removed gently and the slides were then immersed in cold, freshly made lysing solution composed of 89 ml of a stock solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH set to 10.0 with ~8 g solid NaOH, 890 ml of distilled water and 1% sodium lauroyl sarcosine), plus 1 ml of Triton X-100 (Merck) and 10 ml of DMSO. Protected from light, the slides were placed at 4°C for 1 h. They were then placed in the gel box, positioned to the anode end, and left in a high pH (>13) electrophoresis buffer (300 mM NaOH per 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM, pH 10.0, EDTA) at 4°C for 20 min before electrophoresis to allow the DNA to unwind. The electrophoresis ran in an ice bath (4°C) for 20 min under 25 V and 300 mA. Afterwards, the slides were submerged in a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 15 min, dried at room temperature and fixed at 100% ethyl alcohol for 10 min. The slides were dried and stored, at least overnight, before staining. To stain, the slides were briefly rinsed in distilled water, covered with 30 μl of 1× ethidium bromide staining solution prepared from a 10× stock (200 μg/ml) and covered with a coverslip. The material was evaluated immediately at 400× magnification, using a fluorescence microscope (Nikon) with an excitation filter of 515–560 nm and a barrier filter of 590 nm.

2.5. Micronucleus assay

Test agents were added to 5 ml of fresh medium at 28 h (two cell cycles) after the initiation of culture of V79 cells which had been previously seeded in complete culture medium and stabilized in 25 cm^2 flasks, as described. Each one of the substances used in the six already cited treatments (negative control, positive control, mushroom extracts, simultaneous-, pre- and post-treatments) were applied for 2 h. At the end of the treatments, cells were washed twice with 5 ml ice-cold PBS (pH 7.4), and Cyt-B was added to fresh complete culture medium to get a final concentration of 3 μg/ml. It was continually used for 18 h to block cytokinesis and to yield binucleated cells. All assays described here were repeated independently three times.

The procedures employed for harvesting, slide preparation and staining were based on Salvadori et al. [11]. At harvest time, the cells were twice

rinsed with 5 ml PBS (pH 7.4), trypsinized (0.025% trypsin) and centrifuged for 5 min at 900 rpm. The pellet was resuspended in ice-cold hypotonic solution (1% sodium citrate) plus one drop of 40% formaldehyde and then carefully homogenized with a Pasteur pipette. This cell suspension was centrifuged under the same conditions and the pellet resuspended in methanol:acetic acid 3:1, and once more homogenized with a Pasteur pipette. The cell suspension then was centrifuged and resuspended in some drops of methanol:acetic acid 3:1. Fixed cells were then dropped onto slides previously cleaned and covered with a film of ice-cold distilled water. They were stained in 2% Giemsa dissolved in phosphate buffer (0.06 M Na_2HPO_4 and 0.039 M KH_2PO_4, pH 7.0) for 10 min, washed with water, dried and kept at 4°C until microscopic analysis.

2.6. Frequencies and scoring procedures and data evaluation

The extent and distribution of DNA damage indicated by the comet assay was evaluated by examining at least 50 randomly selected and non-overlapping cells on the slides per treatment. These cells were scored visually according to tail size into four classes: (1) class 0: undamaged, without tail; (2) class 1: with a short tail of length smaller than the diameter of the head (nucleus); (3) class 2: with a tail length between 1 and 2× the diameter of the head; and (4) class 3: with a long tail of length superior to 2× the diameter of the head. Comets with no heads and images with nearly all DNA in the tail, or with a very wide tail, were excluded from evaluation because they may represent dead cells [12]. The total score for 50 comets was obtained by multiplying the number of cells in each class by the damage class, ranging from 0 (all undamaged) to 150 (all maximally damaged). Differences between negative or positive controls and treated samples were considered important when they were equal to, or superior to, 50%.

To evaluate micronucleus induction, a minimum of one thousand binucleated cells with well preserved cytoplasm were scored per coded slide in each experimental repetition. The criteria for the identification of binucleated cells and micronuclei [13,14] were as follows: (a) both nuclei and micronuclei should be round; (b) micronuclei should be smaller than one-third of the main nuclei; (c) micronuclei must not touch the main nuclei; (d) micronuclei must not be refractive and should be the same color and intensity as the main nuclei. The results from the three independent tests were summed and the statistically significant differences between controls and treated values were determined with the χ^2-test [15].

