National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Exposure to genotoxic carcinogens at young age: experimental studies to assess children's susceptibility to mutagenic effects of environmental chemicals

RIVM report 2014-0008

Mirjam Luijten et al.

National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Exposure to genotoxic carcinogens at young age: experimental studies to assess children's susceptibility to mutagenic effects of environmental chemicals

RIVM Report 2014-0008

Colophon

Mirjam Luijten
Lya G. Hernández
Edwin P. Zwart
Harry van Steeg
Peter M. Bos
Jan van Benthem

Contact:
Mirjam Luijten
Centre for Health Protection
Mirjam.Luijten@rivm.nl

This investigation has been performed by order and for the account of the Netherlands Food and Consumer Product Safety Authority, within the framework of project V/090123 'CMRI stoffen in het jonge kind'.

This is a publication of:

National Institute for Public Health and the Environment
P.O. Box 1 | 3720 BA Bilthoven
The Netherlands
www.rivm.nl/en

Abstract

Young animals do not appear to be more susceptible than adult animals to mutagenic effects of environmental chemicals

Experimental animals can be more susceptible at a young age to the adverse effects induced upon exposure to environmental chemicals in comparison to adult animals. In research performed at the RIVM, the susceptibility to DNA mutations in young and adult animals was investigated for a selected set of substances. The results from this research suggest that increased susceptibility to mutagenic effects is dependent on the specific mechanism of action.

Environmental chemicals may invoke DNA mutations through a variety of mechanisms. Usually, potential adverse human health effects of environmental chemicals are evaluated in toxicity studies using adult laboratory animals. Children and adults, however, may differ in sensitivity to these adverse effects.

In previous research we found that exposure to benzo[*a*]pyrene, a chemical commonly found in grilled and broiled foods, tobacco smoke and automobile exhaust fumes, induced DNA mutations at a higher frequency in animals exposed at a young age in comparison to animals exposed at adult age. For the other three chemicals investigated in the present study we did not find any age-related differences in genotoxicity. These findings suggest that increased susceptibility to mutagenic effects is dependent on the specific mechanism of action, which then is to be taken into account in chemical risk assessments of children.

Key words:
children, genotoxic chemicals, risk assessment, genotoxicity, carcinogenicity

Publiekssamenvatting

Jonge dieren lijken niet gevoeliger dan volwassen dieren voor schadelijke effecten van DNA-beschadigende stoffen

Op heel jonge leeftijd kunnen proefdieren gevoeliger zijn voor de schadelijke effecten van chemische stoffen dan op volwassen leeftijd. Sommige stoffen veroorzaken in de jonge levensfase meer schade aan het DNA, maar dat is niet altijd het geval. Een hogere gevoeligheid op jonge leeftijd lijkt af te hangen van de manier waarop de stof het erfelijk materiaal beschadigt. Dit blijkt uit onderzoek van het RIVM.

Chemische stoffen kunnen op verschillende manieren veranderingen aan het erfelijk materiaal veroorzaken. Normaal gesproken worden mogelijke schadelijke effecten van chemische stoffen in kaart gebracht door studies met volwassen proefdieren uit te voeren. Kinderen en volwassenen kunnen echter verschillen in de mate waarin ze gevoelig zijn voor chemische stoffen.

Eerder was een hogere gevoeligheid voor schadelijke effecten bij jonge proefdieren waargenomen in onderzoek van het RIVM naar benzo[*a*]pyreen. Deze stof, die voorkomt in voeding, zoals gebraden vlees, en in tabaksrook en uitlaatgassen, geeft meer schadelijke effecten op jonge leeftijd dan op volwassen leeftijd. Nadien is dit effect getoetst voor drie andere stoffen. Bij stoffen die op een andere manier DNA beschadigen, heeft leeftijd veel minder invloed. Bij de risicobeoordeling van chemische stoffen zal per stof beoordeeld moeten worden of de standaard veiligheidsfactor voldoende is of aanpassing behoeft.

Trefwoorden: kinderen, DNA schade, risicobeoordeling, genotoxiciteit, carcinogeniteit

Contents

Summary

Exposure to chemicals may have adverse impacts on human health and the environment. Potential risks associated with exposure are assessed before chemicals are allowed on the market. These safety assessments are carried out based on data obtained from *in silico*, *in vitro* and *in vivo* studies, and include adjustment for safety factors such as extrapolation from animals to humans and/or inter-individual variation in susceptibility. High-risk groups and children, however, are not explicitly accounted for: it is assumed that susceptible groups within the population are covered by the assessment factors. Children, however, differ from adults in many ways. They have different, and sometimes unique, exposures to environmental chemicals, and their physiology is different from those of adults. Consequently, they may be at a higher risk of exposure to a given environmental chemical and/or be more susceptible to a given disease. In the present report, we investigated whether the susceptibility to acquire DNA mutations or chromosomal damage depends on age, and whether this depends on the mutagenic mechanism of action. A selected set of substances was tested in an animal study with different age groups, as representatives of children and adults. The set consisted of three genotoxic substances that each induce DNA breaks but with different mechanisms of action: acrylamide, cisplatin, and etoposide. For these substances, we did not observe an increased susceptibility to mutagenic effects when exposure occurred early in life. This is in contrast with findings from a previous study, in which benzo[*a*]pyrene was observed to induce elevated mutant frequencies in mice exposed at a very young age compared to animals exposed at an adult age. Our results suggest that increased susceptibility to mutagenic effects at a young age is dependent on the specific mechanism of action. This should be taken into account in chemical risk assessments of children. Future research on a larger set of substances is needed to support this conclusion. Ideally, these additional studies should also focus on the question whether thresholds, commonly accepted for some mutagenic mechanism of action, differ between age groups.

