

# Benzidine- and diaminobenzidine-induced micronuclei in mice after intraperitoneal and oral single or multiple treatment

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## ABSTRACT

The capacity of benzidine and its derivative diaminobenzidine to induce micronuclei in bone marrow polychromatic erythrocytes was tested in two mouse strains, C57BL/6 and BALB/c, after intraperitoneal injection or gavage on one, two or three successive days. Results observed after multiple intraperitoneal dosages were inconclusive for both compounds, when tested in C57BL/6 mice. On the other hand, benzidine induced a marked increase in the micronucleated polychromatic erythrocyte frequency after a single intraperitoneal treatment in BALB/c mice. Oral treatment with both chemicals had a genotoxic effect in both mouse strains, although in BALB/c mice a significant increment in micronucleated cell frequency was observed only after two or three consecutive doses. We conclude that diaminobenzidine has a genotoxic activity comparable to benzidine, presenting specific strain and administration route effects.

## INTRODUCTION

Benzidine (BZD), an industrial chemical, is recognized as a carcinogen in both humans and laboratory animals. After intraperitoneal (*ip*) treatment of ICR mice, BZD-DNA adducts are correlated with BZD-induced chromosome aberrations in the liver. As the liver is the target organ in mice, these results suggest that induction of chromosome aberrations can be a mechanism of BZD carcinogenicity (Talaska *et al.*, 1987). Other aromatic amines, such as 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine, have structural alerts to DNA reactivity, and are active in most of the tissues associated earlier with genotoxic carcinogenesis (Tennant and Ashby, 1991; Ashby and Tennant, 1991). The 3,3'-diamino derivative of BZD, diaminobenzidine

(DAB), is used as a laboratory reagent and can probably also be metabolized *in vivo* and produce DNA adducts.

As regards DAB clastogenic activity, only one report (You *et al.*, 1993) was found. This study reports that a single 100 mg/kg *ip* treatment with DAB induced a significant increase in chromosome aberrations at the same potency as BZD in bone marrow cells of an unspecified mouse strain.

The results of the micronucleus (MN) test after BZD treatment are conflicting. When *ip* treatment was employed BZD induced MN in polychromatic erythrocytes (PE) of bone marrow cells of male Swiss albino mice after one, two or three days of 100 mg/kg *ip* treatment (Ribeiro *et al.*, 1993). Induction of micronucleated PE (mPE) was also observed in male B6C3F1 mice after two and three *ip* BZD injections, 200 mg/kg, but not at lower doses, but this effect was not detected with a single treatment, even at 300 mg/kg (Tice *et al.*, 1990a,b). After a single *ip* dose (102 mg/kg), mPE

frequency was enhanced in the bone marrow of pregnant Swiss albino female mice and in the liver of their fetuses (Sanderson and Clark, 1993).

On the other hand, oral administration of BZD did not cause a statistically significant increment of mPE in rats (Trzos *et al.*, 1978) and adult ICR mice bone marrow cells, or in fetal liver after a single 100 or 200 mg/kg dose (Harper *et al.*, 1989). Nevertheless, induction of MN has been demonstrated after oral BZD administration in ICR male mice after one, two or three days of 40 or 200 mg/kg (Parton *et al.*, 1990) or after a single 300 mg/kg dose in male bone marrow and in fetal liver, but not in the bone marrow of pregnant females (Cihák and Vontorková, 1987). Significant effects also were found in C57BL/6 and CBA male mice bone marrow after oral 300 or 900 mg/kg BZD single treatment and with a 150 or 300 mg/kg triple-dose test protocol (Mirkova and Ashby, 1988; Mirkova, 1990) and in Wistar male rats after two consecutive BZD (100, 200 or 300 mg/kg) doses (Cihák, 1979).

Salamone and Heddle (1983) reported that multiple treatment improved the sensitivity of the MN test. Tice *et al.* (1990a) indicated that three *ip* treatments eliminate the need for multiple sample times, minimize the number of animals required and simplify data analysis (BZD induced a significant increase in mPE frequency only 72 h after the first injections). Mirkova (1990) demonstrated that a positive response was enhanced by the BZD triple-oral dose protocol. Parton *et al.* (1990), however, showed that the effect of BZD is not influenced by the number of administrations.

Taking into account these findings, the present study was aimed at testing the capacity of different treatment protocols with BZD and its derivative DAB to induce MN in bone marrow PE of two different mouse strains.

