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Letinula edodes (Berk.) Pegler (Shiitake) modulates genotoxic and mutagenic effects induced by alkylating agents in vivo

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Abstract

We evaluated the antimutagenic effect of *Letinula edodes* (Berk.) Pegler (Shiitake) on the frequency of micronuclei in mice treated with *N*-ethyl-*N*-nitrosourea (ENU) or cyclophosphamide (CP). Mice were orally (gavage) pretreated for 15 consecutive days with solutions of Shiitake (0.6 ml per day, gavage) prepared at three different temperatures: 4, 21 (RT), and 60°C. Then, the animals were intraperitoneally injected on day 15 with CP (25 or 50 mg/kg) or ENU (50 mg/kg) and killed 24 or 48 h after treatment for evaluation of micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow and micronucleated reticulocytes (MNRETs). A mixture of *L. edodes* lineages (LE 95/016, 96/14, 96/17, 96/22, 96/23, 97/27, and 97/28) significantly decreased the frequencies of MNPCEs and MNRETs induced by CP (25 and 50 mg/kg). When a single lineage from the mixture (LE 96/17) was tested we also found a significant reduction in the frequencies of MNPCEs and MNRETs induced by both CP or ENU (50 mg/kg). The comet assay was also performed 3 h after ENU treatment using mice pretreated with the single lineage (LE 96/17) of *L. edodes*. The results showed a high degree of variability with some indications of an antigenotoxic effect. Taken together, our data show that solutions from Shiitake inhibit in vivo mutagenicity of CP and ENU.

Keywords: Letinula edodes; Antimutagenicity; *N*-ethyl-*N*-nitrosourea; Cyclophosphamide; Micronucleus test; Comet assay

1. Introduction

Chemoprevention is a strategy used to reduce the incidence of human cancer either by inhibiting the initiation and spread of carcinogenesis or by preventing exposure to high levels of carcinogens [1,2]. Many dietary constituents markedly influence or alter the adverse effects of carcinogenic agents [3–6]. Therefore, attempts to prevent mutation-related diseases such as cancer have focused on identifying dietary agents that can inhibit mutagenesis or carcinogenesis produced by chemical and physical agents in vivo [5,7].

Because extracts of other kinds of mushrooms that are also frequently used in folk medicine have been shown to possess antimutagenic properties [8], we were interested in investigating a possible antimutagenic effect of *Letinula edodes* in vivo. *Letinula edodes* has been shown to contain compounds that exert

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a protective effect against carcinogenesis [9–13]. The first antitumor compound isolated from this mushroom was a large polysaccharide, identified as lentinan [12]. Although the mechanism of its antitumor action is still not completely clear, it is suggested that lentinan inhibits the tumorigenesis mainly by acting as a host-defense potentiator activating immune cells and inducing gene expression of immunomodulatory cytokines and their receptors [14,15]. An antimutagenic effect of lentinan in combination with antineoplastic agents in vivo was also reported. Lentinan is not only useful for cancer treatment as an immunopotentiator in combination with anti-cancer drugs, but may also prevent the increase of chromosomal damage induced by anti-cancer drugs in vivo [16]. KS2, a polysaccharide also isolated from the mushroom, has been shown to have antitumor activity against induced sarcoma 180 and Ehrlich ascite tumors in mice [17]. Another polysaccharide which has been isolated from Shiitake mycelium, designated LAP1, suppressed liver cancers and ascite tumors in rats [18]. Experimental evidence which defines the interrelationship between mutagen exposure, Shiitake intake, and mutation frequency is very limited. Thus, the importance of evaluating the effects of Shiitake on mutations induced by chemical or physical agents is warranted. Information regarding the antimutagenic effects of Shiitake in vivo are crucial to the understanding of its chemopreventive role in carcinogenesis. Therefore, the present study was designed to investigate the ability of *L. edodes* to modulate the genotoxic effects induced by the alkylating agents CP and ENU in mice using both the micronucleus test and the comet assay.