1 Introduction

The evaluation of potential adverse effects of environmental chemicals to which humans are exposed on a daily basis is a challenging task for risk assessors, especially when dealing with carcinogenic substances. The exact procedures depend on the regulatory framework. In general, cancer risk assessment comprises hazard identification, dose-response assessment, exposure assessment and risk characterization. The carcinogenic potential of a substance is usually assessed based on multiple lines of evidence that are analyzed in a weight-of-evidence approach. Evidence considered may include physicochemical properties, comparability with other structurally related carcinogens, and toxicity assays that address carcinogenic processes and mode of action. The latter include genotoxicity tests, either *in vitro* or *in vivo*, and carcinogenicity studies (1-3). Human data from epidemiologic studies, if available, should always be taken into account. The starting point for dose-response assessment is a dose associated with a carcinogenic endpoint, such as the T25 dose (the dose giving a 25% incidence of cancer in an appropriately designed animal experiment), the no-observed-adverse-effect level (NOAEL; *i.e*. for threshold carcinogens) or, preferably, a benchmark dose (BMD) (1, 2, 4-6). Extrapolation of such data to exposure conditions likely to be encountered by humans requires integration of toxicokinetic and toxicodynamic information, understanding of the mode of action, and consideration of potential susceptible subpopulations and life stages.

Responses to environmental agents can vary widely among individuals and between population groups. Population groups and life stages with potentially increased susceptibility should therefore be given consideration in risk assessment. In recent years there has been an increasing focus on children as a potentially susceptible population in environmental risk assessment (7-10). Children may have vulnerabilities that are distinct from those of adults as their bodies are developing rapidly, their metabolic pathways are immature, and their remaining life expectancy is longer than for adults. They also have a unique exposure pattern as they breathe more air relative to body weight, consume more food and water relative to body weight, and spend more time indoors and closer to the ground than adults (11, 12). Increased awareness of differences between children and adults in terms of toxicokinetics, toxicodynamics and exposure has resulted in the development of lifestage-specific approaches to risk assessment (12-16). A recent OECD survey showed that various methodologies and tools are currently applied in the assessment of risks associated with the exposure of children to environmental chemicals (17). However, as indicated by the survey, there still is a significant need for additional guidance, harmonization of definitions and risk characterization methodologies (*e.g.* extrapolation from adults to children), and tools for exposure assessment.

Cancer risk assessment is normally based on a lifetime daily exposure scenario. However, exposure to a toxic substance may be acute or short-term. Studies in which animals were exposed at different life stages suggest that animals are at a higher risk at a relatively young age (18, 19). When assessing the risks of acute exposure to genotoxic carcinogens, increased susceptibility at specific life stages, including children, needs to be considered (20). The U.S. Environmental Protection Agency (EPA) has evaluated cancer susceptibility associated with early-life exposures by reviewing published animal studies that compared tumour incidence between early-life and adult-only exposures or between early-

life-and-adult and adult-only exposures (15). Both the acute and repeated dose studies support the concept that early-life exposure to chemicals with a genotoxic mode of action would lead to an increased tumour incidence compared to adult exposure. The U.S. EPA therefore recommended implementation of so-called 'age-dependent potency adjustment factors' for carcinogens with a mutagenic mode of action (12). In Europe, different approaches are proposed by, for instance, ECHA and EFSA. According to ECHA, an intraspecies assessment factor is considered not necessary in the derivation of a derived minimal effect level (DMEL), if based on animal experiments. The reason for this is that the linear model used for high to low dose extrapolation is considered sufficiently conservative to also cover differences in intraspecies susceptibility. When a DMEL is based on human epidemiological data, as a general rule an intraspecies factor is also not applied unless the available data indicate otherwise, for example if the study population is not representative (5). EFSA (4) proposes a Margin of Exposure (MoE) approach and considers an overall factor of 100 sufficient for interspecies and intraspecies differences without further specification. So, overall application of higher assessment factors is guided by, if at all, age of the exposed population. In the present study, we investigated whether the susceptibility to acquire DNA mutations or chromosomal damage depends on age, and whether this difference is associated with a specific mutagenic mechanism of action. Four different genotoxic substances (benzo[*a*]pyrene, cisplatin, acrylamide, and etoposide) were tested in an animal study with different age groups, as representatives of children and adults. In addition, for benzo[*a*]pyrene we explored whether the window of exposure affects tumour type, incidence, and severity.