## MATERIAL AND METHODS

### Animals

Adult (12-14 weeks old) male and female C57BL/6 and BALB/c mice were used. The animals were housed in a room with controlled temperature and light, in the animal house facilities at Instituto de Biociências, Universidade Federal do Rio Grande do Sul. All animals received commercial standard mouse food and water *ad libitum*.

### Chemical agents

BZD (4,4'-Diaminodiphenyl, CAS No. 92-87-5) and DAB (3,3',4,4'-Tetraaminobiphenyl, CAS No.

91-95-2) were purchased from Sigma Chemical Company, St. Louis, MO. Cyclophosphamide (CPA) was purchased from Abbott Laboratórios do Brasil Ltda.

### Doses, times for sample collection and micronucleus test

To evaluate the effect of *ip* treatment, C57BL/6 mice were tested for both chemicals, BZD and DAB, after 1, 2 or 3 x 100 mg/kg *ip* injections 24-h apart. Before use, these chemicals were ground in corn oil and mixed. Mirkova (1990) reported that in C57BL/6 mice triple-dose treatment with 150 or 300 mg/kg BZD prevents depression of erythropoiesis. For a comparison of strain-specific effects, the dose and treatment schedule were based on our previous study (Ribeiro *et al.*, 1993). BALB/c mice were treated with a single *ip* dose of 100 or 200 mg/kg of each compound. To test the oral effect, C57BL/6 or BALB/c mice were treated with 100 mg/kg BZD or DAB in a one, two or three gavage protocol. Negative control animals were treated with corn oil and positive controls were treated *ip* with 40 mg/kg of CPA diluted in distilled water. In all treatment regimes the administered volume was 10 ml/kg of body weight.

The animals were killed by cervical dislocation 24 h after the last treatment. The bone marrow cells were prepared as recommended by Salamone *et al.* (1980). The slides were coded, fixed with methanol and stained with May Grünwald-Giemsa solution. All treated groups contained a minimum of three surviving animals of each sex, and at least 2000 PE of each animal were scored for MN presence. The ratio of PE to normochromatic erythrocytes (NE) was based on 1000 NE, 500 observed in each slide. Statistical analyses were performed by the Mann-Whitney test to evaluate MN induction and by the *t*-test to compare the number of PE among 1000 NE.

## RESULTS AND DISCUSSION

The PE/NE relation is used as a toxicity reference. A high reduction in PE can lead to a false negative result in the micronucleus test. In our data, using the original output, a statistically significant reduction of the PE/NE ratio was not detected in any test group, in relation to the negative control group (Tables I to III). These results indicate that BZD and DAB did not significantly modify bone marrow activity. In all experimental approaches (Tables I to III) the positive control groups showed a significant increase in mPE frequency induced by CPA.

Table I shows the results of MN induction on bone marrow PE of C57BL/6 mice after one, two or three *ip* treatment with 100 mg/kg BZD or DAB. Only one BZD treatment (2 x 100 mg/kg) showed a significant effect in males and females while DAB elicited a positive result only in males with 3 x 100 mg/kg.

Tice *et al.* (1990a,b) also demonstrated that a single *ip* treatment (100, 200 or 300 mg/kg) of BZD failed to induce a significant increase of mPE frequency

in the bone marrow of B6C3F1 mice. Following a multiple administration protocol (50, 100 or 200 mg/kg), only the 200 mg/kg treatment induced an increase in mPE frequency, 48 h after two injections and 24 h after three injections. BZD *ip* treatment in Swiss albino mice induces an increase of mPE in male bone marrow after one, two or three x 100 mg/kg dose protocols (Ribeiro *et al.*, 1993). Our data are also in contrast to those of Mirkova and Ashby (1988) and Mirkova (1990), who reported a positive response 24

Table I - Results of C57BL/6 bone marrow micronucleus analyses in polychromatic erythrocytes after intraperitoneal benzidine or diaminobenzidine treatment.