2. Material and methods

2.1. Animals

Male Swiss mice aged 7–8 weeks (weighting 35–40 g), were obtained from the breeding colonies of Universidade Estadual Paulista, UNESP, Botucatu, SP, Brazil for the first experiment and from Centro de Bioterismo, Universidade de Campinas, SP, Brazil for the second experiment. The animals were kept in plastic cages in an experimental room under controlled conditions of temperature $(23 \pm 2)^{\circ}$C, humidity (50 ± 10)%, 12 h light/dark cycle, and with ad libitum access to diet and water. The animals were randomized at the beginning of the experiment.

2.2. Chemicals

The chemical agents used were cyclophosphamide (CP) — Genuxal, Asta Médica, Lot. No. 807034, dissolved in 0.9% NaCl and *N*-ethyl-*N*-nitrosorea (ENU) — Sigma Chemical Co., St. Louis, MO, Lot. No. 107HO389, dissolved in phosphate buffer (pH 6.0) and 2% Tween 80.

2.3. Mushroom

In experiment 1, we used a mixture of *L. edodes* lineages (LE 95/016, 96/14, 96/17, 96/22, 96/23, 97/27, and 97/28) in order to evaluate the effect of this mushroom because it is widely used as a complex mixture as food or folk medicine. In experiment 2, one single lineage from the mixture was studied to evaluate the contribution of this lineage to the total effect induced by the mixture.

2.4. Preparation of mushroom solutions

The mushroom *L. edodes* (Shiitake) was obtained from Faculdade de Ciências Agronômicas, Botucatu, SP, Brazil. *Letinula edodes* aqueous solution (2.5%) was prepared at three different temperatures (5 g of powdered mushroom was added to 200 ml of filtered water). For solution 1, cold (4°C) water was used and was left resting for 1 h before filtering. For solution 2, room temperature (21°C) water was used for the extraction with a resting period of 2 h. In solution 3, water was warmed to (60°C) after which the powder was added and the temperature was maintained (thermometer) for 15 min before filtering. All solutions were used at room temperature. The extracts were administered p.o., by gavage (0.6 ml per day per animal) once a day, daily, during the experimental period.

The total amount of mushroom extract delivered per animal per day was about 0.005 g. The extracts were prepared in the same way for the different experiments.

2.5. Micronucleus test (MNT)

The micronucleus test was conducted according to the methodologies described by MacGregor et al.

[19] for bone marrow, and by Hayashi et al. [20] for peripheral blood. The number of micronucleated cells was determined among 1000 polychromatic erythrocytes (PCEs) and 1000 retyculocytes (RETs) per animal. The slides were scored blindly using a light microscope with a 100× immersion objective for PCEs, and a fluorescent microscope — the combination of a blue excitation (e.g. 488 nm) and a yellow to orange barrier filter (e.g. 515 nm long pass) with a 40× objective for RETs.

The percentage of reduction in the frequency of MN after treatments with the mushroom solutions, was calculated according to Manoharan and Banerjee [21] and Waters et al. [22], by the following formula:

$$\text{reduction (\%)} = \frac{\text{frequency of MN in A} - \text{frequency of MN in B}}{\text{frequency of MN in A} - \text{frequency of MN in C}} \times 100$$

where A is the group treated with CP (positive control), B the group treated with mushroom solutions plus CP and C the group treated with NaCl (negative control).

2.6. Comet assay (single-cell gel electrophoresis assay)

Peripheral blood samples were collected with a microcapillary from a periorbital vein plexus 3 h after the ENU treatment. The comet assay was performed according to Speit and Hartmann [23], using 20 min each for alkali denaturation (pH > 13) and electrophoresis. Fifty randomly selected cells per animal were evaluated at 200× magnification in a Olympus epi-fluorescence microscope equipped with a green filter. Using an image-analysis system (Comet Assay II, Perceptive Instruments), two parameters were selected as indicators of DNA damage: tail length and tail moment.

