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FISH DETECTION OF CHROMOSOME 1 ABERRATION IN HUMAN INTERPHASE AND METAPHASE LYMPHOCYTES AFTER EXPOSURE TO BENZENE

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Abstract: Benzene is a relatively common environmental and occupational contaminant with carcinogenic and clastogenic properties. Therefore, further understanding of the adverse effect of benzene is still a matter of interest. In the present study, induction of aberrations in the pericentromeric region of chromosome 1 (1q12) was examined by fluorescence *in situ* hybridisation (FISH) in both interphase and metaphase human lymphocytes after *in vitro* exposure to benzene at two concentrations (50 and 100 µmol/l). A weak but not significant increase of interphase cells micronuclei frequency was recorded at 100 µmol/l concentration in both donors examined (χ^2 test, $p > 0.05$). No fluorescent signal indicating the presence of chromosome 1 was observed in adjacent micronuclei. In metaphase cells, hypoploidy (monosomy) and polyploidy (tetraploidy) were the types of numerical aberrations most often exhibiting classical satellite probe signal. Chromosome breakage in the investigated pericentromeric region was assumed in lymphocyte metaphase cultures of donor 2 exposed to a dose of 100 µmol/l.

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INTRODUCTION

Benzene is a well-recognized human carcinogen and clastogen. Chronic exposure to benzene in humans is associated with an increased incidence of myelodysplastic syndrome (MDS) and usually proceeds to acute myelogenous leukaemia (AML). The mechanism of leukaemogenesis by benzene has not been fully elucidated despite half a century of investigations [12, 13]. Previous studies have indicated that benzene itself is unlikely to be the actual toxicant, but rather is converted to bioactive metabolites, which cause toxicity to the bone marrow [10, 11]. It was also shown that benzene *in vitro* gave mixed positive and negative results without activation assays for aneugenicity and clastogenicity. The positive findings were primarily obtained in studies employing marrow cells or leukocytes [15].

Recent studies on benzene genotoxicity in humans have exploited progress in molecular cytogenetics to focus the analysis on benzene-induced alterations [6]. According to Li and Yin [4] a number of potential biomarkers are related to benzene exposure including benzene oxide-protein adducts, chromosome aberrations of lymphocytes, GPA mutations in erythrocytes, a decrease in B cell and CD4 T cell counts in peripheral blood and altered expression of some genes in lymphocytes.

Fluorescence *in situ* hybridization (FISH) with probes targeting centromeric or pericentromeric satellite sequences is being increasingly used to detect chromosome aberrations induced by chemical and physical agents *in vitro* and *in vivo*. It has been demonstrated that centromeric heterochromatin of human chromosome 1 (1cen-1q12 region) is one of the important regions prone to breakage [9].

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Moreover, centromeric DNA probes have been shown to be a valuable tool for the identification of aneuploidy occurring in interphase nuclei [2].

The present study deals with evaluation of possible *in vitro* adverse effect of benzene on pericentromeric human chromosome 1 region by means of interphase and metaphase FISH.

MATERIAL AND METHOD

Cell culture and chemical treatment. The blood from two healthy female donors (27 and 46 years old) was used in the experiment. The heparinized blood specimens (0.5 ml) were cultivated for 72 h at 37°C in 5 ml of RPMI 1640 medium supplemented with L-glutamine, 15 µmol/l HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum (Sigma, Chemical Co. St. Louis, MO, USA), antibiotics (penicillin 250 U/ml and streptomycin 250 µg/ml) and phytohaemagglutinin (PHA, 180 µg/ml, Welcome, Dartford, England). Benzene (>99% purity, Lachema Brno, The Czech Republic) was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA). On the basis of previous results [7, 14] two benzene concentrations (50 and 100 µmol/l) were selected and added to lymphocyte cultures for the last 24 hours of incubation. The slides with metaphase spreads were prepared by the standard cytogenetic method; 2 h before harvesting cell proliferation was stopped in metaphases by colchicine (Merck, Darmstadt, Germany) at a concentration of 5 µg/ml. The cells were collected by centrifugation, resuspended in hypotonic solution (0.075 M KCl) and fixed repeatedly in methanol/acetic acid (3:1). The lymphocyte cultures with interphase nuclei were prepared according to procedure of Guttenbach *et al.* [3]. Hypotonic treatment of the cells was performed in 0.075 M KCl for 4 min. at 37°C. The cells were fixed in methanol: glacial acetic acid (3:1) overnight and, after being washed in fixative, they were dropped onto clean slides.

