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Antimutagenic effect of *Lentinula edodes* (BERK.) Pegler mushroom and possible variation among lineages

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Abstract

This study was performed to evaluate the efficiency of four different lineages (95/01, L1, 96/22 and JABK) of *Lentinula edodes* (BERK.) Pegler mushroom (shiitake) for inhibiting the *N*-ethyl-*N*-nitrosourea (ENU) clastogenicity in vivo. Male Swiss mice (10 animals/group) were treated during 15 consecutive days with dried mushroom added to basal diet under three different concentrations (1, 5 and 10%). At day 15, mice were intraperitoneally injected with ENU (50 mg/kg body weight) and sacrificed 24 h later for evaluation of micronucleated bone marrow polychromatic erythrocytes (MNPCE). Negative and positive controls (10 animals each), receiving basal diet and saline or ENU ip injection, respectively, were also evaluated. Results showed that pretreatments with diets containing the lineages 95/01, L1 and 96/22 reduce the frequencies of MNPCE induced by ENU. The absence of an antimutagenic activity for the lineage JABK might be related to intrinsic differences among the lineages such as biochemical composition. Taken together, our data show that the differences in protective activities of the mushrooms need to be clarified in further studies and the mechanisms for such activities need to be investigated.

Keywords: *Lentinula edodes*; Shiitake; Antimutagenicity; *N*-ethyl-*N*-nitrosourea; Micronucleus test

1. Introduction

In the past few decades the cultivation of mushroom expanded all over the world becoming an economically important agricultural industry (Kues and Liu, 2000).

Owing to the high levels of protein in mushrooms, their cultivation has been indicated as an alternative for protein source in developing countries with high nutritional deficiencies (Chang and Hayes, 1980; Wuest et al., 1987; Chang et al., 1992). Nowadays, mushrooms have been incorporated into tonics, teas, soups and healthy food dishes, as well as herbal formulas. Shiitake mushroom (*Lentinula edodes*) is the second most popular edible mushroom in the global market. Several important bioactive compounds such as protein (26% of dry weight), lipids (primarily linoleic acid), carbohydrates, fiber, minerals, vitamins (B1, B2 and C) and ergosterol have been isolated from this fungus (Ying et al., 1987; Terashita et al., 1990).

The possible use of basidiomycetes fungi fruiting bodies in cancer chemotherapy began in Austria with an observation that in small, somewhat isolated villages, self-sufficient in their food sources and with many residents older than 90 years, cancer was a non-prevailing disease (Volz, 1999). Other significant epidemiological evidence showed the correlation between daily mushroom consumption and a low rate of cancer mortality in Japan (Borchers et al., 1999).

In the last decades, many studies have pointed out that shiitake also contains active substances, which inhibit a number of different transplanted tumors in vivo. Polysaccharides with antitumor activity such as

Abbreviations: ENU, *N*-ethyl-*N*-nitrosourea; LEM, *L. edodes* mycelium; MNPCE, micronucleated polychromatic erythrocyte

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lentinan, *L. edodes* mycelium (LEM), and KS-2 (Wasser and Weiss, 1999) have been isolated from this fungus.

Lentinan, a β-glucan, was the first antitumor compound isolated from shiitake (Chiraha et al., 1970). Although its mechanism of action is not completely clear, lentinan inhibits tumorigenesis mainly by activating the immune system and inducing gene expression of immunomodulatory cytokines and their receptors (Borchers et al., 1999; Ooi and Liu, 2000). The protective effects of lentinan on DNA damage induced by antineoplastic agents in vivo were also reported (Hasegawa et al., 1989). Antitumoral activities of two polysaccharides, LEM and KS-2, extracted from the mushroom were also identified in rodents (Fujii et al., 1978; Wasser and Weiss, 1999).

Although some studies have reported the anticarcinogenic activity of shiitake, experimental evidence regarding its action on DNA is still limited. Recently we have described the antigenotoxicity and antimutagenicity of a *L. edodes* lineage (96/17) on chemically-induced mutagenesis, in vivo (Alves de Lima et al., 2001). Based on these findings, we sought to investigate whether other morphologically distinct *L. edodes* lineages (95/01, L1, 96/22 and JABK) exhibit the same antimutagenic effect. The micronucleus test in mouse bone marrow was chosen for the evaluation.