3. Experimental designs

3.1. Experiment 1

3.1.1. MNT with a mixture of lineages from Letinula edodes

In order to investigate the antimutagenic effect of Shiitake (mixture of lineages LE 95/016, 96/14, 96/17, 96/22, 96/23, 97/27, and 97/28) in animals after acute exposure to two different concentrations of an indirect alkylating agent (CP 25 and 50 mg/kg b.w.), animals were divided into 12 groups with 12 animals per group (Fig. 1).

In group 1 (a and b) mice received only drinking water (0.6 ml per day, by gavage) for 2 weeks before treatment with 0.9% NaCl by intraperitoneal (i.p.) injection. Groups 2 (a and b) and 3 (a and b) also received drinking water (0.6 ml per day, by gavage) for 2 weeks, but were treated on day 15 with CP (group 2: 25 mg/kg b.w., i.p.; group 3: 50 mg/kg b.w., i.p.). Groups 4, 5, and 6 (a and b), and 7, 8, and 9 (a and b) received solutions of Shiitake administered orally (0.6 ml per day, by gavage) prepared at three different temperatures: 4°C (groups 4, 7), 21°C (groups 5 and 8), and 60°C (groups 6 and 9), for 2 weeks before the treatment with CP (groups 4, 5, and 6: 25 mg/kg b.w., i.p.; groups 7, 8, and 9: 50 mg/kg b.w., i.p.). Groups 10, 11, and 12 (a and b) received only solutions of Shiitake administered orally (0.6 ml per day, by gavage) prepared at three different temperatures: 4, 21, and 60°C, respectively, before the 0.9% NaCl i.p. injection on day 15. The animals were killed either 24 h (groups 1a, 2a, 3a, 4a, 5a, 6a, 7a, 8a, 9a, 10a, 11a, and 12a) or 48 h after treatment (groups 1b, 2b, 3b, 4b, 5b, 6b, 7b, 8b, 9b, 10b, 11b, and 12b) for evaluation of micronucleated polychromatic erythrocytes (MN-PCEs) in bone marrow and micronucleated retyculocytes (MNRETs) in peripheral blood, respectively.

3.2. Experiment 2

3.2.1. MNT with a single lineage from Letinula edodes

This experiment was performed to determine the antimutagenic effect of one single lineage of Shiitake (LE 96/17) contained in the mixture. In these tests ENU and CP were used as directly and indirectly-acting DNA alkylating agents, respectively. The animals were divided into 12 groups with 20 animals per group (Fig. 2).

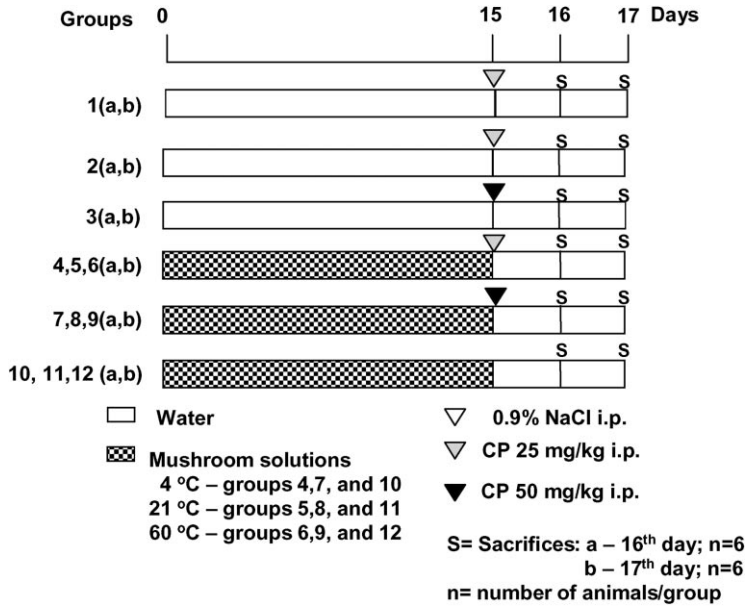

Fig. 1. Experimental design to evaluate the effects of *Letinula edodes* mushroom (mixture of different lineages) on the induction of micronuclei by cyclophosphamide (Experiment 1).

