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

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Abstract

Young animals are more sensitive than adult animals to benzo[*a*]pyrene-induced genotoxic stress

Experimental animals are more sensitive at a young age to the adverse effects induced upon benzo[*a*]pyrene exposure compared to adult animals. In research performed at the RIVM, higher amounts of DNA damage were observed when benzo[*a*]pyrene was given to experimental animals at a young age. 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.

Benzo[*a*]pyrene is commonly found in grilled and broiled foods, tobacco smoke and automobile exhaust fumes. Exposure to benzo[*a*]pyrene may cause DNA damage, and, at very high doses, even induce tumors. Chronic exposure of animals at a very young age did not increase the incidence of tumors, but tumors were primarily found in a different organ compared to animals exposed at an adult age. Further research is ongoing to assess whether these findings are specific for benzo[*a*]pyrene, or representative for genotoxicants in general. Those studies will provide an insight as to what extent children should be regarded as a high-risk group in human health risk assessment.

Keywords: children, benzo[*a*]pyrene, risk assessment, genotoxicity, carcinogenicity

Rapport in het kort

Jonge dieren zijn gevoeliger dan volwassen dieren voor de schadelijke effecten van benzo[*a*]pyreen

Op heel jonge leeftijd zijn proefdieren gevoeliger voor de schadelijke effecten van de stof benzo[*a*]pyreen dan op volwassen leeftijd. Er ontstaat in de jonge levensfase meer schade aan het DNA. Dit blijkt uit onderzoek van het RIVM. 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.

Benzo[*a*]pyreen is een stof die voorkomt in voeding, zoals gebraden vlees, en in tabaksrook en uitlaatgassen. Het is een stof die veranderingen in het erfelijk materiaal kan veroorzaken en bij blootstellingen aan zeer hoge doses tot kanker kan leiden. Langdurige blootstelling op jonge leeftijd leidde niet tot een hoger aantal dieren met tumoren, maar wel tot tumoren in andere organen dan bij volwassen dieren. Momenteel wordt in aanvullende studies onderzocht of deze resultaten specifiek zijn voor benzo[*a*]pyreen of kenmerkend zijn voor DNA-beschadigende stoffen in het algemeen. Dat onderzoek zal uitwijzen of bij de risicobeoordeling van chemische stoffen meer rekening moeten worden gehouden met kinderen als risicogroep.

Trefwoorden: kinderen, benzo[*a*]pyreen, risicobeoordeling, genotoxiciteit, carcinogeniteit

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Summary

Risk assessment of chemical substances is performed to determine whether substances pose a significant risk to human health and/or the environment and, if so, under what circumstances and at which dose. Many substances are therefore extensively tested before they are marketed. Chemical safety assessments are carried out based on data obtained from *in silico*, *in vitro* and *in vivo* studies. This assessment includes adjustment for safety factors such as extrapolation from animals to humans and/or inter-individual variation in sensitivity. High-risk groups such as children, however, are not explicitly accounted for: it is assumed that sensitive populations are covered by these assessment factors. Age-specific assessment factors are not applied, at least not in Europe.
Children, however, do differ from adults in many ways. They have different, and sometimes unique, exposures to environmental hazards, and their physiology is different from those of adults. Consequently, they may be at a higher risk of exposure to a given agent, and/or be more susceptible to a given disease. In the present report we addressed the question whether children are more susceptible to B[*a*]P-induced genotoxic stress than adults by performing two animal studies. In both studies, three different age groups were used, as representatives of children, young adults and adults. Results from the carcinogenicity study show that the overall tumor incidence in very young animals did not increase and was comparable to the tumor incidence in young adult animals. We did observe, however, a shift in the main target tissue from forestomach in older animals to esophagus in younger animals. The mutagenicity study revealed elevated mutant frequencies in mice exposed at a very young age compared to animals exposed at an adult age. These findings indicate that children may indeed be more sensitive to exogenous genotoxic stress. Further studies with a broader panel of chemicals will be needed to demonstrate whether the present findings are applicable to genotoxic carcinogens in general, and whether age-related safety factors should be implemented in human health risk assessment. Thus, although relevant information is provided in the current study, we cannot be conclusive on whether the intra-species factor of 10 is sufficient to protect children as a sensitive population from carcinogen exposure yet.