2. Material and methods

2.1. Animals

Male Swiss mice aged 7–8 weeks (35–40 g body weight) were obtained from the Centro de Bioterismo, UNICAMP, SP, Brazil. Animals were kept in plastic cages in an experimental room under controlled conditions of temperature (23±2 °C), humidity (50±10%), 12-h lightldark cycle, and ad lib. access to diet and water.

2.2. Chemical

The direct-acting alkylating agent, *N*-ethyl-*N*-nitrosourea (ENU) (Sigma Chemical Co., St Louis, MO, USA, Lot. No. 107HO389), dissolved in phosphate buffer (pH 6.0) and 2% Tween 80, was used to induce micronucleus in mouse bone marrow cells (positive control)

2.3. Mushroom

Four *L. edodes* lineages selected by phenotypic and geographic differences were supplied by the Faculdade de Ciências Agronomicas (UNESP, Botucatu, São Paulo, Brazil). The origin and agronomic characteristics (phenotypes) are listed below:

95/01—originated from Londrina, PR, Brazil, it is cultivated during the winter, is generally precocious and presents small fruit bodies (5–7 cm), is smooth and has a dark center and a white border.
96/22—re-isolated from the 95/01 basidiocarp and cultivated in eucalyptus log in Botucatu, SP, Brazil. It presents large fruit bodies (>9 cm), white border and center, thick stipes without peripheral white exfoliates, and it is also generally precocious.
L1—originated from Jaboticabal, SP, Brazil, it grows on logs in the summer, and presents medium-sized dark fruit bodies (7–9 cm), with a white substance, and it is generally precocious.

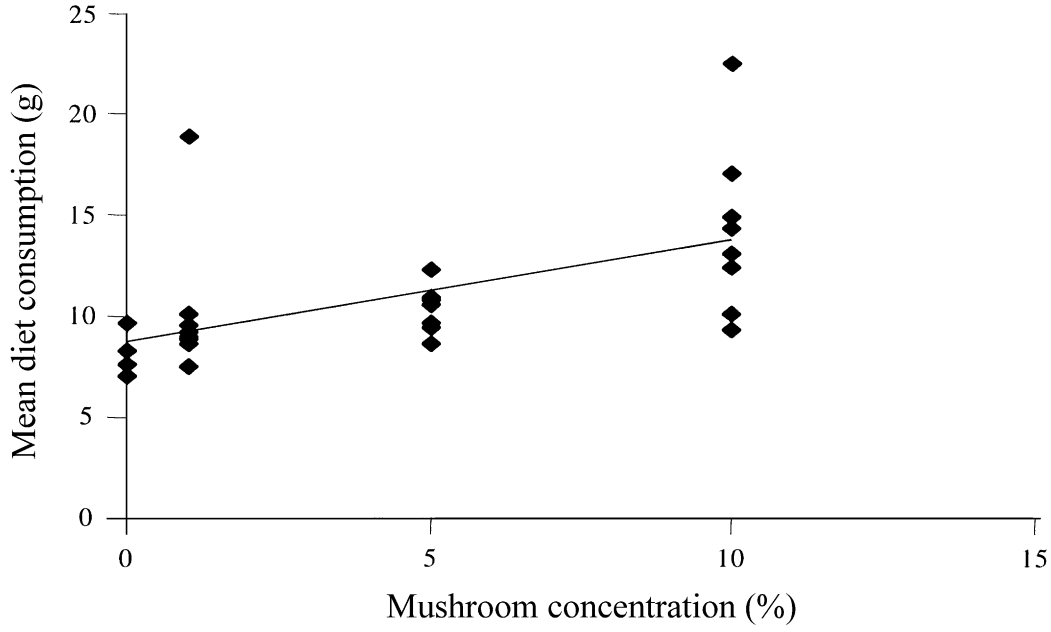

Fig. 1. Relationship between mushroom concentration in the diet and mean diet consumption.

