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Long-lasting persistence of elevated sister-chromatid exchange frequencies induced by perinatal benzo(a)pyrene treatment in rat bone-marrow cells

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Abstract. In this work the possibility that a mutagenic factor acting in utero or in the perinatal period might lead to elevated mutagenic rates in bone-marrow cells after a considerable period of time was examined. An aromatic hydrocarbon, benzo(a)pyrene was used as the test substance. Benzo(a)pyrene treatments resulted in significantly higher sister-chromatid exchange (SCE)-frequencies in both fetal and neonatal groups in both sexes, even four months after exposure. In a second experiment we examined whether mutagenic exposure suffered in utero could make the individual more susceptible to mutagenic effects in adulthood. Preliminary results indicate that such a possibility could exist.
Key words. Benzo(a)pyrene; sister-chromatid exchange; bone-marrow; sensitisation.

Increasing environmental pollution, including the release of considerable quantities of mutagenic agents, is one of the potential risk-factors that can be involved in the pathogenesis of numerous diseases, and especially in carcinogenesis[1]. Although many examples of the short-term mutagenicity of various compounds are known, less is known about whether lesions elicited by such compounds can be detected a long time after exposure. Considering that the development of diseases that may include steps involving mutagenic impacts – especially carcinogenesis – is a lengthy process, the importance of long-lasting lesions brought about by mutagenic effects can even be greater than that of short-term mutagenicity.

We use the term 'long-lasting' for lesions that can be detected more than one month after treatment, and the term 'short-term' for lesions that cannot be detected any more after one month has elapsed since the treatment. For the detection of long-lasting lesions elicited by mutagenic effects, the sister-chromatid exchange (SCE)-test seems to be the most suitable method to apply, although the mechanism eliciting of lesions is unknown. There are a number of experiments demonstrating that SCE-forming lesions can persist during several cell divisions[2–4]. Most of the evidence for long-lasting persistence of elevated SCE-frequencies comes from studies using ethylnitrosourea (ENU) as the test compound. For example, Stetka et al.[5], using ENU on CHO cells, demonstrated that the elevated SCE-frequency decreased with time, but did not return to control levels for up to 7 days of culturing.

There are also findings in non-dividing cells demonstrating long-lasting increases in SCE-frequencies. Jones et al.[6] demonstrated that SCE-forming lesions induced by ENU could persist in spleen lymphocytes for at least 40 days. Tucker et al.[7] found that SCE-frequencies were significantly higher in mice exposed to ENU than in controls, even two or three months after exposure. They returned to control levels six months after treatment, both in spleen and blood lymphocytes.

The possibility that mutagenic effects in utero or during neonatal life could lead to enhanced SCE-frequencies a considerable period later, even in adulthood, could be of particular significance.

In the present study we examined the question of the persistence of elevated SCE-frequencies in continuously dividing bone-marrow cells using an animal model, in which animals were treated either in utero or neonatally with the known short-term mutagen and widespread environmental pollutant benzo(a)pyrene[8]. Four months later SCE-frequencies were determined. In a second set of experiments we tested the possibility whether mutagenic exposure (benzo(a)pyrene treatment) in utero could lead to increased sensitivity to mutagenic effects in adulthood.

Materials and methods

Animals. Wistar rats were used for both experiments. The offspring of 6 dams were used for each experiment, 18 males and females for the first and 12 males for the second. Each dam had an average number of 8 pups. The offspring were weaned from their mothers 4 weeks after birth, and separated according to their sex. The animals used for the experiments were selected randomly. They were provided with food and water ad libitum, and housed 3 per cage under regular conditions.

Test compound. Benzo(a)pyrene was purchased from Fluka (Buchs, Switzerland). It was dissolved in sterile sunflower oil before use.

Genotoxicity tests. In both experiments two genotoxicity tests were applied, the SCE-test and the micro-

nucleus-assay (MN-assay). The MN-assay, being a short-term test, serves as an internal control in both experiments, although its sensitivity and specificity are different from that of the SCE-test.