The reduction percentage in the number of cells with micronuclei in the treatments with the mushroom extracts was calculated according to Manoharan and Banerjee [16] and Waters et al. [17] by the following formula:

$$\text{reduction (\%)} = \frac{\text{number of cells with micronuclei in A} - \text{number of cells with micronuclei in B}}{\text{number of cells with micronuclei in A} - \text{number of cells with micronuclei in C}}$$

where A is the group of cells treated with MMS (positive control); B the group of cells with the extract plus MMS; and C the negative control.

3. Results

3.1. Mutagenicity assays of the A. blazei extracts

Table 1 shows the effects of a 1 h treatment with the mushroom teas on DNA migration in the comet assay in V79 cells. No effects on DNA migration were found in the three kinds of extracts or in their three concentrations. The score of treated cells was similar or increased less than 50% when compared with the negative control. When cells were exposed to three concentrations of the three test extracts, the majority of cells seen in slides was of undamaged cells and there was a reduced number of cells with a large amount of damage. In addition, when cells were exposed to concentrations of 0.1 and 0.15% of the ice-cold tea and to 0.1% concentration of the room temperature tea, the smaller scores were produced, with values under that of negative control, and cells with the maximum DNA migration were not observed on these slides. The biological significance of these results was not completely clear, but might characterize a protective effect

Table 1
DNA migration in the comet assay for genotoxicity assessment of *A. blazei* teas in V79 cells in vitro[a]

Treatments	Comet class				Scores
	0	1	2	3	
PBS (negative control)	38	5	3	4	23
MMS (positive control)	0	27	14	9	82
A. blazei					
Tea 1 (0.05%)	34	11	2	3	24
Tea 1 (0.1%)	33	11	1	5	28
Tea 1 (0.15%)	32	11	4	3	28
Tea 2 (0.05%)	41	5	1	3	16
Tea 2 (0.1%)	49	1	0	0	1
Tea 2 (0.15%)	42	6	2	0	10
Tea 3 (0.05%)	33	8	4	5	31
Tea 3 (0.1%)	46	4	0	0	4
Tea 3 (0.15%)	39	8	0	3	17

[a] *A. blazei*: tea 1, warm (60°C); tea 2, ice-cold (2–8°C); tea 3, at room temperature (20–25°C).

of these substances against the DNA damage yield at a basal level. MMS used as positive control demonstrated the sensitivity of the comet assay and gave a clear positive response in the concentration used.

In accordance with the negative effect in the comet assay, a clear negative mutagenic effect on induction of micronuclei was also found. The results of a 2 h treatment with each one of the tested *A. blazei* teas, after three replications, are summarized in Table 2. Significantly reduced micronuclei frequencies were obtained in all cases compared to the positive control level. They showed no statistically significant differences from the negative control. The data clearly showed that the tested mushroom teas had no effect on the induction of micronuclei in V79 cells.

3.2. Antimutagenicity assays of the A. blazei extracts

Table 3 shows the effects of 1 h simultaneous treatment of MMS and each one of *A. blazei* extracts in the comet assay. While DNA migration was clearly enhanced with MMS over the negative control, no relevant reduction of DNA damaging effects of MMS occurred in the presence of the three mushroom extracts in the range of concentrations tested. These data showed that co-treatment with these extracts did

Table 2
MN frequencies in V79 cells in vitro for the assessment of mutagenicity in *A. blazei* teas[a]

Treatments	Number of cells analyzed	MN	
		Number	%
PBS (negative control)	3000	14	0.47
MMS (positive control)	3000	53	1.77
A. blazei			
Tea 1 (0.05%)	3088	20*	0.65
Tea 1 (0.1%)	3000	24**	0.80
Tea 1 (0.15%)	3000	12*	0.40
Tea 2 (0.05%)	3030	12*	0.40
Tea 2 (0.1%)	3018	5***	0.16
Tea 2 (0.15%)	3031	11*	0.36
Tea 3 (0.05%)	3020	14*	0.46
Tea 3 (0.1%)	3003	13*	0.43
Tea 3 (0.15%)	3012	10*	0.33