JABK—originated from Jaboticabal, SP, Brazil, it is cultivated in hot climates, and presents large, fleshy and smooth fruit bodies (at least 9 cm), with a dark center and a light border, it has thick and short stipes, and late production.

2.4. Mushroom diets

L. edodes diets were prepared at three different concentrations 1, 5 and 10% of powdered mushroom added to basal diet. The diet was administered ad lib. for 15 days. Food consumption and body weight were evaluated during the experimental period.

2.5. Micronucleus test

The bone marrow micronucleus test was performed according to MacGregor et al. (1987). One thousand polychromatic erythrocytes were analyzed per animal. Slides were scored blindly using a light microscope with a 100× immersion objective.

The percentage of reduction in the frequency of micronucleated polychromatic erythrocytes (MNPCEs) was calculated according to Manoharan and Banerjee (1985) and Waters et al. (1990), using the following formula:

$$\%\ \text{Reduction} = \frac{\text{frequency of MNPCEs in A} - \text{frequency of MNPCEs in B}}{\text{frequency of MNPCEs in A} - \text{frequency of MNPCEs in C}} \times 100$$

where: A = group treated with ENU (positive control)
B = group treated with mushroom plus ENU
C = group treated with NaC1 (negative control)

Table 1
Food consumption (means±S.D.) after 15 days of treatment with three different concentrations of *Lentinula edodes*

Treatments	No. of animals	Body weight (g) mean±S.D.	Body weight gain (g) mean±S.D.	Ingested mushroom (g/kg body weight)	Food consumption (g/day/animal) mean±S.D.[a]
Basal diet + 0.9%NaCl[b,c]	10	34.1±3.67	1.6±1.41	–	7.02±0.59
Basal diet + ENU[d]	10	34.8±1.48	2.0±0.58	–	7.65±0.63
96/22 + ENU: 1%	10	37.6±1.83	2.6±1.46	2.39	9.54±0.67
5%	10	38.2±1.76	2.4±1.30	12.30	9.40±1.16
10%	10	35.1±1.27	1.8±1.18	35.00	12.38±3.58
L1 + ENU: 1%	10	34.9±1.13	1.6±1.42	5.15	18.85±2.16
5%	10	32.2±0.28	−0.2±1.23**	14.90	9.68±2.08
10%	10	35.5±1.69	2.4±0.92	36.61	13.08±0.99
JABK + ENU: 1%	10	34.2±2.26	3.1±0.77	2.04	7.47±1.42
5%	10	34.0±2.33	3.3±1.09*	13.80	9.47±1.73
10%	10	35.0±1.83	2.5±2.11	28.57	10.06±0.96
96/22: 1%	10	36.7±1.55	2.1±1.33	2.17	8.61±1.01
5%	10	35.6±1.48	2.0±0.98	11.79	8.59 ±0.47
10%	10	37.9±1.34	1.8±1.07	37.46	14.29±1.85
L1: 1%	10	30.8±1.20	1.7±0.91	2.59	8.89 ±0.61
5%	10	33.6±1.06	1.5±0.71	16.07	10.91±1.04
10%	10	33.0±0.70	1.0±1.13	68.18	22.52±8.16
JABK: 1%	10	33.5±1.48	2.1±1.42	2.68	9.02±3.18
5%	10	33.7±1.55	2.1±1.60	15.72	10.60±0.46
10%	10	34.0±1.69	2.4±1.11	27.35	9.37±0.85
Basal diet + 0.9%NaCl[b,c]	08[e]	33.4±0.63	0.8±0.74	–	8.31±0.76
Basal diet + ENU[d]	10	33.8±1.13	1.3±1.27	–	9.64±1.08
95/01 + ENU: 1%	10	34.9±0.77	1.1±0.60	2.57	9.18±0.61
5%	10	33.7±0.84	1.2±1.88	16.02	10.81±0.96
10%	10	33.8±1.13	1.6±0.82	50.29	17.01±3.59
95/01: 1%	10	32.6±0.42	0.6±1.01	3.06	10.16±3.12
5%	10	36.1±1.06	1.5±0.61	16.89	12.32±2.02
10%	10	32.5±0.84	1.2±0.84	45.53	14.85±2.66