2 Materials and methods

2.1 Chemicals

Etoposide (ETO; CAS No. 33419-42-0), *cis*-diammineplatinum(II) dichloride (CPPD; CAS No. 15663-27-1), acrylamide (AA; CAS No. 79-06-1), and cyclophosphamide (as positive control) (CPA; CAS No. 6055-19-2) were purchased from Sigma-Aldrich (Zwijndrecht, NL). ETO, CPPD and CPA were dissolved in DMSO and further diluted in phosphate buffered saline (PBS). Each of these three chemicals was administered by intraperitoneal injection (*i.p.*). AA was dissolved in PBS and administered by oral gavage. In the AA study, CPA was dissolved in PBS and administered by oral gavage.

2.2 Mice

C57BL/6J wild type mice were obtained from Harlan Laboratories (Blackthorn, UK). All mice were maintained under specific pathogen-free conditions in a climate-controlled room with a 12h on/off light cycle. Feed and water were available *ad libitum*. The studies were approved by the institute's Ethical Committee on Experimental Animals, in accordance with national legislation.

2.3 *In vivo* micronucleus test

Animals were three or ten weeks of age at the beginning of treatment; treatment groups consisted of six animals. Ten-week-old animals were acclimated for two weeks prior to start of the experiment. Because of differences in toxicokinetic and toxicodynamic properties (see below), two different study designs were used: one for ETO and CPPD, and one for AA. In both studies, CPA served as positive control. The dose-range selection for these chemicals was based on previous experiments performed at our institute or on published studies. For ETO, CPPD, and the positive control CPA we used three males and three females per group. Animals were administered a single dose of vehicle, 0.25, 0.50 or 1.0 mg/kg body weight of ETO or CPPD. CPA was tested at only one dose, *i.e.* 50 mg/kg body weight. Exactly 48 hours after treatment, mice were anesthetized with CO_2/O_2, blood was extracted by eye puncture and the animals were killed by cervical dislocation.
For AA, only female mice were used because of a higher bioavailability of AA in female animals (21). Animals were given by oral gavage on three consecutive days either AA in concentrations of 12.5, 25.0, and 50 mg/kg body weight per day, or CPA (50 mg/kg body weight per day), or PBS. Exactly forty hours after the last treatment, peripheral blood samples were obtained by tail puncture. Animals were killed by cervical dislocation.
Peripheral blood samples were processed immediately upon collection as described in MicroFlow® BASIC kit (Litron Laboratories, Rochester, NY). Fixed blood samples were stored at −80°C until flow cytometric analysis was conducted. Flow cytometric evaluation of micronucleated reticulocytes (MN-RETs) was performed by Covance Laboratories (Harrogate, UK).

2.4 Statistical analyses

All parameter values are represented as means ± SD, where appropriate. Statistical analyses were performed using GraphPad Prism version 6.02 for

Windows (GraphPad Software, La Jolla, California, USA). Comparisons between groups were performed with one-way ANOVA or two-way ANOVA followed by either a Dunnett's test or a Tukey's test. P-values smaller than 0.05 were considered statistically significant.

3 Genotoxicity studies

Four different genotoxic substances (benzo[*a*]pyrene (B[*a*]P), cisplatin (CPPD), acrylamide (AA), and etoposide (ETO)) were tested in an animal study with different age groups, as representatives of children and adults. The results for B[*a*]P have been described previously (22). In brief, B[*a*]P was tested in a gene mutation assay, using three different age groups (3, 10, and 26-week-old mice) as representatives of children, young adults and adults. The group of 10-week-old mice was considered the reference group, since mice used in conventional genotoxicity tests commonly are 6-15 weeks of age at start of the experiment. Both wild type (WT) and *Xpc* mice, all carrying the *LacZ* reporter gene, were tested. The results showed that mutant frequencies in the liver of WT mice treated at the age of 3 weeks were significantly higher as compared to those of mice treated at 10 weeks of age.

Each of the other three substances (AA, CPPD, and ETO) induces DNA breaks, but with different mechanisms of action. AA produces DNA adducts via its metabolite glycidamide, CPPD is an alkylating-like agent that forms DNA cross links, whilst ETO poisons type II topoisomerases thereby inhibiting religation of cleaved DNA molecules (23-28). For all three substances an *in vivo* micronucleus (MN) assay in peripheral blood was performed. Since we did not find substantial differences in the severity of genotoxic effects between 10-week-old and 26-week-old mice in the B[*a*]P study (22), we now only used two age groups: animals of either 3 or 10 weeks of age at the beginning of treatment. For ETO and CPPD, flow cytometric measurements of MN-RETs in peripheral blood samples from 3-week-old and 10-week-old animals showed positive, dose-dependent increases (Figure 1). All dose groups differed significantly from the control group (see Tables 1 and 2 for ETO and CPPD, respectively). We did, however, not observe a statistically significant difference in MN-RETs between the two age groups. For both substances, cytotoxicity was observed for the highest dose tested in both age groups. Exposure to ETO at a dose of 1.0 mg/kg body weight reduced relative cell counts to 53.7% and 41.4% (compared to controls) in 3-week-old and 10-week-old mice, respectively. A similar dose of CPPD caused relative cell counts of 71.7% and 61.3% (compared to controls) in 3-week-old and 10-week-old mice, respectively.
Exposure to AA resulted in only a minor increase in the frequency of MN-RETs, both in 3-week-old and in 10-week-old mice (Figure 1;Table 3). AA exposure did not result in substantial cytotoxicity in 10-week-old mice: the relative cell count was 83.6% for the highest dose tested (50 mg/kg body weight per day). In contrast to 3-week-old mice, in which this dose of AA reduced the relative cell counts to 59.1% (compared to controls).

