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THE INDUCTION OF MICRONUCLEI IN BOVINE LYMPHOCYTES BY EXPOSURE TO BENZENE AND S-9 MIX

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Abstract: Benzene is a widespread human carcinogen, inducing leukaemia and hematotoxicity. It has been shown to be a multi-organ carcinogen in animals. The effect of benzene was studied using induction of micronuclei (MN) in whole blood lymphocytes cultures after treatment with different concentrations of benzene (5, 10, 50, 100, 500 and 1000 μM) with and without metabolic activation (S-9 mix). A significant elevation in the induction of micronuclei was found after application of benzene at doses of 50 and 100 μM in both donors. Treatment of bovine lymphocytes did not result in the induction of micronuclei in a dose-dependent manner. The addition of an external metabolic factor (10% S-9 mix for 2 h) in blood cultures treated with benzene indicated an increase of the genotoxic activity of benzene (at concentrations ranging from 10–100 μM).

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INTRODUCTION

Animals are often exposed to environmental agents that can increase the likelihood of mutagenicity and carcinogenicity. The risk exposure to these agents can be assessed either from laboratory studies simulating exposure to different agents or from epidemiological studies in relation to actual exposure. The essential importance of genotoxicity testing lies in its ability to evaluate potential animal toxicity so that harmful effects can be prevented [1]. In the case of genotoxic agents, which effect biomonitoring parameters, such as cytogenetic effects in peripheral blood cells are applied [7]. Chromosomal analysis can be carried out using various methods: conventional chromosomal analysis, sister chromatid exchange, and micronucleus frequency detection.

In our study, short-term cultures of bovine lymphocytes were used to investigate the genotoxicity of benzene in the cytokinesis-block micronucleus assay (CBMN). This assay has emerged as one of the preferred methods for assessing chromosome damage [4, 9]. Benzene, an important industrial solvent and constituent of unleaded gasoline, causes leukaemia and aplastic anemia in humans [12]. It is a well-characterized human carcinogen and clastogen still present in both the occupational and general environment [15]. Zhang *et al.* [17] reported that benzene is an established cause of human leukaemia producing chromosomal aberrations and alterations in cell differentiation.

Benzene has been shown to be a multi-organ carcinogen in animals [13]. Many of environmental mutagens are associated with reduced productive and reproductive efficiency of livestock.

The present study evaluates the ability of benzene to cause genetic impairment by the induction of MN in bovine lymphocytes *in vitro*.

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MATERIALS AND METHODS

Chemicals. Benzene (99%, Lachema, Brno, Czech Republic) was dissolved in dimethyl sulphoxyde (DMSO, Sigma, St. Louis, MO, USA) and added to the cultures at the concentrations of 5, 10, 50, 100, 500 and 1000 μM. The highest dose of benzene was chosen on the basis of the reduction in mitotic index by > 50%. Mitomycin C (MMC, Sigma, St. Louis, MO, USA, 0.4 μM), and cyclophosphamide (CP, Jenapharm, Ankerwerk, Rudolfstadt, Germany, 100 μM) were used as positive control agents in the assays in both the absence and presence of the metabolic activation (S-9 mix).

Lymphocyte cultures. Lymphocyte cultures were constituted by adding 0.5 ml of heparinized whole blood to 5 ml of RPMI 1640 medium supplemented with L-glutamine, 15 μM HEPES (Sigma, St. Loius, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 U/ml and streptomycin 250 μg/ml), and phytohaemagglutinin (PHA, 180 μg/ml, Welcome, Dartford, UK). Blood from 2 healthy donors was used (8-month-old Black spotted cattle).

For the CBMN assay the bovine cultures were incubated at 37ºC for 72 h and 44 h from the initiation; cytochalasin B (Cyt-B) at a final concentration of 6 μg/ml was added to arrest cytokinesis. Benzene was added 24 h after PHA stimulation.

The cultures treated for 2 h with S-9 mix (4 ml of chromosome medium with 0.5 ml of S-9) were set up without heat inactivated foetal calf serum. After the treatment, the cultures were washed twice with PBS and reconstituted in the same way as those treated for 48 h. Standard cytogenetic method was used for the slides obtained.

MN analysis. A total of 1000 binucleated cells (BN) were examined for each tested concentration and donor. Cell proliferation was evaluated using the cytokinesis-block proliferation index (CBPI), which indicates the average number of cell cycles undergone by a given cell [11]. Minimum of 500 lymphocytes were scored to evaluate the percentage of cells with 1, 2, 3, and 4 nuclei. CBPI was calculated according to Surrallés *et al.* [14] as follows: CBPI = MI + MII + 3(MIII + MIV)/N, where MI-MIV represent the number of cells with 1–4 nuclei, respectively, and N is the total number of the cells scored.