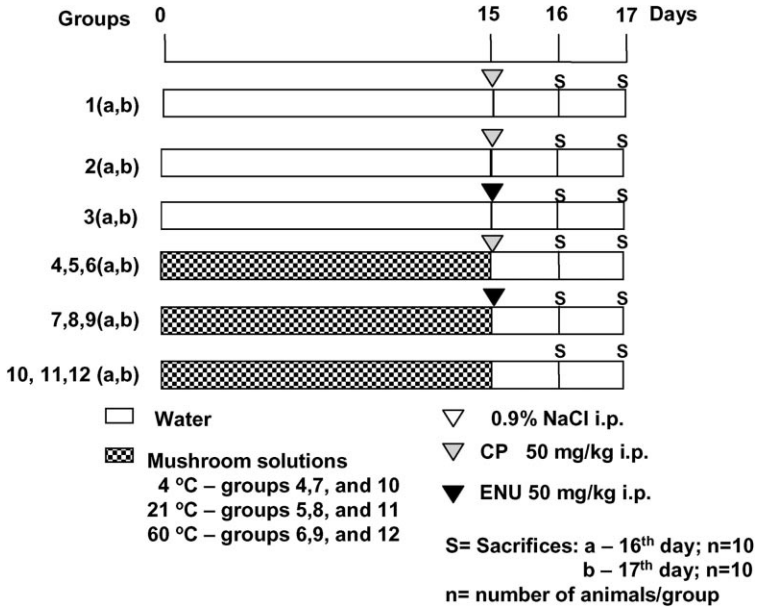

Fig. 2. Experimental design to test the antimutagenicity of mushroom *Letinula edodes* (single lineage) on mutagen-induced micronuclei and comet assay effects (Experiment 2).

Group 1 (a and b) received only drinking water (0.6 ml per day, by gavage) for 2 weeks before treatment with 0.9% NaCl by intraperitoneal (i.p.) injection. Group 2 (a and b) and 3 (a and b) also received drinking water (0.6 ml per day, by gavage) for 2 weeks, and were treated with CP (50 mg/kg b.w., i.p.) or ENU (50 mg/kg b.w., i.p.), respectively, on day 15. Groups 4, 5, and 6 (a and b) and groups 7, 8, and 9 (a and b) received solutions of Shiitake prepared at three different temperatures: 4°C (groups 4 and 7), 21°C (groups 5 and 8) and 60°C (groups 6 and 9) administered orally (0.6 ml per day, by gavage) for 2 weeks before treatment with CP (50 mg/kg b.w., i.p.) or with ENU (50 mg/kg b.w., i.p.), respectively, on day 15. Groups 10, 11, and 12 (a and b) only received treatment with the mushroom solutions prepared at three different temperatures: 4, 21, and 60°C, respectively, before the 0.9%NaCl i.p. injection on day 15.

The animals were killed by cervical dislocation, 24 h (group 1a, 2a, 3a, 4a, 5a, 6a, 7a, 8a, 9a, 10a, 11a, and 12a) or 48 h (groups 1b, 2b, 3b, 4b, 5b, 6b, 7b, 8b, 9b, 10b, 11b, and 12b) after treatment, for the evaluation of MNPCEs in bone marrow and micronucleated reticulocytes (MNRETs) in peripheral blood, respectively.

3.2.2. Comet assay with a single lineage from Letinula edodes

The comet assay was performed in groups 1, 3, 7, 8, 9, 10, 11, and 12. Samples of peripheral blood were collected from the periorbital vein plexus 3 h after treatment with ENU.

4. Statistical analysis

The MNT data were statistically analyzed by the Chi-square test [24], and for the comet assay, the significance level considered was $P < 0.05$ by Student's *t*-test [25].