[a] *A. blazei*: tea 1, warm (60°C); tea 2, ice-cold (2–8°C); tea 3, room temperature (20–25°C).
* $P > 0.5$.
** $P > 0.1$.
*** $P > 0.05$.

not protect the V79 cells against the genotoxic effects of MMS seen in the comet assay. Table 4 shows data on pre-treatment with mushroom extracts. In this case, MMS alone also induced a great increase (above 50%) of comet scores compared to the negative control. However, some tea concentrations (0.1 and 0.15% warm tea, 0.1% ice-cold tea and 0.05% room temperature tea) showed an efficient reduction in damage (above 50%). Although, the DNA damage level had not reached the negative control level in all the conditions and tea concentrations in this pre-treatment, some concentrations showed a potential antigenotoxic action against the MMS effects. The effects of post-treatment with mushroom extracts on DNA migration in the comet assay are shown in Table 5. There was no important difference in results observed with the extracts and concentrations tested versus the MMS treatment, because the reductions obtained were always less than 50% of the positive control level. This observation was suggested that *A. blazei* extracts were an ineffective antigenotoxic agent in post-treatment to MMS.

Table 6 summarizes the influence of mushroom teas on the mutagenic effects of MMS evaluated through

Table 3
Effect of co-treatment with *A. blazei* teas and MMS on DNA migration in the comet assay for antigenotoxicity assessment in V79 cells in vitro[a]

Treatments	Comet class				Scores
	0	1	2	3	
PBS (negative control)	42	7	1	0	8
MMS (positive control)	0	1	12	37	136
A. blazei					
Tea 1 (0.05%) + MMS	0	5	13	32	127
Tea 1 (0.1%) + MMS	0	5	10	35	130
Tea 1 (0.15%) + MMS	0	18	15	17	99
Tea 2 (0.05%) + MMS	0	6	24	19	111
Tea 2 (0.1%) + MMS	0	2	14	34	132
Tea 2 (0.15%) + MMS	0	3	18	29	126
Tea 3 (0.05%) + MMS	0	0	7	43	143
Tea 3 (0.1%) + MMS	0	3	16	31	128
Tea 3 (0.15%) + MMS	0	16	17	17	101

[a] *A. blazei*: tea 1, warm (60°C); tea 2, ice-cold (2–8°C); tea 3, room temperature (20–25°C).

Table 5
Effect of post-treatment with *A. blazei* teas and MMS on DNA migration in the comet assay for antigenotoxicity assessment in V79 cells in vitro[a]

Treatments	Comet class				Scores
	0	1	2	3	
PBS (negative control)	28	15	4	3	32
MMS (positive control)	1	20	4	15	73
A. blazei					
Tea 1 (0.05%) + MMS	4	20	7	19	91
Tea 1 (0.1%) + MMS	11	16	10	13	75
Tea 1 (0.15%) + MMS	15	14	7	14	70
Tea 2 (0.05%) + MMS	8	29	8	5	60
Tea 2 (0.1%) + MMS	6	30	6	8	66
Tea 2 (0.15%) + MMS	23	13	6	8	49
Tea 3 (0.05%) + MMS	21	15	4	10	53
Tea 3 (0.1%) + MMS	22	21	2	5	40
Tea 3 (0.15%) + MMS	24	22	3	11	61

[a] *A. blazei*: tea 1, warm (60°C); tea 2, ice-cold (2–8°C); tea 3, room temperature (20–25°C).

micronuclei induction in simultaneous treatments. An increase of micronuclei frequencies was observed with MMS alone (positive control) but when this was associated with the mushroom extracts, a highly statistically significant decrease of frequencies was found at all concentrations studied. The interaction of both substances was assessed more accurately by quantification of the treatment-specific decrease of micronuclei. The data clearly showed that, all the tested teas have a strong protective effect against micronuclei induction by MMS in simultaneous treatment, by decreasing the micronuclei frequency