1 Introduction

The evaluation of potential adverse effects of 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, the starting point for human cancer risk assessment is a dose associated with a carcinogenic endpoint, such as the T25 dose (the dose giving a 25% incidence of cancer in appropriately designed animal experiment), the no observed adverse effect level (NOAEL) or a benchmark dose (BMD). This assessment also includes adjustment for assessment factors such as extrapolation from animals to humans and/or inter-individual variation in sensitivity (1). An important aspect of cancer risk assessment involves the evaluation of available data to obtain information on the mode of action leading to carcinogenicity. This analysis is important for the assessment of human relevance, existence of thresholds and comparability with other structurally related carcinogens (2).

Carcinogenic substances are therefore classified as either genotoxic or non-genotoxic. Genotoxic carcinogens have the ability to directly react with DNA, thereby inducing DNA mutations. Currently, in risk assessment, this class of carcinogens is considered not to have a threshold: induced increases in DNA damage are considered to be linearly related to the administered dose. Therefore, non-threshold dose-response curves are used for risk assessment of genotoxic carcinogens. Non-genotoxic carcinogens, however, lack genotoxicity as a primary biological activity. There is general agreement that knowledge of the underlying non-genotoxic mechanism of such substances justifies a threshold approach. In risk assessment of non-genotoxic carcinogens a NOAEL (or BMD(L)) and safety factor of 100 (x10 for intra- and x10 for inter-species variation) is used (1, 3).

The regulatory question addressed in this report is whether the intra-species factor of 10 is sufficient to protect sensitive populations such as children from carcinogen exposure. In this regard, the U.S. Environmental Protection Agency (EPA) has developed age-dependent potency adjustment factors (ADAF) to be implemented if a carcinogen is found to have a mutagenic mode of action. The ADAF is an additional 10x assessment factor for children from birth to < 2 years, and an additional 3x assessment factor for children from 2 years of age to < 16 years (4). These additional factors are presumed to account for differences in toxicokinetics and toxicodynamics between children and adults (5, 6).

Children may have increased systemic exposure to respiratory and dermal xenobiotics because they have a higher respiratory activity per kilogram body weight and a higher body surface-body weight ratio, respectively. Children may also have higher exposure to oral xenobiotics because they consume more food and drinks per kilogram body weight than adults. In addition, oral absorption of xenobiotics in children may be affected by differences in gastric pH and emptying rate, and differences in gastric digestive enzyme concentration and composition of bacterial gut flora (5). The clearing of xenobiotics may be hampered in children because of the immaturity of metabolic enzymes in the liver, the absence of first-pass elimination in the liver, and low renal blood flow and glomerular filtration rate in the kidneys below 6 months of age (5). In terms of metabolism, nearly all enzyme activity is at adult levels within 6-12 months of age. The metabolism in neonates is usually impaired, in comparison to adults, and metabolic activity is generally higher in children between 6 months to 12 years of age due to a higher metabolic rate (7). Renal blood flow and glomerular filtration rate is comparable to adults within 6 months of age. Finally, in children, the relative amount of body water is higher and the fat content is

lower, in comparison to adults; thus, resulting in differences in volume distribution of compounds (7).

All the above-mentioned may result in differences in systemic concentration of xenobiotics in children, in comparison to adults. It remains unclear whether xenobiotics elicit irreversible damage to developing organs and systems by disrupting the proliferation, differentiation, migration or maturation of cells (5). Given that risk is dependent on inherent sensitivity and exposure conditions, we investigated if there is a potency difference across life-stages in animals.