Table 1
The effect of *Letinula edodes* (mixture of seven lineages) on the frequencies of MNPCEs in the bone marrow of mice after treatment with CP[a]

Treatment	No. of analyzed cells	MNPCEs		Reduction (%)
		No.	%	
Water + 0.9% NaCl[b]	6000	10	0.16	
Water + CP (25 mg/kg)[c]	5000[d]	69	1.38	
L. edodes				
Solution 1 + CP	5000[d]	29	0.58**	68
Solution 2 + CP	5000[d]	43	0.83*	44
Solution 3 + CP	6000	65	1.08	7
Water + CP (50 mg/kg)[c]	4930[d]	87	1.76	
L. edodes				
Solution 1 +CP	5000[d]	71	1.42	21
Solution 2 + CP	5000[d]	47	0.94**	52
Solution 3 + CP	5000[d]	79	1.58	10
L. edodes				
Solution 1	4000[e]	4	0.10	
Solution 2	6000	7	0.11	
Solution 3	5000[d]	8	0.16	

[a] *L. edodes* — solution 1: 4°C; solution 2: 21°C (RT); solution 3: 60°C.
[b] Negative control.
[c] Positive controls.
[d] One animals died.
[e] Two animals died.
* $P < 0.05$.
** $P < 0.01$.

5. Results

5.1. Experiment 1

Tables 1 and 2 show the frequencies of MN in PCEs and in RETs, respectively, in mice treated with mixtures of lineages of *L. edodes* and, thereafter, with 25 or 50 mg/kg CP. The results show that there was a statistically significant reduction both in the frequencies of MN in PCEs and RETs in animals treated with mushroom solutions. All three solutions prepared at different temperatures promoted protection against clastogenicity induced by CP. This antimutagenic effect was not clearly dependent on the temperature at which the solution was prepared, although the solution prepared at 60°C always had the smallest effect. The results also show that the frequencies of MN in PCEs and RETs of animals treated only with mushroom solutions are not significantly different from those of untreated controls.

5.2. Experiment 2

5.2.1. MNT with a single lineage — LE 96/17

Tables 3 and 4 show the frequencies of MN in PCEs and in RETs, respectively, in mice treated with three solutions prepared at different temperatures from the isolated lineage of *L. edodes*, and thereafter with CP or ENU. The results show that there was a statistically significant reduction of mutagen-induced MN in both PCEs and RETs after treatment with mushroom solutions. Reduced MN frequencies were found for all solutions prepared at three different temperatures, but there seems to be no direct relationship between the temperature and the effect suggesting that all three solutions of this lineage protect against the

Table 2
The effect of *Letinula edodes* mushroom (mixture of seven lineages) on the frequencies of MNRETs in peripheral blood of mice after treatment with CP[a]

Treatment	No. of analyzed cells	MNRETs		Reduction (%)
		No.	%	
Water + 0.9% NaCl[b]	4000[e]	8	0.20	
Water + CP (25 mg/kg)[c]	6000	70	1.16	
L. edodes				
Solution 1 + CP	5000[d]	32	0.64**	61
Solution 2 + CP	5000[d]	38	0.76*	52
Solution 3 + CP	6000	45	0.75*	40
Water + CP (50 mg/kg)[c]	6000	116	1.93	
L. edodes				
Solution 1 + CP	6000	45	0.75***	66
Solution 2 + CP	6000	30	0.50***	80
Solution 3 + CP	5000[d]	61	1.22**	51
L. edodes				
Solution 1	6000	9	0.15	
Solution 2	6000	9	0.15	
Solution 3	6000	8	0.13	

[a] *L. edodes* — solution 1: 4°C; solution 2: 21°C (RT); solution 3: 60°C.
[b] Negative control.
[c] Positive controls.
[d] One animal died.
[e] Two animals died.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 3
The effect of *Letinula edodes* (LE 96/17) on the frequencies of MNPCEs in the bone marrow of mice after treatment with CP or *N*-ethyl-*N*-nitrosourea (ENU)[a]