Treatment protocols. In the first experiment animals were treated either in utero or neonatally using benzo(a)pyrene. Every treatment group consisted of 3 animals of one sex. To bring about fetal exposure, 2.0 mg/kg b.wt benzo(a)pyrene was administered intramuscularly to the dams on days 15, 17 and 19 of gestation. Controls received 0.5 ml sunflower oil in the same way. The neonates received 20.0 µg benzo(a)pyrene per animal subcutaneously on days 0, 3 and 7 postnatally.

In the second experiment animals were treated either in utero or in adult life (i.e. at four months of age), or both. In the fetal treatments the dams received either sunflower oil or benzo(a)pyrene in the same way as in the first experiment. The offspring received in adulthood either a single dose of 2.0 mg/kg b.wt benzo(a)pyrene or sunflower oil in 0.5 ml volume.

The animals were sacrificed four months after fetal or neonatal treatment in the first experiment, and two days after treatment at 4 months of age in the second experiment. Both femurs were removed and the bone-marrow was extracted under sterile conditions (according to the method of Schmid[9]).

SCE-test. After the centrifugation of the bone-marrow suspension, parallel cultures using TC199 medium (National Institute of Public Health, Budapest) containing 10% fetal calf serum (FCS, Sebak GmbH, Aidenbach, Germany) were started. Cultures were incubated for 36–40 h to achieve the highest possible proportion of second mitoses, and for the last 24–28 h of that period in the presence of BrdU (Sigma, St Louis, USA) in 10 µM concentration. Before harvesting, cultures were incubated with Colcemid (Serva, Feinbiochemica, Heidelberg, Germany) in 0.05 µg/ml concentration for 2 h. Cultures were incubated at 37 °C with 5% CO_2/95% air.

The cultures were harvested according to the method of Dean and Danford[10]. After dropping the suspensions onto ice-cold slides, the slides were stained according to the Bisbenzimid-H-33258 (Riedel de Haen AG, Seelze, Hannover, Germany)-Giemsa technique[11].

After staining, 30 s metaphases per animal were counted. The proportion of dividing cells per 1000 cells (the mitotic index) was also determined.

MN-assay. Following the centrifugation of the bone-marrow suspension, the sediment was suspended in a few drops of FCS. Smears were prepared, and then stained according to the method of Heddle et al.[12] After staining, 1000 PCEs (polychromatic erythrocytes) were counted and the number of MN-containing PCEs and the PCE/NCE (normochromatic erythrocytes) ratio was registered.

Statistical methods. In both experiments the MN-values were analysed using the Kruskal-Wallis-test (KW-test). The SCE-frequencies were analysed by the use of Student's t-test. All possible comparisons were made.

Results

First experiment. In this experiment the animals were treated either in utero or neonatally, and MN- and SCE-frequencies were determined four months later in adulthood. Due to the generally large fluctuation of MN-values, average scores under 5.00 are not generally considered to be significant[12], thus we can conclude that MN-values were not changed in any group, either in males or in females.

The SCE-frequencies, however, were significantly higher in both treated groups compared to controls, in both sexes ($p < 0.001$). Animals treated with benzo(a)pyrene in the neonatal period exhibited significantly higher SCE-frequencies than those treated in utero, in both sexes (in female groups: $p < 0.02$; in male groups: $p < 0.005$; table 1).

Though the responses to benzo(a)pyrene were the same in male and female groups concerning the trend of changes, in both fetal and neonatal treatment groups males exhibited slightly higher SCE-frequencies compared to the same groups of females (fetal treatment groups: $p < 0.01$; neonatal treatment groups: $p < 0.005$). These findings were checked using the Kruskal-Wallis-test, and it gave similar results.

Second experiment. The animals in this experiment received either fetal or adult treatment, or both. MN-values in groups that received a first or second benzo(a)pyrene dose in adulthood were significantly higher than those of groups which had not been exposed to benzo(a)pyrene or had been exposed only in utero ($p < 0.05$).

The group that received two benzo(a)pyrene treatments exhibited significantly higher SCE-frequencies com-

Table 1. Results of the first experiment: treatments prenatally or neonatally.