2 Experimental studies

In the present study, we investigated a potential age difference in susceptibility to chemical-induced mutagenicity and carcinogenicity by examining whether young animals are more susceptible than adult animals. Two different animal experiments were performed; in both studies three different age groups were used, as representatives of children, young adults and adults.

2.1 Benzo[*a*]pyrene

In both experimental studies, we used the model compound benzo[*a*]pyrene (B[*a*]P). B[*a*]P is a polycyclic aromatic hydrocarbon (PAH) classified as a human carcinogen by the International Agency for Research on Cancer (8). B[*a*]P has a widespread occurrence and is present in drinking water, food, ambient air, and tobacco smoke (9-11). The primary dietary source of B[*a*]P is charred meat. However, B[*a*]P is also found in grains, oils, fruits, and vegetables. Ingestion is estimated to be the major route of B[*a*]P exposure in humans (cited in (12)). Children may have greater exposure than adults to B[*a*]P, especially via ambient air contamination, and to a lesser extent via food (http://www.epa.gov/teach/chem_summ/BaP_summary.pdf; (13).

2.2 Carcinogenicity

Given that carcinogenicity studies in rodents usually take around two years, we used transgenic *Xpc*$^{-/-}$ mice that have a partial deficiency in one of the main DNA repair pathways, *i.e.* nucleotide excision repair, to shorten the latency period of tumors and to reduce the number of animals needed. Tumor manifestation time is reduced because the XPC protein is responsible for DNA damage recognition (14, 15), an event that is necessary to repair DNA lesions like those induced by B[*a*]P (16).
In the 39-week carcinogenicity study *Xpc*$^{-/-}$ mice (males and females) were exposed to B[*a*]P between weeks 3 and 16, 10 and 23, and 26 and 39 after birth. Mice exposed between 10 and 23 weeks of age were considered as reference, since mice used in classic genotoxicity and carcinogenicity tests usually are 8-15 weeks of age at start of the experiment. A schematic overview of the study design is depicted in Figure 1. All mice were exposed to a dose of 150 ppm of B[*a*]P in the feed. In addition, untreated mice were used as controls. Mice in the control groups were of similar age as the oldest mice in the treatment groups. At 39 weeks after the start of treatment mice were sacrificed.