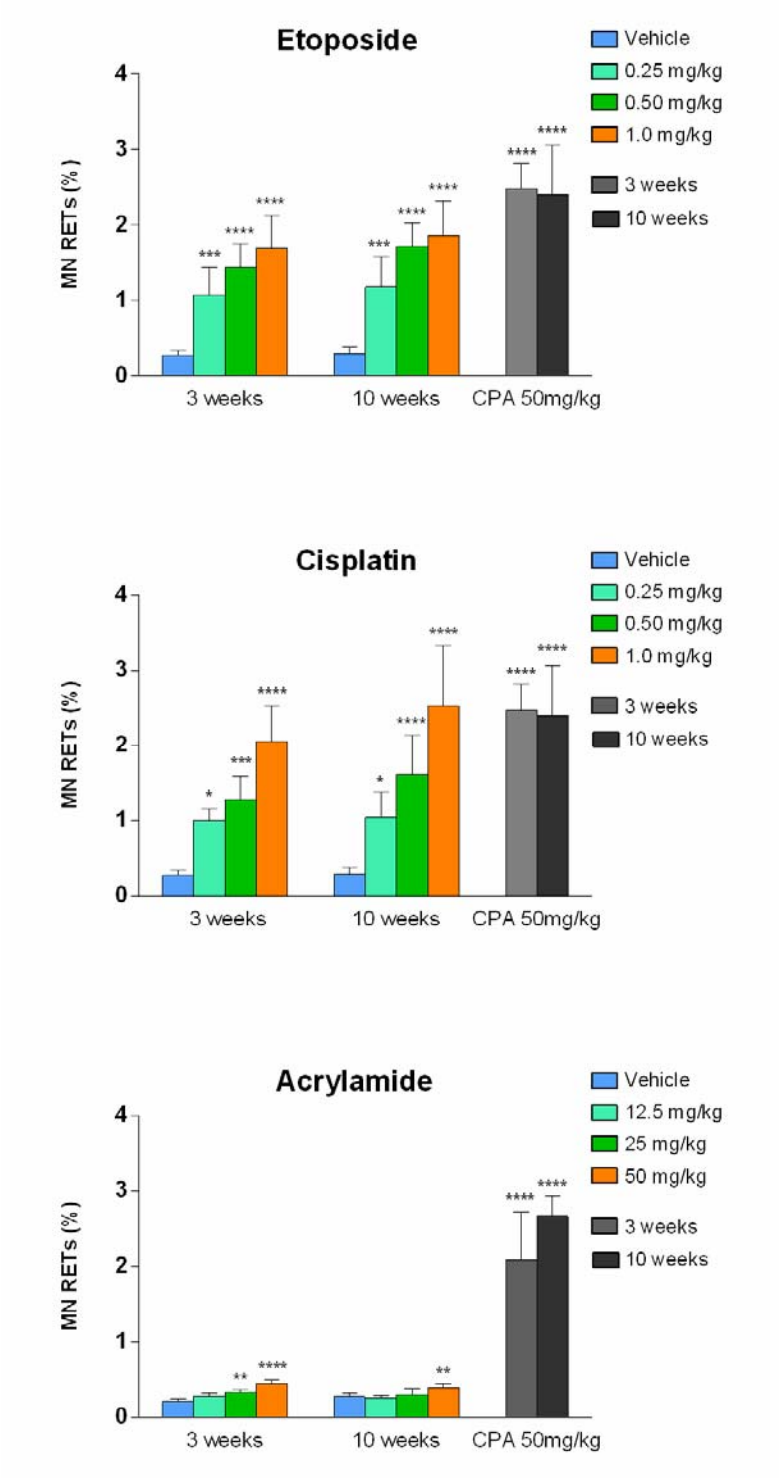

Figure 1. Percentage of micronucleated reticulocytes (MN-RETs) in peripheral blood after exposure to ETO, CPPD or AA
Asterisks indicate a significant difference between the MN-RETs in the exposed groups compared to the unexposed groups of the same age. No significant difference was observed between the two age groups. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$.