FISH procedure. Chromosome 1 classical satellite probe (1q12, green direct labelled, Q-BIOgene, UK), optimised to detect copy numbers of chromosome 1 in metaphase and interphase spreads, was used in FISH. The classical satellite DNA probe hybridises to short AATGG related repeats localised near the centromere of chromosome 1. Hybridization with probe was performed according to manufacturer´s instructions. The slides were counterstained with DAPI/Antifade (4´, 6´-diamino-2-fenolindol, Q-BIOgene, Middlesex, UK).

Slide scoring. In both donors, 3000-interphase nuclei for each benzene concentration, as well as a control group, were scored for associated micronuclei (MN). Cells were classified as micronucleated if the micronuclei were either directly attached to the nucleus or if the distance between nucleus and micronucleus was not obviously larger than the diameter of the micronucleus.

250 metaphase spreads for the first donor and 150–200 metaphases for the second donor were examined for each experimental and control culture. The appearance of an extra green signal on a fragment or signal translocated to another chromosome was taken as evidence of breakage within pericentromeric region of chromosome 1. Metaphase cells with one fluorescence signal were classified as hypoploid; chromosome spreads with three or more signals as hyperploid.

A fluorescent microscope Nikon Labophot 2A/2, equipped with two dual band pass filters DAPI/FITC and FITC/TRITC, was used for probe visualisation. Chromosome 1 aberrations were recorded by means of a Nikon digital camera (Coolpix 4500, Nikon) and Nikon View computer programme. Statistical analysis of the results was performed using chi-square test (χ^2 test).