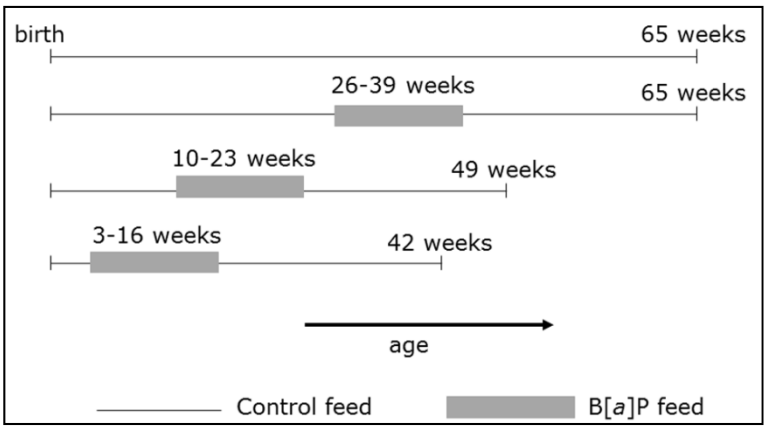

Figure 1 Experimental design of the carcinogenicity study

Comprehensive pathological analyses were performed on the known target tissue, *i.e.* forestomach, as well as esophagus, tongue, liver, lung and spleen to assess possible tumor development due to the exposure. Results of these analyses are shown in Table 1. B[*a*]P exposure clearly induced (pre-) neoplastic lesions in the forestomach and esophagus. The forestomach tumor incidence upon B[*a*]P exposure was significantly increased in comparison to the control group in all treatment groups, with mice treated with B[*a*]P between weeks 10 and 23 after birth having the highest incidence of squamous cell carcinomas (Table 1). Besides tumors also a significant increase in hyperplasia incidence was found in the forestomach of treated mice, regardless of the age at treatment. Furthermore, we found that B[*a*]P exposure induced a marked increase in the incidence of inflammation, a lesion that has long been associated with the development of cancer. For the esophagus, tumor incidences were increased in all age groups compared to the control group. The increase in proportion of mice with malignant tumors, however, was only statistically significant for the group exposed to B[*a*]P early in life. The proportion of mice with benign tumors was only significantly increased in mice exposed between 10 and 23 weeks of age. In the esophagus, B[*a*]P exposure did also induce hyperplasia, but to a lesser extent than in the forestomach. Like the forestomach, there was no association with age of treatment. No statistically significant differences were found in tumor incidences for any of the other organs between control and B[*a*]P-treated mice.

Table 1 Incidence of tumors and hyperplasia upon B[a]P exposure in $Xpc^{-/-}$ mice

Group	**Control**	**3-16 weeks B[*a*]P**	**10-23 weeks B[*a*]P**	**26-39 weeks B[*a*]P**
# Tumor bearing mice / # mice examined	3/31 (10%)	21/40 (53%)***	22/33 (67%)***	16/36 (44%)**
Forestomach	**2 (6%)**	**12 (30%)***	**16 (48%)*****	**11 (31%)***
Inflammation	4 (13%)	26 (65%)***	19 (58%)***	14 (39%)*
Epithelial hyperplasia				
Slight to mild	22 (71%)	8 (20%)***	7 (22%)***	12 (33%)**
Moderate to severe	9 (29%)	31 (78%)***	27 (78%)***	24 (67%)**
Squamous cell papilloma	2 (6%)	9 (23%)	5 (15%)	4 (11%)
Squamous cell carcinoma	0	5 (13%)[a]	12 (36%)***	7 (19%)**
Esophagus	**0**	**14 (35%)*****	**10 (30%)*****	**7 (19%)*****
Epithelial hyperplasia				
Mild	2 (6%)	23 (58%)***	12 (36%)*	13 (36%)*
Moderate to severe	1 (3%)	3 (8%)	5 (15%)	8 (22%)*
Squamous cell papilloma	0	5 (13%)	8 (24%)**	3 (8%)
Squamous cell carcinoma	0	10 (25%)**	3 (9%)	4 (11%)
Other				
Lymphoma	0	0	1 (3%)	2 (6%)
Carcinoma (liver)	1 (3%)	0	0	0

*, **, *** Statistically significantly different from the control group with $P<0.05$, $P<0.01$, or $P<0.001$, respectively (Fisher's exact test)

[a] Statistically significantly different from the 10-23 weeks B[*a*]P group with $P<0.05$ (Fisher's exact test)

2.3 Genotoxicity

To investigate whether the observed differences in tumor incidence between the three age groups are also reflected in a different mutant frequency in the various organs, a short-term genotoxicity study was performed. Wild type (WT) and DNA repair-deficient *Xpc*$^{-/-}$ mice, all carrying the *lacZ* reporter gene, were treated with 0, 75, 150, 300 or 450 ppm of B[*a*]P in the feed for 1 week. All mice were sacrificed six weeks after end of treatment. Mice were 3, 10 or 26 weeks old at the start of treatment. As in the carcinogenicity study, 10-week-old mice were considered as the reference group.

Since esophagus and forestomach were the major target organs in the carcinogenicity study, these were the organs that were subjected to mutant frequency analyses. However, the volume of these tissues, especially those from 3-week-old animals, appeared to be too small for this type of analyses. We therefore analyzed the liver, the major organ for detoxification. The results show that short-term exposure to B[*a*]P induced overall a dose-dependent increase in mutant frequency within an age group (Figure 2). More importantly, 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. Remarkably, mutant frequencies in *Xpc*$^{-/-}$ mice were somewhat lower than higher compared to WT mice.