Treatment	No. of analyzed cells	MNPCEs		Reduction (%)
		No.	%	
Water + 0.9% NaCl[b]	10000	36	0.36	
Water + CP (50 mg/kg)[c]	10000	360	3.60	
L. edodes				
Solution 1 + CP	10000	293	2.93*	21
Solution 2 + CP	10000	324	3.24	11
Solution 3 + CP	10000	220	2.20**	43
Water + ENU (50 mg/kg)[c]	10000	398	3.98	
L. edodes				
Solution 1 + ENU	10000	315	3.15*	23
Solution 2 + ENU	10000	394	3.94	1
Solution 3 + ENU	10000	308	3.08**	25
L. edodes				
Solution 1	10000	32	0.32	
Solution 2	10000	22	0.22	
Solution 3	10000	21	0.21	

[a] *L. edodes* — solution 1: 4°C; solution 2: 22°C (RT); solution 3: 60°C.
[b] Negative control.
[c] Positive controls.
* $P < 0.01$.
** $P < 0.001$.

clastogenicity induced by CP and *N*-ethyl-*N*-nitrosourea (ENU). The results also show that the frequencies of MN in PCEs and RETs of animals treated only with mushroom solutions are not significantly different from those of untreated controls.

5.2.2. Comet assay with a single lineage — LE 96/17

The comet assay results are summarized in Table 5. Mean values for tail moment and tail length are shown for 10 animals per group. Treatment of mice with solutions of *L. edodes* did not significantly influence DNA migration in the comet assay. However, considerable variation among animals was found. The influence of the mushroom solutions on mutagen-induced DNA damage in the comet assay was only tested in combination with ENU, which clearly induced DNA migration in all animals analyzed 3 h after the treatment. Pretreatment with *L. edodes* solutions prepared at 4 and 60°C significantly reduced ENU-induced DNA damage. No significant difference was found for pretreatment with the solution prepared at room temperature and there was considerable variation among the animals in all groups.

6. Discussion

Letinula edodes (Shiitake) has been shown to contain compounds with anticarcinogenic properties [9–17]. The mechanism of its anticarcinogenic action is still unknown but activation of the immune response seems to be involved [14,15]. Because extracts of other kinds of mushrooms that are also frequently used in folk medicine have been shown to possess antimutagenic properties [8], we were interested in investigating a possible antimutagenic effect of *L. edodes* in vivo. Our results clearly indicate that aqueous solutions extracted from *L. edodes* exhibit antimutagenic activity against the in vivo DNA-damaging effect of alkylating agents. A mixture of various lineages of the mushroom inhibited the induction of micronuclei by the indirectly acting alkylating agent

Table 4
The effect of *Letinula edodes* (LE 96/17) on the frequencies of MNRETs in peripheral blood of mice after treatment with CP or *N*-ethyl-*N*-nitrosourea (ENU)[a]

Treatment	No. of analyzed cells	MNRETs		Reduction (%)
		No.	%	
Water + 0.9% NaCl[b]	10000	30	0.30	
Water + CP (50 mg/kg)[c]	10000	341	3.41	
L. edodes				
Solution 1 + CP	10000	228	2.28***	36
Solution 2 + CP	10000	221	2.21***	38
Solution 3 + CP	10000	194	1.94***	47
Water + ENU (50 mg/kg)[c]	10000	226	2.26	
L. edodes				
Solution 1 + ENU	10000	185	1.85*	19
Solution 2 + ENU	10000	192	1.92[d]	16
Solution 3 + ENU	10000	162	1.62**	30
L. edodes				
Solution 1	10000	26	0.26	
Solution 2	10000	27	0.27	
Solution 3	10000	17	0.17	

[a] *L. edodes* — solution 1: 4°C; solution 2: 22°C (RT); solution 3: 60°C.
[b] Negative control.
[c] Positive controls.
[d] Tendency.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Table 5
The effect of *Letinula edodes* (LE 96/17) on DNA migration in the comet assay with peripheral blood of mice after treatment with ENU