[a] Kendall tau = 0.56: $P < 0.001$ to 1% < 5% < 10%, for the four lineages.
[b] Experiments were conducted at different times.
[c] Negative control.
[d] ENU = *N*-ethyl-*N*-nitrosourea (50 mg/kg body weight), positive control.
[e] Two animals died.
* $P < 0.05$.
** $P < 0.01$.

Table 2
Frequencies of micronucleated polychromatic erythrocytes (MNPCE) in mice bone marrow after treatment with diet containing *L. edodes* (96/22, L1, JABK and 95/01)

Treatment	No. of analyzed cells	MNPCE	
		No.	%
Basal diet + 0.9% NaCl[a,b]	10 000	11	0.11
Basal diet + ENU[c]	10 000	174	1.74
96/22: 1%	10 000	09	0.09
5%	10 000	02	0.02
10%	10 000	11	0.11
L1: 1%	10 000	10	0.10
5%	10 000	07	0.07
10%	10 000	06	0.06
JABK: 1%	10 000	14	0.14
5%	10 000	10	0.10
10%	10 000	06	0.06
Basal diet + 0.9% NaCl[a,b]	8000[d]	14	0.17
Basal diet + ENU[c]	10 000	137	1.37
95/01: 1%	10 000	21	0.21
5%	10 000	21	0.21
10%	10 000	22	0.21

[a] Experiments were conducted at different times.
[b] Negative control.
[c] ENU = *N*-ethyl-*N*-nitrosourea (50 mg/kg body weight), positive control.
[d] Two animals died.

2.6. Experimental design

In order to investigate the protective effect of different lineages of *L. edodes* (96/22, L1 JABK and 95/01) against the mutagenicity induced by ENU (50 mg/kg body weight, ip), mice were divided into groups of 10 animals each. Group I mice received only basal diet for 2 weeks. At day 15, they were injected ip with 0.9% NaCl. In group 2, the animals also received basal diet for 2 weeks, but at day 15 they were treated with ENU. Groups 3, 4 and *5* received diets containing different lineages of shiitake (1, 5 and 10%, respectively) for 2 weeks before ENU administration (50 mg/kg). Groups 6, 7 and 8 received only diets containing the different lineages of shiitake (1, 5 and 10%, respectively) for 2 weeks. All animals were sacrificed at day 16, 24 h after NaCl or ENU treatment.

2.7. Statistical analysis

Mean body weight and mean diet consumption were analyzed by ANOVA and post-hoc compared by Tukey HSD test in case of significance. The correlation between diet consumption and mushroom concentration (1, 5 and 10%) was calculated by Kendall tau. The MN data were analyzed by the Chi-square test. For all tests, α for significance was fixed at 0.05.

Table 3
Frequencies of micronucleated polychromatic erythrocytes (MNPCE) in bone marrow of mice treated with *L. edodes* (96/22, L1, JABK and 95/01) plus ENU (50 mg/kg)

Treatments	No. of analyzed cells	MNPCE		Reduction%
		No.	%	
Basal diet + 0.9% NaCl[a,b]	10 000	11	0.11	
Basal diet + ENU[c]	10 000	174	1.74	
96/22 + ENU: 1%	10 000	136	1.36*	23
5%	10 000	113	1.13**	37
10%	10 000	121	1.21**	32
L1 + ENU: 1%	10 000	153	1.53	13
5%	10 000	136	1.36*	23
10%	10 000	170	1.70	2
JABK + ENU: 1%	10 000	146	1.46	17
5%	10 000	145	1.45	18
10%	10 000	152	1.52	13
Basal diet + 0.9% NaCl[a,b]	8000[d]	14	0.17	
Basal diet + ENU[c]	10 000	137	1.37	
95/01 + ENU: 1%	10 000	114	1.14	19
5%	10 000	77	0.77*	50
10%	10 000	123	1.23	11

[a] Experiments were conducted at different times.
[b] Negative control.
[c] ENU = *N*-ethyl-*N*-nitrosourea (50 mg/kg body weight), positive control.
[d] Two animals died.
* $P < 0.05$.
** $P < 0.01$.