Since one of the characteristic differences between age groups is the difference in amount of food consumed, we calculated the amount of feed consumed in the week of treatment for each dose group. The relative feed consumption compared to 10-week-old mice is given in Table 2. As expected, mice of very young age (3 weeks) tended to eat more per kilogram body weight, whereas at an older age (26 weeks) mice ate substantially less in comparison to the young adult mice (10 weeks). Mutant frequencies in mice exposed at a very young age, however, are still elevated, especially in the highest dose groups, even when taking increased feed intake into account.

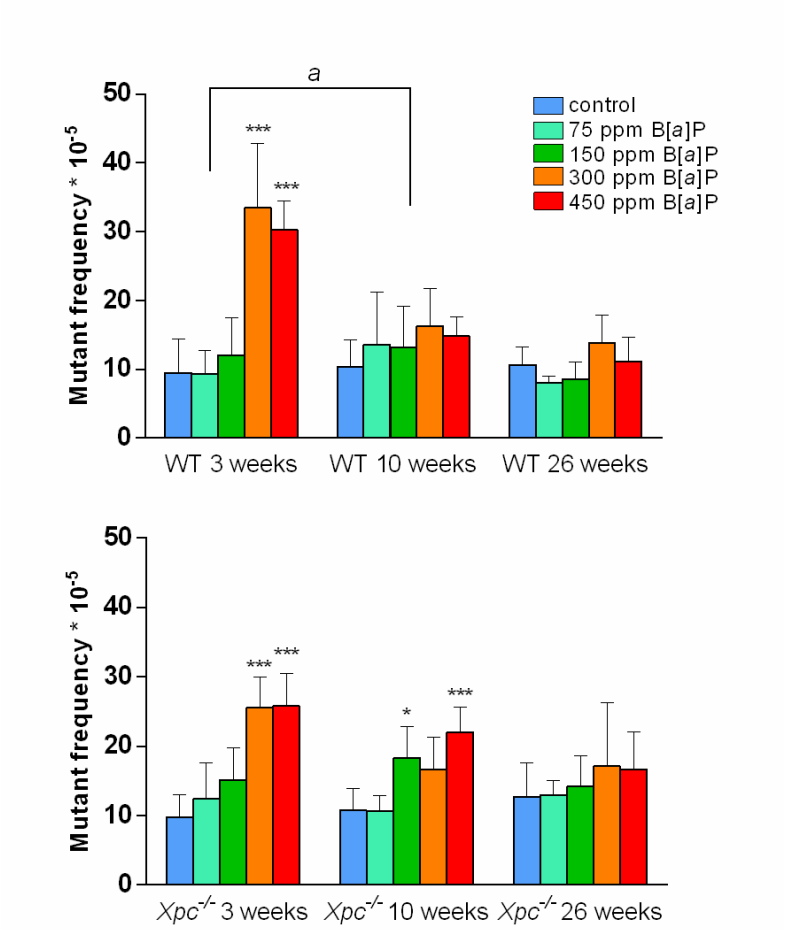

Figure 2 LacZ mutant frequency in livers from mice treated with B[a]P

*** Statistically significantly different from the corresponding control group with P<0.001 (two-way ANOVA); [a] Statistically significantly different from the 10-23 weeks B[a]P group with P<0.001 (two-way ANOVA)

Table 2 Relative feed consumption, compared to 10-week-old mice

		WT	*Xpc*$^{-/-}$
3 weeks	Control	112.6[a]	131.7
	75 ppm B[a]P	122.5	118.1
	150 ppm B[a]P	131.2	139.1
	300 ppm B[a]P	101.2	136.4
	450 ppm B[a]P	117.9	126.4
26 weeks	Control	76.7	78.5
	75 ppm B[a]P	71.3	70.0
	150 ppm B[a]P	81.1	78.7
	300 ppm B[a]P	63.2	67.8
	450 ppm B[a]P	70.4	77.7

[a] Percentage of feed uptake in week of treatment per gram body weight

3 Discussion

In the present study, we investigated whether young animals are more susceptible to B[*a*]P-induced genotoxic stress than adult animals and possible implications for human risk assessment. To this end, a short-term carcinogenicity as well as a genotoxicity study was performed with B[*a*]P exposure during various life stages, mimicking children, young adults and adults in humans. Here, the group of young adult mice served as reference, since this age group is typically used in classic genotoxicity and carcinogenicity studies.