Table 4
Effect of pre-treatment with *A. blazei* teas and MMS on DNA migration in the comet assay for antigenotoxicity assessment in V79 cells in vitro[a]

Treatments	Comet class				Scores
	0	1	2	3	
PBS (negative control)	42	7	1	0	9
MMS (positive control)	4	27	16	3	68
A. blazei					
Tea 1 (0.05%) + MMS	21	21	7	1	38
Tea 1 (0.1%) + MMS	28	19	1	2	27
Tea 1 (0.15%) + MMS	28	16	4	2	30
Tea 2 (0.05%) + MMS	20	19	9	2	43
Tea 2 (0.1%) + MMS	23	22	4	1	33
Tea 2 (0.15%) + MMS	14	19	15	2	55
Tea 3 (0.05%) + MMS	33	13	2	2	23
Tea 3 (0.1%) + MMS	3	22	19	6	78
Tea 3 (0.15%) + MMS	1	35	20	4	87

[a] *A. blazei*: tea 1, warm (60°C); tea 2, ice-cold (2–8°C); tea 3, room temperature (20–25°C).

Table 6
MN frequencies in V79 cells in vitro for antimutagenicity assessment after co-treatment of *A. blazei* teas with MMS[a]

Treatments	Number of cells analyzed	MN		Reduction (%)
		Number	%	
PBS (negative control)	3000	14	0.47	
MMS (positive control)	3000	53	1.77	
A. blazei				
Tea 1 (0.05%) + MMS	3016	17	0.56*	92.3
Tea 1 (0.1%) + MMS	3024	13	0.43*	102.6
Tea 1 (0.15%) + MMS	3019	10	0.33*	110.3
Tea 2 (0.05%) + MMS	3032	14	0.46*	100.0
Tea 2 (0.1%) + MMS	3023	13	0.43*	102.6
Tea 2 (0.15%) + MMS	3056	10	0.33*	110.3
Tea 3 (0.05%) + MMS	3048	14	0.46*	100.0
Tea 3 (0.1%) + MMS	3015	12	0.40*	105.1
Tea 3 (0.15%) + MMS	3035	14	0.46*	100.0

[a] *A. blazei*: tea 1, warm (60°C); tea 2, ice-cold (2–8°C); tea 3, room temperature (20–25°C).
* $P > 0.001$.

obtained for the positive control by at least about 90%, and by reaching the levels corresponding to the spontaneous micronuclei frequency. When used in pre-treatment protocols, the *A. blazei* extracts, at each concentration studied, also produced a statistically significant decrease in the micronucleus frequency induced by the mutagen (Table 7). Under this pre-treatment condition, the percentage of induction reduction was less than simultaneous treatment, ranging between 69.2 and 94.9%. Table 8 shows the results of the post-treatment with the mushroom extracts compared the to DNA damage inducer. Again, all types of extracts and concentrations decreased the frequency of micronuclei induced by the MMS to

Table 7
MN frequencies V79 cells in vitro for antimutagenicity assessment after pre-treatment with *A. blazei* teas with MMS[a]

Treatments	Number of cells analyzed	MN		Reduction (%)
		Number	%	
PBS (negative control)	3000	14	0.47	
MMS (positive control)	3000	53	1.77	
A. blazei				
Tea 1 (0.05%) + MMS	3024	16	0.53*	94.9
Tea 1 (0.1%) + MMS	3020	25	0.83**	71.8
Tea 1 (0.15%) + MMS	3001	18	0.60*	89.7
Tea 2 (0.05%) + MMS	3036	19	0.62*	87.2
Tea 2 (0.1%) + MMS	3036	26	0.86**	69.2
Tea 2 (0.15%) + MMS	3158	21	0.66*	82.1
Tea 3 (0.05%) + MMS	3017	23	0.76*	76.9
Tea 3 (0.1%) + MMS	3013	23	0.76*	76.9
Tea 3 (0.15%) + MMS	3033	19	0.63*	87.2

[a] *A. blazei*: tea 1, warm (60°C); tea 2, ice-cold (2–8°C); tea 3, room temperature (20–25°C).
* $P > 0.001$.
** $0.01 < P < 0.001$.