Treatment	Tail moment ± S.D.	*P*-value	Tail length ± S.D.	*P*-value
0.9% NaCl	0.24 ± 0.08		17.89 ± 2.97	
L. edodes				
Solution 1	0.22 ± 0.12	0.653	15.73 ± 0.87	0.051
Solution 2	0.57 ± 0.64	0.166	17.84 ± 2.16	0.966
Solution 3	0.33 ± 0.19	0.328	16.06 ± 1.48	0.123
ENU (50 mg/kg)	2.70 ± 1.42		45.00 ± 18.73	
L. edodes				
Solution 1 + ENU	1.30 ± 1.17	0.027*	21.67 ± 14.06	0.006*
Solution 2 + ENU	2.67 ± 1.66	0.959	34.54 ± 11.48	0.153
Solution 3 + ENU	1.24 ± 0.95	0.015*	26.70 ± 6.44	0.014*

CP in the bone marrow of mice. This antimutagenic effect was also seen in peripheral blood confirming the results from the bone marrow MNT. Because this mushroom is widely used as food or in folk medicine, teas at different temperatures were tested in order to evaluate the possible influence of the kind of preparation on the antimutagenic effect. However, the results did not show any significant influence of the temperature at which the solutions were prepared on antimutagenic activity. Solutions prepared at three different

temperatures (4, 21, and 60°C) led to reduced micronucleus frequencies without showing systematic differences.

In a further attempt to characterize the antimutagenic potential of *L. edodes*, we studied a single lineage (LE 96/17) that was part of the mixture used in the previous tests. Evaluating the antimutagenic potential of isolated lineages should give an idea about the variability of the effect and open the possibility to use a lineage with a high activity and to characterize the active compound(s). Like the previous mixture, this lineage exhibited antimutagenic activity against the clastogenic action of CP. Again this protective effect seen in bone marrow as well as in peripheral blood cells was not clearly related to the temperature at which the solutions were prepared. Although the antimutagenic effects of the isolated lineage are somewhat smaller than those of the mixture, there seems to be no fundamental difference in the antimutagenic capacity of the two approaches. LE 96/17 was also found to have an antimutagenic effect on the induction of micronuclei by ENU. Because ENU is a directly acting alkylant that does not require metabolic activation, the antimutagenic activity of *L. edodes* is not due to an interaction with activating enzymes.

To further elucidate the mechanism of the antimutagenic action, we performed additional experiments with the comet assay. An antimutagenic effect in the MNT can principally be due to reduced induction of damage or increased repair. The comet assay measures the amount of DNA damage after treatment with a mutagen and thus can indicate if an antimutagenic effect is due to reduced formation of primary DNA damage. Unexpectedly, CP did not induce clear and reproducible DNA effects in the comet assay under the experimental conditions used here. DNA migration in the comet assay is mainly induced by strand breaks and alkali labile sites [23]. However, besides other lesions, CP also induces DNA crosslink lesions which are known to inhibit DNA migration in the comet assay [26]. Induction of DNA effects by CP in the comet assay has been demonstrated in vitro and in vivo [27]; but depending on the experimental conditions, this effect can be masked by CP-induced crosslinks. However, we were able to show a clear induction of DNA effects by ENU and could demonstrate that this effect can be reduced by pretreatment of the animals with aqueous solutions of *L. edodes*. Although a significant reduction of ENU-induced DNA damage was found for only two out of the three solutions tested, this result gives a first indication of the mode of the mushroom's antimutagenic action. It seems likely that pretreatment with *L. edodes* diminishes the induction of DNA damage by alkylating agents.

Taken together, our results suggest that aqueous solutions from *L. edodes* have antimutagenic properties and that the antimutagenic action might be involved in its anticarcinogenic effects. Potential differences in the antimutagenic activity of extracts from different lineages and possible seasonal influences still need to be established. Further studies must be conducted with purified substances to identify the components responsible for this protective effect of *L. edodes* and elucidate the precise mechanism of action.

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