In the carcinogenicity study, we found that mice treated with B[*a*]P between 10-23 weeks of age had a high incidence of forestomach tumors, and some tumors of the esophagus. This is consistent with findings of our previous study, conducted with WT, *Xpa*$^{-/-}$, and *Xpa*$^{-/-}$.*p53*$^{+/-}$ mice of the same age. Oral B[*a*]P exposure at 75 ppm in a similar study design induced mainly benign tumors in the forestomach, and some in the esophagus (17). In the present study, we found a higher incidence of tumors in the esophagus, and a higher proportion of malignant tumors in the forestomach. This is most likely due to the two-fold higher dose of B[*a*]P we employed. Although humans lack a forestomach, these findings are considered relevant for the assessment of the carcinogenic potential of a given substance. In a recent study, molecular evidence was provided supporting the use of the mouse forestomach model to evaluate B[*a*]P-induced gastrointestinal carcinogenesis in humans (18).

Comparison of the youngest age group with the reference group revealed a higher incidence of malignant tumors in the esophagus, but a lower incidence of malignant forestomach tumors. An interesting finding was that the main tumor 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 tumor target tissue may be due to differences in esophageal and gastric motility. Also differences in enzyme activities required for activation of B[*a*]P may play a role. The overall tumor incidence, however, was comparable between the two age groups.
It is noteworthy to mention that mice of young age tend to eat more, which may in the present study have resulted in a somewhat higher exposure as compared to the reference mice. Taking this into account, the observed tumor incidences in the youngest age group may be an overestimation, and are likely to be actually lower. The incidence of malignant tumors in the esophagus in young mice however would most likely still be increased. In mice exposed to B[*a*]P at a relatively old age, a lower incidence of forestomach and esophagus tumors was found, as compared to the reference group. This may be explained by reduced metabolic capacity and/or reduced feed intake (and thus reduced exposure).

Results of the mutagenicity study showed a clear dose-dependent increase in mutant frequency within the youngest age groups (both genotypes) and the reference group of *Xpc*$^{-/-}$ mice. The observed frequencies are in concordance with previous findings (17). WT mice of young age seem to be more susceptible to genotoxic stress in comparison to adult mice, at least in the liver. This was different in *Xpc*$^{-/-}$ mice: mice of young age as well as adult mice exhibited an increase in mutant frequency. *Xpc*$^{-/-}$ mice, deficient in DNA repair, are known to be far more cancer prone than WT mice (19, 20). 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 (21). This suggests that the increased sensitivity of *Xpc*$^{-/-}$ mice towards induction of mutations and tumors only becomes phenotypically apparent in long-term studies.

The implications of the current findings for risk assessment are (very) limited. Although tumors in animals exposed at a young age did primarily arise in a different organ, the overall tumor incidence was comparable to the one found in adult animals. As such, the risk of adverse health effects from B[*a*]P exposure appears not to be greater at a young age. Nevertheless, the shift in target tissue together with the increase in mutant frequencies does indicate that young animals, as representatives of children, may indeed be more sensitive to exogenous genotoxic stress. More studies are needed to conclude whether the present findings are specific for B[*a*]P, or a common feature of genotoxicants in general. To this end, we are currently performing short-term genotoxicity tests with genotoxicants to which humans, especially children, are frequently exposed. Those studies will improve our knowledge as to what extent children should be considered as a high-risk group, and whether age-related assessment factors should be implemented in human health risk assessment. Thus, although relevant information is provided in the current study, we cannot be conclusive on whether the intra-species factor of 10 is sufficient to protect children as a sensitive population from carcinogen exposure yet.

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