Table 8
MN frequencies in V79 cells in vitro for antimutagenicity assessment after post-treatment of *A. blazei* teas with MMS[a]

Treatments	Number of cells analyzed	MN		Reduction (%)
		Number	%	
PBS (negative control)	3000	14	0.47	
MMS (positive control)	3000	53	1.77	
A. blazei				
Tea 1 (0.05%) + MMS	3074	23	0.75*	76.9
Tea 1 (0.1%) + MMS	3014	15	0.50*	97.4
Tea 1 (0.15%) + MMS	3020	13	0.43*	102.6
Tea 2 (0.05%) + MMS	3068	25	0.81*	71.8
Tea 2 (0.1%) + MMS	3035	23	0.76*	76.9
Tea 2 (0.15%) + MMS	3021	14	0.46*	100.0
Tea 3 (0.05%) + MMS	3002	20	0.67*	84.6
Tea 3 (0.1%) + MMS	3050	29	0.95**	61.5
Tea 3 (0.15%) + MMS	3005	23	0.76*	76.9

[a] *A. blazei*: tea 1, warm (60°C); tea 2, ice-cold (2–8°C); tea 3, room temperature (20–25°C).
* $P > 0.001$.
** $0.01 < P < 0.001$.

statistically significant levels. In this post-treatment condition, the protective effects also were somewhat less pronounced than simultaneous treatment (reduction percentage ranged from 61.5 to 102.6%). There were no statistically significant differences in micronucleus frequencies obtained on cell cultures treated with the mushroom extracts in all three treatment conditions to which the cells were exposed compared to the negative control cultures.

4. Discussion

Most of results of the micronucleus assay clearly showed that all tested mushroom extracts have strong antimutagenic potential regardless of the combination form with the mutagen. The most effective protective activity against micronucleus production, however, was detected in the simultaneous treatment. This higher protection may be explained by the continuous presence of the extract compounds in cells throughout the action time (2 h) of the mutagenic agent. The results of this protocol may also include some effect of pre- or post-treatments. A protective effect of the same *A. blazei* teas has been demonstrated previously in vivo against the clastogenicity induced by cyclophosphamide [7].

Furthermore, the results in the present study clearly showed that the mushroom extracts had a partial antigenotoxic potential. The mushroom teas repressed the genotoxic potency of MMS, as measured by the comet assay, only in the pre-treatment, at some preparations and concentrations. It might be assumed that the exposure time to the mutagen or extracts (1 h, in this assay) was insufficient for the cells to express the effects of each type of treatment. However, even though the antigenotoxic effects of the extracts seemed to be under expressed, the results between negative and positive controls were clearly different, indicating that the genotoxic effect occurred within this period.

According to De Flora [18], it is essential not only to assess the efficacy and safety of putative inhibitors by using a variety of test systems, but also to understand the mechanisms involved, for a rational implementation of chemoprevention strategies. In this context, in the present study, three experimental designs (simultaneous-, pre- and post-treatments) were applied in relation to MMS to better investigate the possible antimutagenicity/antigenotoxicity mechanisms of the *A. blazei* extracts. The post-treatment could show its antimutagenic potential, by playing a role in optimization of DNA repair. The results obtained in the pre-treatment could reflect the effects of the teas on the prevention of DNA damage,

by affecting metabolic pathways, being antioxidant or acting on DNA replication. These action mechanisms, occurring in both post- and pre-treatments, were called bio-antimutagenicity [19,20] or fidelogenesis [21]. The use of a simultaneous treatment appeared to identify mechanisms with direct action on the mutagen by inactivating it, which were classified as of desmutagenicity effects according to Kada and collaborators [19,20]. The highest reductions in micronuclei produced by simultaneous treatment and the most effective action of pre-treatment on comet production suggested that both direct or indirect protective mechanisms might be involved in countering MMS-induced mutagenesis.

The obtained data indicated that natural products such as mushroom extracts may yield a wealth of commercially available antimutagenic agents.

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