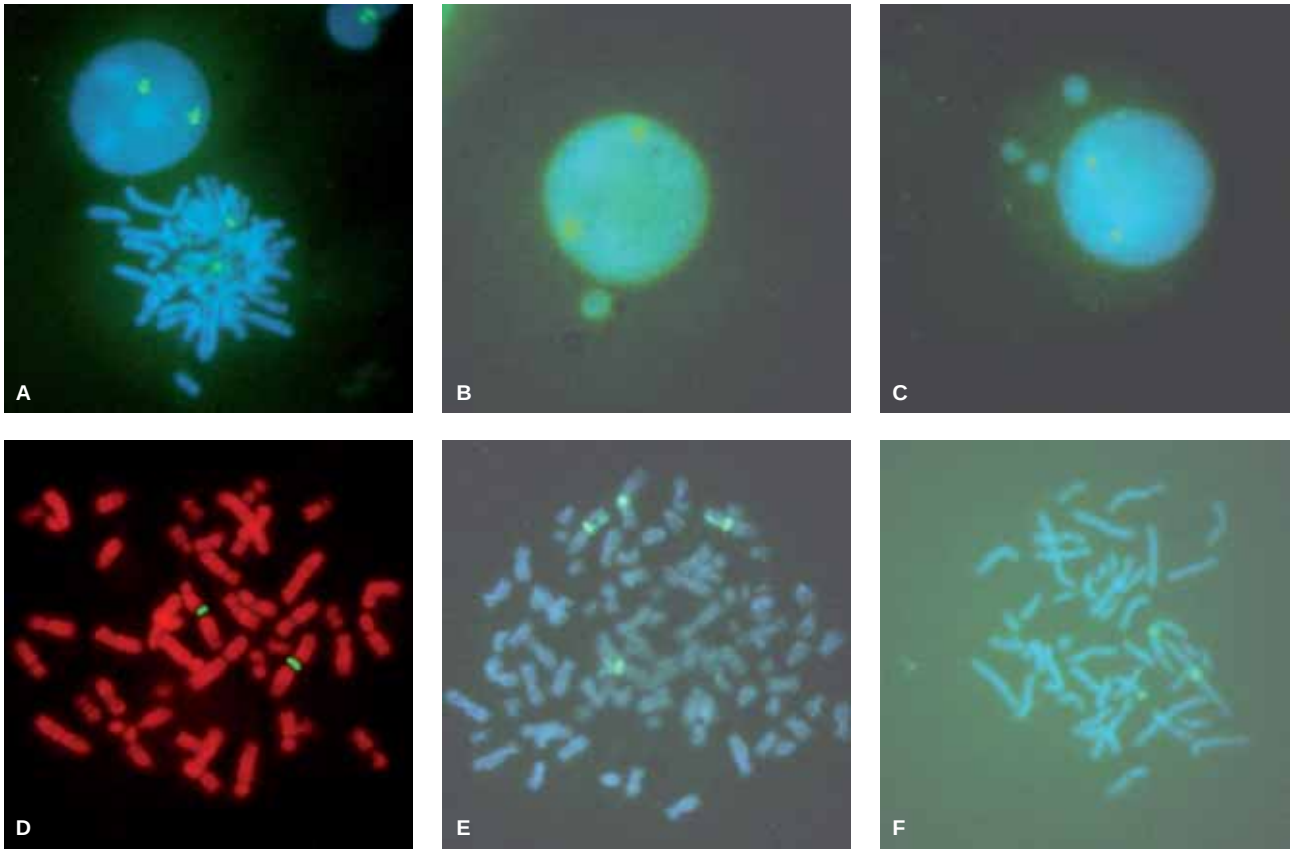
RESULTS

The frequencies of micronuclei (MN) in interphase cells as well as chromosome 1 aberrations in metaphase cells detected by FISH following 24 h *in vitro* benzene exposure of lymphocyte cultures are shown in Table 1. An increase in micronuclei frequency was recorded at 100 µmol/l

Table 1. FISH analysis of chromosome 1 aberrations in interphase and metaphase cells of human lymphocyte cultures after 24 h benzene treatment.

Donor	Benzene concentration	Cells scored	Micronuclei analyzed	Rate per 1000 cells	Metaphases	Chromosome 1 aberrations		
						monosomies	tetraploidies	break in 1q12 region
	µmol/l	n	n		n	n (%)	n (%)	n (%)
1	0 (DMSO)	3,000	9	3.0	250	1 (0.4)	1 (0.4)	0
	50	3,000	10	3.3	250	2 (0.8)	2 (0.8)	0
	100	3,000	12	4.0[a]	250	2 (0.8)	2 (0.8)	0
2	0 (DMSO)	3,000	7	2.3	200	0	1 (1.0)	0
	50	3,000	8	2.7	200	2 (1.0)	1 (1.0)	0
	100	3,000	11	3.7[a]	150	2 (1.3)	1 (0.7)	1 (0.7)

[a] – no statistically significant data (χ^2 test)



A – Normal interphase cell without micronucleus (two green signals; dividing cell randomly present)
B – Interphase cell with a signal-negative micronucleus
C – Interphase nucleus with associated micronuclei without hybridisation signal
D – Normal human metaphase plate (filter FITC/TRITC, two green signals)
E – Human metaphase with tetraploidy (filter DAPI/FITC, four green signals)
F – Human metaphase with presumable breakage in chromosome 1 pericentromeric region (three green signals)

Figure 1. Interphase and metaphase FISH of human peripheral lymphocytes with a classical satellite DNA probe for the pericentromeric 1q12 region (green signals).

concentrations in interphase cells of both donors in comparison with control cultures. However, no statistical significance was recorded (χ^2 test, $p > 0.05$). The green FISH signals were observed in interphase nuclei indicated the presence of chromosomes 1. In adjacent micronuclei, no fluorescence spot was detected for each tested concentration and donor (Fig. 1: A, B, C). To ensure that the structures identified as micronuclei were not artifactual we used two types of dual filters for red/green and blue/green fluorescence.

Depending on the richness of the slides, from 150–250 metaphases per benzene concentration were analysed by FISH using the same chromosome 1 classical satellite probe as in MN assay. Monosomy and tetraploidy of chromosome 1 were observed at both concentrations as well as in both donors, but the significant differences in the above-mentioned numerical aberration induction compared with controls were not determined. In chromosome 1 pericentromeric region (1q12), presumable breakage was observed after exposure to a benzene dose of 100 µmol/l (donor 2) (Fig. 1: D, E, F).