Table 1. MN induction in peripheral blood cells in mice administered etoposide[a]

Age (weeks)	Dose (mg/kg bw)	No. RETs scored[b]	MN-RETs	MN-RETs (%) ± SD
3	0	119,659	324	0.27 ± 0.07
	0.25	118,713	1287	1.07 ± 0.37***
	0.50	118,268	1733	1.44 ± 0.31****
	1.0	98,308	1694	1.69 ± 0.43****
10	0	99,708	293	0.29 ± 0.09
	0.25	118,587	1418	1.18 ± 0.40***
	0.50	117,951	2055	1.71 ± 0.32****
	1.0	117,801	2232	1.86 ± 0.46****
CPA 3	50	117,016	2974	2.48 ± 0.34****
CPA 10	50	58,885	1123	2.40 ± 0.66****

[a] A single dose of etoposide was administered by *i.p.* injection.
[b] Per group, three male and three female mice were used.
Asterisks indicate a significant difference between the MN-RETs in the exposed groups compared to the unexposed groups of the same age; *** $P<0.001$; **** $P<0.0001$.

Table 2. MN induction in peripheral blood cells in mice administered cisplatin[a]

Age (weeks)	Dose (mg/kg bw)	No. RETs scored[b]	MN-RETs	MN-RETs (%) ± SD
3	0	119,659	324	0.27 ± 0.07
	0.25	118,803	1199	1.00 ± 0.16*
	0.50	118,466	1534	1.28 ± 0.31***
	1.0	117,533	2469	2.06 ± 0.47****
10	0	99,708	293	0.29 ± 0.09
	0.25	118,750	1253	1.04 ± 0.33*
	0.50	118,067	1936	1.61 ± 0.53****
	1.0	116,967	3038	2.53 ± 0.81****
CPA 3	50	117,016	2974	2.48 ± 0.34****
CPA 10	50	58,885	1123	2.40 ± 0.66****

[a] A single dose of cisplatin was administered by *i.p.* injection.
[b] Per group, three male and three female mice were used.
Asterisks indicate a significant difference between the MN-RETs in the exposed groups compared to the unexposed groups of the same age.
* $P<0.05$; *** $P<0.001$; **** $P<0.0001$

Table 3. MN induction in peripheral blood cells in mice administered acrylamide[a]

Age (weeks)	Dose (mg/kg bw/day)	No. RETs scored[b]	MN-RETs	MN-RETs (%) ± SD
3	0	99,787	215	0.21 ± 0.03
	12.5	99,729	276	0.28 ± 0.04
	25	99,674	328	0.33 ± 0.03**
	50	99,556	452	0.45 ± 0.05****
10	0	99,740	267	0.27 ± 0.05
	12.5	99,756	246	0.25 ± 0.04
	25	99,706	302	0.30 ± 0.08
	50	99,615	389	0.39 ± 0.05**
CPA 3	50	98,367	1760	1.76 ± 0.53****
CPA 10	50	97,953	2805	2.78 ± 0.22****

[a] Acrylamide was administered on three consecutive days by oral gavage.
[b] Per group, five female mice were used.
Asterisks indicate a significant difference between the MN-RETs in the exposed groups compared to the unexposed groups of the same age. ** $P<0.01$; **** $P<0.0001$

4 Discussion

The results obtained from the *in vivo* genotoxicity studies differed for the four chemicals tested. ETO clearly induced MN-RETs in peripheral blood from exposed mice, in a dose-dependent manner, but without significant differences between the two age groups. The findings for the 10-week-old mice are in concordance with published literature: ETO has been reported to increase the frequency of micronucleated polychromatic erythrocytes (MN-PCEs) in the bone marrow of mice of 10-14 weeks of age (29). The frequency of MN-PCEs in bone marrow (3.44%), as observed by Attia et al. (29), was somewhat higher than the frequency of MN-RETs in our study (1.86%), a common observation consistent with highly efficient splenic scavenging (30). Whether the generally acknowledged threshold concept for type II topoisomerase poison-induced genotoxicity (31) also applies for animals at young age cannot be concluded from the current data. However, this is in our view to be expected since the mechanism underlying topoisomerase inhibitor-induced clastogenicity is the formation of topoisomerase II-stabilized cleavage complexes (31). Future research should focus on the question whether this threshold is different for various age groups.

Like ETO, CPPD exposure increased significantly the frequency of MN-RETs in the peripheral blood, but these levels are not significantly different between 3-week-old and 10-week-old mice. Previous studies in mice have shown that CPPD exposure gives rise to micronuclei in bone marrow (32, 33) and peripheral blood (34). However, direct comparison of the frequencies of micronucleated cells observed in those studies with our findings is not possible due to differences in doses tested.

In contrast to ETO and CPPD, exposure to AA resulted in only a minor increase in the frequency of MN-RETs, in comparison to the controls. The magnitude of the increase observed is in concordance with a previous study, in which similar doses were tested (35). AA is generally considered to be a weak clastogen *in vivo*, and several other investigations have demonstrated that both short-term and long-term exposure to AA increases the frequency of micronucleated cells, even at low doses, in rodents (24, 36, 37). In another study that also focused on the question whether young animals are more susceptible to genotoxicity, young animals appeared to be slightly more susceptible to AA exposure (38).