Compound (mg/kg)	Number of mice	Sex	Number of PE counted	mPE/1000 PE per animal	mPE/1000 PE Mean $\pm$ SD	PE/500 NE Mean $\pm$ SD	
Negative Control (0)	3	M	6,000	0.5; 0.5; 2.5	1.2 $\pm$ 1.17	0.46 $\pm$ 0.24	
	3	F	6,000	1.5; 2; 0.5	1.3 $\pm$ 1.03	0.81 $\pm$ 0.25	
BZD (1 x 100)	3	M	6,000	3.5; 1; 1.5	2.0 $\pm$ 1.17	0.26 $\pm$ 0.03	
	3	F	6,000	2; 0.5; 0	0.8 $\pm$ 1.17	0.75 $\pm$ 0.44	
	(2 x 100)	3	M	6,000	3; 4.5; 3	3.5 $\pm$ 1.22*	0.69 $\pm$ 0.31
		3	F	6,000	2.5; 3.5; 3.5	3.2 $\pm$ 1.17*	0.48 $\pm$ 0.27
	(3 x 100)	3	M	6,000	0.5; 2; 1.5	1.3 $\pm$ 1.72	0.37 $\pm$ 0.29
		3	F	6,000	2; 1.5; 1.5	1.7 $\pm$ 0.75	0.60 $\pm$ 0.45
DAB (1 x 100)	3	M	6,000	2.5; 2.5; 3.5	2.8 $\pm$ 1.72	0.42 $\pm$ 0.18	
	3	F	6,000	1.5; 1.5; 0.5	1.2 $\pm$ 0.75	0.46 $\pm$ 0.17	
	(2 x 100)	3	M	6,000	2.5; 2; 1	1.8 $\pm$ 1.60	0.50 $\pm$ 0.23
		3	F	6,000	2.5; 4; 2	2.8 $\pm$ 1.17	1.12 $\pm$ 0.27
	(3 x 100)	3	M	6,000	2.5; 4; 4	3.3 $\pm$ 1.75*	0.53 $\pm$ 0.43
		3	F	6,000	2.5; 1; 1	1.5 $\pm$ 1.52	0.64 $\pm$ 0.03
Positive control	5	M	10,000	23; 19; 31; 18; 28	23.8 $\pm$ 5.63**	-	
CPA (1 x 40)	5	F	10,000	33; 18; 20; 40; 27	27.0 $\pm$ 9.12**	-	

PE = Polychromatic erythrocytes; mPE = micronucleated PE; NE = normochromatic erythrocytes; BZD = benzidine; DAB = diaminobenzidine; CPA = cyclophosphamide; \*P < 0.05; \*\*P < 0.01.

Table II - Results of BALB/c bone marrow micronucleus analyses in polychromatic erythrocytes after intraperitoneal benzidine or diaminobenzidine treatment.

Compound (mg/kg)	Number of mice		Sex	Number of PE counted	mPE/1000 PE per animal	mPE/1000 PE Mean $\pm$ SD	PE/500 NE Mean $\pm$ SD	
	T	S						
Negative control (0)	5	5	M	10,000	2.5; 0; 1.5; 1; 2.5	1.5 $\pm$ 1.35	0.42 $\pm$ 0.19	
	5	5	F	10,000	1.5; 2; 1; 4; 1.5	2.0 $\pm$ 1.33	0.72 $\pm$ 0.29	
BZD (1 x 100)	5	3	M	6,000	12; 10; 11	11.3 $\pm$ 1.89**	0.41 $\pm$ 0.21	
	5	4	F	8,000	8.5; 10; 8; 9.5	9.0 $\pm$ 1.31**	0.81 $\pm$ 0.68	
	(1 x 200)	5	3	M	7,000	13.5; 11.5; 16	13.7 $\pm$ 2.19**	0.34 $\pm$ 0.15
		5	3	F	7,000	6; 5.7; 4	5.3 $\pm$ 1.38**	0.41 $\pm$ 0.16
DAB (1 x 100)	5	5	M	10,000	2.5; 4; 2.5; 2.5; 4.5	3.2 $\pm$ 1.23*	0.59 $\pm$ 0.04	
	5	4	F	8,000	1.5; 3; 3; 2.5	2.5 $\pm$ 0.93	0.99 $\pm$ 0.43	
	(1 x 200)	5	5	M	10,000	2.5; 2; 2; 2.5; 2	2.2 $\pm$ 0.63	0.53 $\pm$ 0.29
		5	5	F	10,000	2.5; 2.5; 2.5; 1; 3	2.3 $\pm$ 1.06	0.56 $\pm$ 0.31
Positive control	5	5	M	10,000	12; 19.5; 24.5; 28; 16.5	20.1 $\pm$ 6.67**	-	
CPA (1 x 40)	5	5	F	10,000	18; 25.5; 20.5; 11.5; 18.5	18.7 $\pm$ 4.83**	-	

T = Treated; S = surviving; for other abbreviations see Table I.