The statistical evaluation of the results was carried out using Fisher's exact test for micronucleated cells and χ^2 test for CBPI.

RESULTS

The ability of benzene to induce micronuclei in cytokinesis-blocked cells is illustrated in Tables 1 and 2. In the treatments lasting for 48 h without S-9 mix, the cultures from both donors showed a statistical significance. Donor A induced an increase in the frequency of BNMN at concentrations tested 50 and 100 μM ($p < 0.001$ and $p < 0.1$, respectively). Donor B was able to induce an increase in the frequency of BNMN at the same concentrations, but produced different statistical significance ($p < 0.1$ and $p < 0.01$, respectively). The other concentrations tested were increased but without statistical significance. The CBPI showed no positive results.

Table 1. Induction of micronuclei in bovine lymphocyte cultures exposed to benzene, donor A.

Treatment	Concentration μM	CBPI	Total BNMN
48 h (-S9)	Control	1.62	24
	5	1.74	39
	10	1.53	37
	50	1.69	54[c]
	100	1.73	40[a]
	500	1.73	36
	1000	1.63	34
MMC	0.4	1.42	56[c]
2 h (+S9)	Control	1.57	24
	5	1.52	38
	10	1.41	57[c]
	50	1.48	50[b]
	100	1.40	48[b]
	500	1.60	29
	1000	1.45	26
CP	100	1.47	53[c]

1000 binucleated cells of each concentration were determined; Statistical significance: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$

Table 2. Induction of micronuclei in bovine lymphocyte cultures exposed to benzene, donor B.

Treatment	Concentration μM	CBPI	Total BNMN
48 h (-S9)	Control	1.78	22
	5	1.52	33
	10	1.50	34
	50	1.61	37[a]
	100	1.69	45[b]
	500	1.58	31
	1000	1.69	33
MMC	0.4	1.65	53[c]
2 h (+S9)	Control	1.66	20
	5	1.69	34
	10	1.52	39[b]
	50	1.54	40[b]
	100	1.53	31
	500	1.62	28
	1000	1.52	34
CP	100	1.50	47[c]

1000 binucleated cells of each concentration were determined; Statistical significance: [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$

Some genotoxic chemicals can produce their effects directly, whereas others require metabolism to mutagenically activate intermediates [10]. Aroclor 1254-induced rat liver homogenate supernatant is routinely used as the exogenous metabolic activation system for the evaluation of mutagenicity of xenobiotics [5]. The addition of the S-9 mix induced a positive response at the concentrations tested 10, 50, 100 μM ($p < 0.001$ and $p < 0.01$, respectively) in donor A. In the cultures from donor B, addition of the S-9 mix attained a statistical significance at the concentrations tested 10 and 50 μM ($p < 0.01$). The CBPI value was not affected by the treatments in these cultures.

DISCUSSION

Multifactorial risk factors are responsible for many diseases. They can be categorized as environmental, genetic, as well as life style factors in human life. Au [2] has hypothesized that chronic exposure to mutagenic chemicals causes cellular abnormalities that can reduce the capacity of cells to repair DNA damage, and thus increase the risk of environmental disease.

The present study provides the evidence of genotoxic potential of benzene on *in vitro* cultures of bovine lymphocytes from 2 subjects randomly chosen. Our results indicate the statistically significant increase of micronucleated cells (BNMN) in exposed cultures when compared to the controls. In contrast to those results, Zarani *et al.* [16] did not present a significant increase in the number of micronuclei in BN human lymphocytes after 48 h of *in vitro* treatment. Their experiments were performed in the presence of benzene at doses from 0.1–5 mM. Lower concentrations of benzene were used in our study.

We have demonstrated that the frequency of micronuclei was higher at low levels of exposure than that at high levels of exposure. Similarly, Au *et al.* [3] observed the higher frequency of chromosomal aberrations at low levels of benzene. The explanation suggested by Au *et al.* [3] for this result was that glutathione-S-transferase, which counteracts the toxicity, could be induced by high levels of exposure.

Eastmond *et al.* [6] obtained in mice, dose- and time-dependent benzene-induced increases in chromosomal alterations. However, the results of human biomonitoring studies were not as clear-cut: chromosomal alterations in a population of Chinese workers highly exposed to benzene did not differ from control levels, a smaller group of Estonian workers exposed to lower levels of benzene showed chromosomal changes.

In recently published study, Kaneko *et al.* [8] found out that while the relatively low doses of benzene have been confirmed as inducing chromosomal aberrations, sister chromatid exchanges and micronuclei in rats and mice, it has not been proved that similar changes occur in humans.

CONCLUSIONS

The results of this study demonstrate the suitability of CBMN assay for biomonitoring animal exposure to genotoxic agents. Our findings do not aim be exhaustive in determining the genetic risk of benzene. Mainly, the limited number of experiments performed and only one sampling time used delineated the present study.

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