In our previous study on B[*a*]P (22), we found that exposure to B[*a*]P resulted in a dose-dependent increase in *LacZ* mutant frequencies in the liver of mice in the youngest age groups (both genotypes) and the reference group of *Xpc* mice. In WT mice, mutant frequencies were significantly higher in mice exposed at 3 weeks of age as compared to mice exposed at 10 weeks of age. This could be explained in part by a higher level of exposure due to increased feed consumption per kilogram bodyweight. However, even when taking this difference in exposure into account, mutant frequencies were still elevated in mice exposed at young age. It is quite unlikely that this phenomenon is due to differences in DNA repair capacity, because multiple studies in a variety of human cells have shown a decline in DNA repair capacity with age (39, 40). A possible explanation could be that the remaining difference in mutant frequency is caused by differences in xenobiotic metabolism. Metabolism is considered to be immature in neonates and said to mature rapidly during postnatal development (11, 41), but the extent of this maturation in 3-week-old mice

remains unknown. This possible difference in metabolism together with other differences in toxicokinetics and toxicodynamics may result in an increased susceptibility towards B[*a*]P-induced genotoxic stress, at least in the liver. The increased frequencies observed in WT mice exposed during early life was not noted in *Xpc* mice: mice of young age as well as adult mice exhibited a comparable increase in mutant frequency. *Xpc* mice, deficient in DNA repair, are known to be far more cancer prone than WT mice (42, 43). As such, we hypothesized that mutant frequencies would be more elevated in *Xpc* mice than in WT mice. A similar lack of response has been observed in germ cells of male *Xpc* mice upon B[*a*]P exposure (44). This suggests that the increased sensitivity of *Xpc* mice towards induction of mutations and tumours only becomes phenotypically apparent in long-term studies.

In a chronic study with B[*a*]P, using the same age groups as in the gene mutation assay, we found a higher incidence of malignant tumours in the esophagus, but a lower incidence of malignant forestomach tumours in the youngest age group in comparison to the reference group (22). An interesting finding was that the main tumour target organ in younger mice was the esophagus, in comparison to the forestomach observed in older mice exposed to B[*a*]P. This shift in tumour target tissue may be due to differences in esophageal and gastric motility. The overall tumour incidence, however, was comparable between the two age groups. In mice exposed to B[*a*]P at a relatively old age, a lower incidence of forestomach and esophagus tumours was found, as compared to the reference group. This may be explained by reduced metabolic capacity and/or reduced feed intake relative to body weight (and thus reduced exposure).

5 Implications for risk assessment

The aim of the present study was to investigate whether the susceptibility to acquire DNA mutations or chromosomal damage upon exposure to an environmental chemical was increased when exposure occurs early in life, and if the particular mechanism of action underlying mutagenicity plays a role in this susceptibility. Our results indicated that this is indeed the case. Exposure to B[*a*]P at a young age increased mutant frequencies in WT mice, but not in *Xpc* mice, pointing towards an increased susceptibility for young mice. A fold increase in susceptibility cannot be derived from these studies due to the fact that exposure levels were not similar in all groups. Additional studies would be needed to assess this fold increase as well as to learn whether this increased vulnerability applies also for other mutagens with a mechanism of action similar to B[*a*]P. The consequences of the increased susceptibility to the mutagenic effects of B[*a*]P did not become apparent in terms of tumour formation in our chronic B[*a*]P study. The overall tumour incidence in animals exposed at young age was comparable to the one found in adult animals. This discrepancy with the gene mutation assay can be explained in several ways. First, mutant frequencies were measured in the liver, an organ that is representative of systemic exposure, whereas the tumours found in esophagus and forestomach are rather a local effect of the B[*a*]P treatment. Next, there might be differences in biotranformation capacity between the various organs. The observed shift in the main target tissue from forestomach in older animals to esophagus in younger animals is in our view indicative for a difference in site of exposure rather than a difference in vulnerability to the effects of B[*a*]P. We consider this an important finding, supportive of a life stage-specific approach when assessing exposure for risk assessment of environmental chemicals, but beyond the scope of the present study. So, based on the B[*a*]P data, we conclude that an increased susceptibility to mutagenic effects of a given chemical does not automatically imply an increased susceptibility to carcinogenic effects. Given that carcinogenesis is a multi-step process, this is by no means a surprising finding. For risk assessment, this may imply however that increased cancer risks due to early-life exposure cannot be derived from *in vivo* genotoxicity studies. Obviously, in order to draw such a conclusion more data for a larger number of genotoxic substances are needed. Preferably, genotoxic effects of a given chemical should then be measured in tissues that are target for carcinogenesis. For the other genotoxic substances tested, we did not observe an increased susceptibility to mutagenic effects when exposure occurred early in life. These findings suggest that increased susceptibility to mutagenic effects is indeed dependent on the specific mechanism of action. Future research on a larger set of substances is needed to support this conclusion. Ideally, these additional studies should also focus on the question whether thresholds, commonly accepted for some mutagenic mechanism of action, differ between age groups.