Treatment groups	MN-values		PCE/NCE ratio (mean)	SCE-frequencies/cell (mean ± SD)
	mean	range		
Males				
control	1.66	1–3	0.915	4.39 ± 1.11
N-BP	2.00	1–3	0.669	8.74 ± 1.93*
F-BP	3.33	2–6	1.291	7.77 ± 1.94*
Females				
control	2.00	1–3	1.452	4.23 ± 1.21
N-BP	1.33	1–3	1.874	7.71 ± 1.27*
F-BP	2.75	2–4	1.558	7.06 ± 1.60*

Treatment groups: N = neonatal, F = fetal, BP = benzo(a)pyrene. PCE = polychromatic erythrocyte; NCE = normochromatic erythrocyte.
*significant difference from controls.

Table 2. Results of the second experiment: double treatments.

BP treated groups	MN-values		PCE/NCE ratio (mean)	SCE-frequencies/cell (mean ± SD)
	mean	range		
−/−	3.66	2–6	1.988	6.84 ± 2.08
+/−	3.33	3–4	0.964	7.48 ± 1.73*
−/+	6.66*	5–9	2.413	8.26 ± 2.73
+/+	7.66*	7–8	1.056	10.46 ± 3.53*

Treated groups: BP = benzo(a)pyrene. The first symbol represents the fetal, the second the adult treatments: + = BP treatment; − = no BP treatment.
PCE = polychromatic erythrocyte; NCE = normochromatic erythrocytes.
*significant difference from controls.

pared to the groups treated once in utero or as adults, and the untreated controls ($p < 0.001$). The group receiving benzo(a)pyrene treatment in adulthood exhibited significantly higher SCE-frequencies than the untreated control group and the fetally treated group ($p < 0.001$ to the absolute control; $p < 0.05$ to the fetal treatment group). The fetal treatment group showed significantly higher SCE-frequencies compared to the untreated controls ($p < 0.05$; table 2).

The toxicity indices (PCE/NCE ratio and mitotic index), which indicate the harmful effects of a tested compound on the dividing capacity of cells, did not show substantial differences between treated and non-treated groups in either experiment (data of mitotic index are not shown.)

Discussion

Benzo(a)pyrene, an agent known to possess short-term mutagenic capacities[1,8], produced elevated SCE-frequencies that persisted for four months in continuously dividing bone-marrow cells in both fetal and neonatal treatment groups of both sexes.

Micronucleus-values, however, were not changed in any group in the first experiment compared to the controls. The formation of micronuclei can be the result of spindle dysfunctions and chromosome breakages, that result in chromosome pieces or whole chromosomes being unable to move to the cell poles in the anaphase of mitosis, and forming distinct bodies apart from the cell nucleus. It was shown that in PCEs (polychromatic erythrocytes) micronuclei can be detected for up to 5 days following treatment[12]. By applying this test we can obtain information about the short period (i.e. 5 days) preceding processing. Elevated MN-frequencies in the first experiment would indicate unforeseen mutagenic effects in the short period preceding processing, and that would render the interpretation of the data of the SCE-test difficult. In the second experiment, however, elevated MN-values indicate the effectiveness of treatment in adulthood. MN-values in the first experiment do not indicate any mutagenic effect of this kind taking place within the 5 days preceding processing.

It has been proved that elevated SCE-frequencies can persist through several cell divisions[2–4], though the mechanism of this is unknown. In the experiments described here, the period of the persistence of the elevated SCE-frequencies is even longer, which makes it even more difficult to explain. Neonatal treatments resulted in significantly higher SCE-frequencies than fetal exposure. This finding is consistent with results of experiments examining the inducibility of microsomal enzymes and the sexual behaviour of adult animals treated with benzo(a)pyrene in the fetal or neonatal period[13,15]. The greater effect of neonatal treatment could be explained by the fact that the animals were exposed directly, and not through the mother.

Males presented slightly higher SCE-frequencies than females in both fetal and neonatal treatment groups, which indicates that they were more susceptible to benzo(a)pyrene treatments than females. This could be explained by the disparate development of the microsomal enzyme system in males and females[16–18]. This system has a significant role in the transformation of aromatic hydrocarbons[8].