**Table III** - Results of C57BL/6 and BALB/c bone marrow micronucleus analyses in polychromatic erythrocytes after oral benzidine or diaminobenzidine treatment.

Compound (mg/kg)	Number of mice	Sex	Number of PE counted	mPE/1000 PE per animal	mPE/1000 PE Mean $\pm$ SD	PE/500 NE Mean $\pm$ SD
<b>C57BL/6</b>						
Negative control (0)	3	M	6,000	6.5; 3; 5.5	5.0 $\pm$ 2.19	0.34 $\pm$ 0.06
	3	F	6,000	2.5; 3; 3	2.8 $\pm$ 1.33	0.77 $\pm$ 0.36
BZD (1 x 100)	3	M	6,000	7.5; 8.5; 8	8.0 $\pm$ 1.55*	0.52 $\pm$ 0.15
	3	F	6,000	10; 7.5; 5.5	7.7 $\pm$ 2.58**	0.38 $\pm$ 0.06
DAB (1 x 100)	3	M	6,000	6; 13; 8.5	9.5 $\pm$ 3.33*	0.80 $\pm$ 0.23*
	3	F	6,000	7.5; 9; 7.5	8.0 $\pm$ 1.41**	0.88 $\pm$ 0.12
Positive control	5	M	10,000	23; 19; 31; 18; 28	23.8 $\pm$ 5.63**	-
CPA (1 x 40)	5	F	10,000	33; 18; 20; 40; 27	27.0 $\pm$ 9.12**	-
<b>BALB/c</b>						
Negative control (0)	3	M	6,000	5; 2; 2	3.0 $\pm$ 1.65	0.65 $\pm$ 0.38
	3	F	6,000	1; 0; 1	0.7 $\pm$ 0.52	0.89 $\pm$ 0.35
BZD (1 x 100)	5	M	10,000	6; 5; 3; 3; 1.5	3.7 $\pm$ 2.10	0.67 $\pm$ 0.15
	5	F	10,000	1.5; 2; 2; 1.5; 2.5	1.9 $\pm$ 0.74	0.93 $\pm$ 0.20
(2 x 100)	5	M	10,000	8; 10; 10.5; 8.5; 12	9.8 $\pm$ 1.60**	0.55 $\pm$ 0.29
	5	F	10,000	13; 12; 11; 8.5; 9.5	10.8 $\pm$ 1.79**	0.42 $\pm$ 0.18
(3 x 100)	5	M	10,000	11; 14; 13; 9.5; 14	12.1 $\pm$ 2.89**	0.60 $\pm$ 0.12
	5	F	10,000	11; 10.5; 9; 8; 6.5	8.9 $\pm$ 2.41**	0.61 $\pm$ 0.22
DAB (1 x 100)	5	M	10,000	2; 2.5; 2; 3; 1	2.1 $\pm$ 0.70	0.65 $\pm$ 0.12
	5	F	10,000	2.5; 0.5; 1; 2.5; 2	1.7 $\pm$ 1.22	0.99 $\pm$ 0.03
(2 x 100)	4	M	8,000	8.5; 9; 7; 8; 6	7.7 $\pm$ 1.63**	0.37 $\pm$ 0.04
	5	F	10,000	8.5; 3.5; 5.6; 5	5.7 $\pm$ 2.58**	0.58 $\pm$ 0.14
(3 x 100)	5	M	10,000	5.5; 3.5; 4; 4; 3.5	4.1 $\pm$ 1.00	0.52 $\pm$ 0.18
	5	F	9,000	2; 3; 2; 2; 2	2.1 $\pm$ 0.78**	0.32 $\pm$ 0.09
Positive control	5	M	10,000	12; 19.5; 24.5; 28; 16.5	20.1 $\pm$ 6.67**	-
CPA (1 x 40)	5	F	10,000	18; 25.5; 20.5; 11.5; 18.5	18.7 $\pm$ 4.83**	-

For abbreviations see Tables I.

and 48 h after a single 300 mg/kg BZD oral treatment and a greater effect after three daily oral doses (150 or 300 mg/kg) than after a 900 mg/kg single administration. Sanderson and Clark (1993) detected positive mPE induction after a single 102 mg/kg *ip* administration in pregnant Swiss mice bone marrow and in the liver of their fetuses. The authors suggest that the differences in results are due to different administration routes.