6 References

1. European Commission. Technical guidance document on risk assessment in support of Commission Directive 93/67 EEC on risk assessment for new notified substances and Commission Regulation (EC) No. 1488/94 on risk assessment for existing substances. 2003
2. U.S. Environmental Protection Agency. Guidelines for Carcinogen Risk Assessment. Risk Assessment Forum, Washington, DC. 2005
3. European Commission. SCHER/SCCP/SCENIHR scientific opinion on the risk assessment methodologies and approaches for genotoxic and carcinogenic substances. 2009
4. European Food Safety Authority. Opinion of the Scientific Committee on a request from EFSA related to a harmonised approach for risk assessment of substances which are both genotoxic and carcinogenic. The EFSA Journal. 2005;282:1-31
5. European Chemicals Agency. Guidance on information requirements and chemical safety assessment. Chapter R.8: Characterisation of dose [concentration]-response for human health. 2012
6. Sanner T., Dybing E., Willems M.I., Kroese E.D. A simple method for quantitative risk assessment of non-threshold carcinogens based on the dose descriptor T25. Pharmacol Toxicol. 2001;88(6):331-41
7. Graeter L.J., Mortensen M.E. Kids are different: developmental variability in toxicology. Toxicology. 1996;111(1-3):15-20
8. Bruckner J.V. Differences in sensitivity of children and adults to chemical toxicity: the NAS panel report. Regul Toxicol Pharmacol. 2000;31(3):280-5
9. SchwenkM., Gundert-Remy U., Heinemeyer G., Olejniczak K., Stahlmann R., Kaufmann W., et al. Children as a sensitive subgroup and their role in regulatory toxicology: DGPT workshop report. Arch Toxicol. 2003;77(1):2-6
10. Makri A., Goveia M., Balbus J., Parkin R. Children's susceptibility to chemicals: a review by developmental stage. J Toxicol Environ Health B Crit Rev. 2004;7(6):417-35
11. De Zwart L.L., Haenen H.E., Versantvoort C.H., Wolterink G., Van Engelen J.G., Sips A.J. Role of biokinetics in risk assessment of drugs and chemicals in children. Regul Toxicol Pharmacol. 2004;39(3):282-309
12. U.S. Environmental Protection Agency. Child-Specific Exposure Factors Handbook. National Center for Environmental Assessment, Washington, DC. 2008
13. Brown R.C., Barone S., Jr., Kimmel C.A. Children's health risk assessment: incorporating a lifestage approach into the risk assessment process. Birth Defects Res B Dev Reprod Toxicol. 2008;83(6):511-21
14. Makris S.L., Thompson C.M., Euling S.Y., Selevan S.G., Sonawane B. A lifestage-specific approach to hazard and dose-response characterization for children's health risk assessment. Birth Defects Res B Dev Reprod Toxicol. 2008;83(6):530-46
15. U.S. Environmental Protection Agency. Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens. Risk Assessment Forum, Washington, DC. 2005
16. Cohen Hubal E.A., De Wet T., Du Toit L., Firestone M.P., Ruchirawat M., Van Engelen J., et al. Identifying important life stages for monitoring and assessing risks from exposures to environmental contaminants: Results of a World Health Organization review. Regul Toxicol Pharmacol. 2014;69(1):113-24
17. Organisation for Economic Co-operation and Development (OECD). Assessing the risk of chemicals to children's health: An OECD-wide survey. 2013
18. Drew R.T., Boorman G.A., Haseman J.K., McConnell E.E., Busey W.M., Moore J.A. The effect of age and exposure duration on cancer induction by a