DISCUSSION

Micronuclei (MN) are small, extranuclear bodies formed during mitosis from acentric chromosomal fragments or whole chromosomes that are not included in each daughter nucleus. A combination of the micronucleus assay and fluorescence *in situ* hybridization technique using centromeric probes is useful for detecting aneuploidy, as well as for characterizing the origin of MN occurring either spontaneously, or following exposure to various chemical agents. According to Chung *et al.* [2], a centromeric fluorescent signal in an MN indicates either an entire chromosome resulting from chromosome mal-segregation (non-disjunction), or a chromosome fragment originating from a breakage within the centromeric region. MN containing acentric fragments outside the centromeric region does not exhibit a centromeric signal.

In general, an important reason for using interphase analysis is that it can be performed on rarely or non-dividing cells allowing relatively simple detection of chromosome alterations. In our *in vitro* experiment, a classical satellite

probe that hybridizes near the centromere in the heterochromatic area of chromosome 1 (pericentromeric region) was used to detect possible alterations of the region under study after benzene treatment. Since no FISH signals were detected in adjacent micronuclei, our results suggest that either no chromosome 1 or only a chromosome fragment originating from outside the pericentromeric region were included in MN.

Nowadays, limited data have been presented about chromosome 1 aberrations after *in vitro* exposure to benzene. In experiments *in vivo*, Marcon *et al.* [5] used two different chromosome 1 specific probes to detect chromosome alterations in peripheral blood cells of benzene-exposed shale oil workers. No significant difference in the incidence of breakage was detected in the nucleated cells of blood smears of exposed versus control subjects. In contrast, the authors observed significantly increased frequencies of breakage affecting chromosome 1 in the cultured lymphocytes. Considering that the 1 cen-q12 region includes about 0.5–0.7% of the human genome, the positive results could indicate that this region of chromosome 1 is quite sensitive to the induction of chromosomal breakage. In a later analysis, Marcon *et al.* [6] concluded that signal displacement in 1cen-1q12 regions of human chromosome 1 might be a marker of chemical exposure.

We have demonstrated only a low incidence of breaks in the fluorescent labelled area of chromosome 1 pericentromeric region in metaphase cells. This could be influenced by the setting of our experimental conditions, such as treatment and sampling time, as well as the relatively small number of metaphases examined. It is well known that benzene depress B and T lymphocytes as well as their mitotic responses [8].

In our study, hypoploidy (monosomy) and polyploidy (tetraploidy) were the most often observed numerical aberrations including chromosome 1 in the cultured metaphases. These types of aberrations were demonstrated also in other human chromosomes [1, 16]. The reason for the selective sensitivity of specific chromosomes to benzene is still unclear. One possible explanation for the preferential effect of benzene metabolite on specific chromosomes may be suggested by the fact that only cells with non-lethal chromosome aberration could survive to be detected. The elimination of telomere on a particular chromosome has been reported to lead to selective chromosome gain and loss [16]. Zhang *et al.* [16] have reported that benzene and its metabolites can lead to microtubule toxicity, consequently spindle disruption. If spatial positioning of certain chromosomes is more susceptible to this spindle disruption effect, then specific chromosome aberration will result. According to Whysner [15], aneugenesis due to damage to components of the mitotic apparatus is one of the four specific mechanisms to explain benzene genotoxicity.

Our observations were in general consistent with those of Zhang *et al.* [16]. The authors concluded that metaphase FISH was more sensitive than interphase FISH in detecting both monosomy and trisomy in the lymphocytes of exposed workers. Thus, whereas interphase FISH allows nondividing cells to be analysed, metaphase FISH can detect structural changes on specific chromosomes, permitting the more detailed analysis of damage affecting specific chromosome regions.

CONCLUSION

In the present study, simultaneous detection of aberration in different phases of the cell cycle was performed after *in vitro* treatment of human lymphocytes by benzene. In spite of the modest benzene-related *in vitro* effect on the pericentromeric region of the human chromosme 1 examined in interphase and metaphase lymphocytes, a combination of interphase and metaphase FISH seems to be a good choice for chromosome damage studies.

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