The finding that fetal or neonatal treatment with benzo(a)pyrene could lead to increased SCE-frequencies even after 4 months, in adulthood, is further evidence for the potential danger of increasing environmental pollution. Since we are exposed to the actions of mutagenic substances all our lives long, this could mean that these compounds can exert their harmful effects not only through adult, but also through perinatal exposure. Considering that the life-span of humans is far longer than that of rats, this could mean that mutations suffered in the pre- and postnatal period may manifest themselves years later.

The differences between the SCE-frequencies of the control groups of the two experiments could be explained by the inherent individual differences between animals[19–21].

The preliminary results of the second experiment confirm the findings of the previous one, demonstrating that mutagenic effects in utero could lead to increased SCE-frequencies in adulthood. The striking point in this experiment is that the group receiving benzo(a)pyrene treatments both in utero and in adulthood exhibited significantly higher SCE-frequencies than groups receiving treatments only once. This might mean that somehow a mechanism of sensitisation [or addition][14,17] could exist, and exposure to mutagenic effects in utero could lead to increased susceptibility to the same (or other) mutagenic impacts in the adulthood. This phenomenon would imply great risk for the population, considering the high level of aromatic hydrocarbon pollution, but more research is needed to achieve a better understanding of the mechanisms involved.

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1 Hall, M., and Grover, P. L., in: Chemical Carcinogenesis and Mutagenesis, vol. I, p. 327. Eds C. S. Cooper and P. L. Grover. Springer Verlag, Heidelberg 1990.
2 Conner, M. K., and Cheng, M., Cancer Res. *43* (1983) 965.
3 Morales-Ramirez, P., Rodríguez-Reyes, R., and Vallarino-Kelly, T., Mutat. Res. *232* (1990) 77.
4 Schvartzman, J. B., Goyanes, V. J., Campos, A., Lage, A. M., Veiras, C., Silva, M. C., and Ramos, S., Chromosoma *92* (1985) 7.
5 Stetka, D. G. Jr., Bleicher, W. T., and Brewen, J. G., Envir. Mutagen. *7* (1985) 233.
6 Jones, I. M., Burkhart-Schultz, K., and Carrano, A. V., Mutat. Res. *143* (1985) 245.
7 Tucker, J. D., Strout, C. L., Christensen, M. L., and Carrano, A. V., Envir. Mutagen. *8* (1986) 345.
8 Gelboin, H. V., Physiol. Rev. *60* (1980) 1107.
9 Schmid, W., Mutat. Res. *31* (1975) 9.
10 Dean, B. J., and Danford, N., in: Mutagenicity Testing. A Practical Approach, p. 187. Eds S. Venitt and J. M. Parry. IRL Press, Oxford-Washington DC 1984.
11 Perry, P., and Wolff, S., Nature *352* (1974) 156.
12 Heddle, J. A., Stuart, E., and Salamone, M. F., in: Handbook of Mutagenicity Test Procedures, p. 441. Eds B. J. Kilbey, M. Legator, W. Nichols and C. Ramel. Elsevier Science Publishers, Amsterdam 1984.
13 Csaba, G., Karabélyos, C., and Dalló, J., J. devl Physiol. *19* (1993) 67.
14 Csaba, G., Biol. Rev. *55* (1980) 47.
15 Csaba, G., Dobozy, O., and Szeberényi, S., Acta physiol. hung. *70* (1987) 87.
16 Finnen, N. J., and Hassal, K. A., Biochem. Pharmac. *29* (1980) 3133.
17 Haake-McMillan, J. M., and Safe, S. H., J. Biochem. Toxic. *5* (1990) 203.
18 Lamartiniere, J. M., and Nicholas, J. N., Biochem. Pharmac. *33* (1981) 4092.
19 Hosomi, J., Nemoto, N., and Kuroki, T., Cancer Res. *43* (1983) 3643.
20 Harper, P. A., Golas, C. L., and Okey, A. B., Molec. Pharmac. *40* (1991) 818.
21 Manchester, E., Gordon, S. K., Golas, C. L., Roberts, E. A., and Okey, A. B., Cancer Res. *47* (1987) 4861.