known carcinogen in rats, mice, and hamsters. Toxicol Appl Pharmacol. 1983;68(1):120-30
19. Kari F.W., Foley J.F., Seilkop S.K., Maronpot R.R., Anderson M.W. Effect of varying exposure regimens on methylene chloride-induced lung and liver tumors in female B6C3F1 mice. Carcinogenesis. 1993;14(5):819-26
20. Bos P.M., Baars B.J., Van Raaij M.T. Risk assessment of peak exposure to genotoxic carcinogens: a pragmatic approach. Toxicol Lett. 2004;151(1):43-50
21. Doerge D.R., Young J.F., McDaniel L.P., Twaddle N.C., Churchwell M.I. Toxicokinetics of acrylamide and glycidamide in Fischer 344 rats. Toxicol Appl Pharmacol. 2005;208(3):199-209
22. Luijten M., Hernandez L.G., Zwart E.P., Bos P.M.J., Van Steeg H., Van Benthem J. The sensitivity of young animals to benzo[a]pyrene-induced genotoxic stress. RIVM Report No. 340701002. 2013
23. Ghanayem B.I., McDaniel L.P., Churchwell M.I., Twaddle N.C., Snyder R., Fennell T.R., et al. Role of CYP2E1 in the epoxidation of acrylamide to glycidamide and formation of DNA and hemoglobin adducts. Toxicol Sci. 2005;88(2):311-8
24. Yener Y., Dikmenli M. Increased micronucleus frequency in rat bone marrow after acrylamide treatment. Food Chem Toxicol. 2009;47(8):2120-3
25. Pinto A.L., Lippard S.J. Binding of the antitumor drug cis-diamminedichloroplatinum(II) (cisplatin) to DNA. Biochim Biophys Acta. 1985;780(3):167-80
26. Kartalou M., Essigmann J.M. Recognition of cisplatin adducts by cellular proteins. Mutat Res. 2001;478(1-2):1-21
27. Anderson R.D., Berger N.A. International Commission for Protection Against Environmental Mutagens and Carcinogens. Mutagenicity and carcinogenicity of topoisomerase-interactive agents. Mutat Res. 1994;309(1):109-42
28. Choudhury R.C., Palo A.K., Sahu P. Cytogenetic risk assessment of etoposide from mouse bone marrow. J Appl Toxicol. 2004;24(2):115-22
29. Attia S.M., Kliesch U., Schriever-Schwemmer G., Badary O.A., Hamada F.M., Adler I.D. Etoposide and merbarone are clastogenic and aneugenic in the mouse bone marrow micronucleus test complemented by fluorescence in situ hybridization with the mouse minor satellite DNA probe. Environ Mol Mutagen. 2003;41(2):99-103
30. Fiedler R.D., Weiner S.K., Schuler M. Evaluation of a modified CD71 MicroFlow method for the flow cytometric analysis of micronuclei in rat bone marrow erythrocytes. Mutat Res. 2010;703(2):122-9
31. Lynch A., Harvey J., Aylott M., Nicholas E., Burman M., Siddiqui A., et al. Investigations into the concept of a threshold for topoisomerase inhibitor-induced clastogenicity. Mutagenesis. 2003;18(4):345-53
32. Choudhury R.C., Jagdale M.B., Misra S. Cytogenetic toxicity of cisplatin in bone marrow cells of Swiss mice. J Chemother. 2000;12(2):173-82
33. Attia S.M. The impact of quercetin on cisplatin-induced clastogenesis and apoptosis in murine marrow cells. Mutagenesis. 2010;25(3):281-8
34. Oliveira R.J., Sassaki E.S., Monreal A.C., Monreal M.T., Pesarini J.R., Mauro M.O., et al. Pre-treatment with glutamine reduces genetic damage due to cancer treatment with cisplatin. Genet Mol Res. 2013;12(4):6040-51
35. Witt K.L., Livanos E., Kissling G.E., Torous D.K., Caspary W., Tice R.R., et al. Comparison of flow cytometry- and microscopy-based methods for measuring micronucleated reticulocyte frequencies in rodents treated with nongenotoxic and genotoxic chemicals. Mutat Res. 2008;649(1-2):101-13
36. Zeiger E., Recio L., Fennell T.R., Haseman J.K., Snyder R.W., Friedman M. Investigation of the low-dose response in the in vivo induction of micronuclei and adducts by acrylamide. Toxicol Sci. 2009;107(1):247-57
37. YenerY. Effects of long term low dose acrylamide exposure on rat bone marrow polychromatic erythrocytes. Biotech Histochem. 2013;88(6):356-60

38. Koyama N., Yasui M., Kimura A., Takami S., Suzuki T., Masumura K., et al. Acrylamide genotoxicity in young versus adult gpt delta male rats. Mutagenesis. 2011;26(4):545-9
39. Goukassian D., Gad F., Yaar M., Eller M.S., Nehal U.S., Gilchrest B.A. Mechanisms and implications of the age-associated decrease in DNA repair capacity. FASEB J. 2000;14(10):1325-34
40. Gorbunova V., Seluanov A., Mao Z., Hine C. Changes in DNA repair during aging. Nucleic Acids Res. 2007;35(22):7466-74
41. Klinger W. Developmental pharmacology and toxicology: biotransformation of drugs and other xenobiotics during postnatal development. Eur J Drug Metab Pharmacokinet. 2005;30(1-2):3-17
42. Melis J.P., Wijnhoven S.W., Beems R.B., Roodbergen M., Van den Berg J., Moon H., et al. Mouse models for xeroderma pigmentosum group A and group C show divergent cancer phenotypes. Cancer Res. 2008;68(5):1347-53
43. Melis J.P., Kuiper R.V., Zwart E., Robinson J., Pennings J.L., Van Oostrom C.T., et al. Slow accumulation of mutations in Xpc mice upon induction of oxidative stress. DNA Repair (Amst). 2013; 12: 1081-1086
44. Verhofstad N., Van Oostrom C.T., Zwart E., Maas L.M., Van Benthem J, Van Schooten F.J., et al. Evaluation of benzo(a)pyrene-induced gene mutations in male germ cells. Toxicol Sci. 2011;119(1):218-23

RIVM

Committed to health and sustainability