3. Results

Table 1 presents the means of body weight, body weight gain, food consumption and ingested mushroom during the experiment. The results show no relationship between the concentration of ingested mushroom and body weight gain. However, significant differences were observed when the body weight gain in groups L1 (5%) + ENU and JABK (5%) + ENU were compared with the negative control. An increased diet consumption related to concentration of mushroom in the diet, independently of the lineage was also observed ($P < 0.001$, Kendall $\tau = 0.56$; Fig. 1).

Table 2 shows that the frequencies of MN in PCEs of animals treated only with the mushroom diets are not significantly different from those of untreated controls. Thus, the data indicate the absence of mutagenic effect by the different mushroom diets regardless of concentration. Table 3 shows the frequencies of MN in PCEs in mice treated with different lineages of *L. edodes* (96/22, L1, 95/01 and JABK) and with 50 mg/kg ENU. The results show a statistically significant reduction in the frequencies of MN for the animals treated with lineages 96/22, L1 and 95/01. However, this protective effect was not related to mushroom concentration.

4. Discussion

Owing to the increasing consumption of shiitake in many countries, the present study evaluated its effect on DNA, since possible a relationship between this mushroom and antitumoral activity has been reported. First, our results showed an increased mean food consumption related to the mushroom concentration in the diet. Better palatability promoted by the mushrooms diet, or caloric compensation due to the lower concentration of basal diet, or perhaps the mushroom fiber content could explain this positive correlation. It is known that dietary fiber is a heterogeneous group of substances that resist digestion by the endogenous enzymes of the human gut and apparently reduce the digestibility of nutrients (Bijlani, 1985).

Shiitake mutagenic potential evaluated in mice bone marrow cells by the micronucleus test indicates that the lineages (96/22, L1, JABK and 95/01) did not cause chromosome breaks or damage to the mitotic apparatus. The results are in accordance with our previous in vivo study in which a lack of mutagenic effect was observed for the 96/17 *L. edodes* lineage (Alves de Lima et al., 2001).

As no deleterious effect on DNA was detected, we evaluated the protective action of shiitake against ENU-induced genetic damage. Three (96/22, L1 and 95/01) of the four lineages tested, exhibited antimutagenic activity by decreasing the frequencies of MNPCE induced by a direct-acting alkylating agent. Although no dose–response relationship was observed these findings agree with those of our previous study, where a 15-day treatment with aqueous solutions of lineage 96/17 reduced the frequencies of mouse bone marrow MNPCEs induced by direct and indirect alkylating agents (ENU and CP, respectively) (Alves de Lima et al., 2001).

The lack of antimutagenic activity for the JABK lineage could be due to variation in biochemical composition among lineages. Similar results were found when the antimutagenic action of the mushroom *Agaricus blazei* Murrill was evaluated (Delmanto et al., 2001).

Taken together, our results showed that the antimutagenic effect of *L. edodes* may vary among lineages. Grüter et al. (1991) have demonstrated that season, geographic or intraspecies differences may influence the chemical composition of mushrooms and consequently modulate their effects. In conclusion, further studies must be conducted to establish under which conditions shiitake modulates DNA damage in mammalian cells. In this way, chemical identification of the components that characterize those effective *L. edodes* lineages and their respective mechanisms of action should be evaluated.

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References

Alves de Lima, P.L., Delmanto, R.D., Sugui, M.M., da Eira, A.F., Salvadori, D.M.F., Speit, G., Ribeiro, L.R., 2001. *Lentinus edodes* (Berk.). Pegler (Shiitake) modulates the mutagenic effects induced by alkylating agents *in vivo*. Mutation Research 496, 23–32.