Table II presents the results of MN induction after *ip* BZD and DAB administration (100 or 200 mg/kg) in BALB/c bone marrow. After *ip* BZD injection the mice presented convulsive behavior during about 15 min, and nearly 18 h later, some animals were found dead. These results were not observed in C57BL/6 mice after *ip* treatment or in the subsequent experiment (oral treatment). In BALB/c, a single *ip* BZD treatment induced an increase of mPE frequency at both doses and in both sexes. Our results confirmed those of Mirkova and Ashby (1988), Mirkova (1990), Sanderson and Clark (1993) and Ribeiro *et al.* (1993). After this treatment

protocol DAB induced significant results only in one situation, i.e., males treated with 1 x 100 mg/kg.

Table III summarizes the data obtained after gavage administration of BZD or DAB in both C57BL/6 and BALB/c strains. Our results indicate that after a single 100 mg/kg oral administration to C57BL/6 mice, both compounds increase the mPE frequency in male and female bone marrow cells. These results are in contrast with Harper *et al.* (1989), who found no significant increase in mPE frequency, after a single BZD (100 or 200 mg/kg) oral treatment, in the bone marrow of ICR males, pregnant females or in the livers of their fetuses. The data are however in agreement with those obtained in ICR (Cihák and Vontorková, 1987 and Parton *et al.*, 1990), in C57BL/6 (Mirkova and Ashby, 1988 and Mirkova, 1990) and in Swiss albino mice (Ribeiro *et al.*, 1993). On the other hand, in BALB/c a single 100 mg/kg oral administration of BZD did not increase the mPE frequency, while two or three treatment protocols were effective in both sexes.

In conclusion, we consider that our sporadic positive results after *ip* BZD treatment of C57BL/6 mice are inconclusive (Table I) whereas results from the *ip* BZD treatment of BALB/c mice (Table II) or oral BZD administration to both mouse strains (Table III) indicate a clear genotoxic effect of this compound. So, the contradictory data obtained by the MN test after BZD treatment could be due to the different administration routes, as suggested by Sanderson and Clark (1993), to the number of administrations as discussed by Tice *et al.* (1990a), Mirkova (1990) and Parton *et al.* (1990), or based on our previous data (Ribeiro *et al.*, 1993) and the present results, to mouse strain differences.

Taking into account the results obtained after *ip* DAB administration (Tables I and II - significance only after 3 x 100 mg/kg in C57BL/6 males and 1 x 100 mg/kg in BALB/c males), we consider that the *ip* treatment demonstrated inconclusive results.

After oral treatment (Table III) the results of DAB administration were very similar to those induced by BZD. The difference was related to the DAB triple dose effect. Although a DAB 3 x 100 mg/kg treatment induced a weak increment in mPE frequency in males, the response of that treatment was statistically significant only in females.

Despite the fact that a clear positive response was found only after oral treatment, our results support the clastogenic activity observed in murine bone marrow by You *et al.* (1993) after 100 mg/kg BZD or DAB *ip* treatment, when the endpoint was chromosomal aberrations. Talaska *et al.* (1987) observed a clear dose-related increase in chromosome aberrations caused by BZD in the livers of partially hepatectomized mice. Most of the detected damage was chromatid breaks. Although MN analysis reflects structural or numerical chromosome aberrations, our results indicate that BZD and DAB have similar clastogenic activity.

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## RESUMO

No presente trabalho investigamos a capacidade da benzidina e de seu derivado, a diamino benzidina, usada como reagente laboratorial, na indução de micronúcleos em eritrócitos policromados na medula óssea de duas linhagens de camundongos, C57BL/6 e BALB/c, após uma, duas ou três

injeções intraperitoneais ou administrações intragástricas separadas em 24 h. Após administração intraperitoneal (simples ou múltipla) nossos resultados foram inconclusivos para ambos os compostos quando testados na linhagem C57BL/6. Entretanto, uma única dose intraperitoneal de benzidina induziu um claro aumento na frequência de eritrócitos policromados micronucleados na linhagem BALB/c. Após tratamento oral ambas as drogas demonstraram efeito genotóxico nas duas linhagens, embora nos camundongos BALB/c o aumento de células micronucleadas foi observado somente após duas ou três administrações consecutivas. Nossos resultados permitiram concluir que a diamino benzidina possui genotoxicidade comparável a da benzidina, apresentando efeitos linhagem e rota de administração específicos.

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