Bijlani, R.L., 1985. Dietary fibre: consensus and controversy. Progress in Food and Nutritional Science 9 (3–4), 343–393.

Borchers, A.T., Stern, J.S., Hackman, R.M., Keen, C.L., Gershwin, M.E., 1999. Mushrooms, tumors, and immunity. Proceedings of the Society Experimental Biology and Medicine 221, 281–293.

Chang, S.T., Hayes, W.A., 1980. The Biology and Cultivation of Edible Mushrooms. Academic Press, New York.

Chang, S.T., Larque Saavedra, A., Martinez Carrera, D., Morales, M., Sobal, M., 1992. The cultivation of edible mushrooms as a boost for rural development: a case study from Cuetzalan Ara, México. At Source. Mexico 20 (1), 22–25.

Chiraha, G., Hamuro, J., Maeda, Y.Y., Arai, Y., Fukuoka, F., 1970. Fractionation and purification of the polysaccharides with marked antitumor activity, especially Lentinan, from *Lentinus edodes*. Cancer Research 30, 2776–2781.

Delmanto, R.D., Alves de Lima, P.L., Sugui, M.M., da Eira, A.F., Salvadori, D.M.F., Speit, G., Ribeiro, L.R., 2001. Antimutagenic

effect of *Agaricus blazei* Murrill mushroom on the genotoxicity induced by cyclophosphamide. Mutation Research 496, 15–21.

Fujii, T., Maeda, H., Suzuki, F., Ishida, N., 1978. Isolation and characterization of a new antitumor polysaccharide, KS-2, extracted from culture mycelia of *Lentinus edodes*. Journal of Antibiotics 31 (1), 1079–1090.

Grüter, A., Friederich, U., Wurgler, F.E., 1991. The mutagenicity of edible mushrooms in a histidine-independent bacterial test system. Food and Chemical Toxicology 29 (3) 159–165.

Hasegawa, J., Hosokawa, M., Okada, F., Kobayashi, H., 1989. Inhibition of mitomycin C-induced sister chromatid exchanges in mouse bone marrow cells by the immunopotentiators Krestin and Lentinan. Mutation Research 226, 9–12.

Kues, U., Liu, Y., 2000. Fruiting body production in basidiomycetes. Applied Microbiology and Biotechnology 54 (2), 141–152.

MacGregor, J.T., Heddle, J.A., Hite, M., Margolin, B.H., Ramel, C., Salamone, M.F., Tia, R.R., Wild, D., 1987. Guidelines for the conduct of micronucleus assay in mammalian bone marrow erythrocytes. Mutation Research 189, 103–112.

Manoharan, K., Banerjee, M.R., 1985. β-carotene reduces sister chromatid exchange induce chemical carcinogens in mouse mammary cells in organ culture. Cell Biology International Reports 9, 783–789.

Ooi, V.E., Liu, F., 2000. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. Current Medicine and Chemistry 77, 715–729.

Terashita, T., Kono, M., Mishima, N., Obata, T., Yamauchi, M., 1990. The proximate components, free and protein bound amino acids in protein and 5 GMP in fruit bodies of *Lentinus edodes* Singer Shiitake mushroom grown on artificial bed blocks. Journal of the Japanese Society of Food Science and Technology 37, 528–532.

Volz, P.A., 1999. Early historic cancer chemotherapy work involving basidiomycetous mushrooms. International Journal of Medicinal Mushrooms 1, 191–194.

Wasser, S.P., Weiss, A.L., 1999. Therapeutic effects of substances occurring in higher basidiomycetes mushrooms: a modern perspective. Critical Reviews in Immunology 19, 65–96.

Waters, M.D., Brady, A.L., Stack, H.F., Brockman, H.E., 1990. Antimutagenicity profiles for some model compounds. Mutation Research 238, 57–85.

Wuest, D.J., Royse, D.J., Beelman, R.B., 1987. Cultivating Edible Fungi. Elsevier, Amsterdam.

Ying, J., Mao, X., Ma, Q., Zong, Y., Wen, H., 1987. Icons of Medicinal Fungi from China. Science Press, Beijing.