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Fluoranthene and phenantrene, two predominant PAHs in heat-prepared food, do not influence the frequency of micronucleated mouse erythrocytes induced by other PAHs

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) occur in complex mixtures present in the human environment. Because of the carcinogenic properties of some of these PAHs, they raise concerns regarding health and food safety. Because the occurrence of benzo[a]pyrene, chrysene, benz[a]anthracene, and benzo[b]fluoranthene (PAH4) are considered markers for other genotoxic PAHs in foodstuffs, the European Union has put a maximum level of PAH4 in some foodstuffs. Fluoranthene (Flu) and phenanthrene (Phe), two other PAHs, are not classified as genotoxic and are abundant at rather high concentrations in food. Inasmuch as PAH4, Flu, and Phe are metabolized by the same cytochrome P450 pathway system, it is important to clarify whether Phe and Flu influence the genotoxicity of PAH4. We have analyzed the genotoxic response of Phe and Flu, separately and together, as well as in combination with different low doses of PAH4. In all experiments we used the flow cytometer-based micronucleus test *in vivo*. Phe and Flu, when administered separately, did not show any dose-related effect on the frequency of micronucleated polychromatic erythrocytes (fMNPCE). Nor did a mixture of Phe and Flu change the fMNPCEs. Phe and Flu did not significantly change the fMNPCE of PAH4-exposed FVB and BALB/c mice.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) belong to the class of organic pollutants containing two or more fused aromatic rings made up of carbon and hydrogen atoms. They are chemically stable and poorly degradable [14]. The main exposure to PAHs occurs through food and inhaled air. Concerning food, studies have shown that food preparation plays a major role for PAH concentration, which leads to wide variation in the concentration data for a given PAH in food [8,18,31]. For example, in the barbecuing of food it is the high temperature and especially the exposure to open flames that determine the number, concentration, and proportion of different PAHs [4,8,10,15]. Perelló et al. [21] demonstrated that the cooking procedure (*e.g.*, frying, grilling, and the use of oil) has considerable impact on PAH levels, including phenanthrene (Phe) and fluoranthene (Flu), two predominant PAHs in food prepared by heating. The proportion of different PAHs, carcinogenic and non-carcinogenic, varies significantly in food. Numerous studies have shown that the two volatile PAHs, Flu and Phe, occur at levels several times higher than other PAHs [21,25]. In a study on different PAH levels in the edible part of smoked fish the Phe and Flu levels were about a hundred times higher than benzo(a) pyrene (BaP) [15]. In that study the mean levels of Phe and Flu in fish were about 150 μg/kg and 30 μg/kg, respectively.

A persuasive body of evidence suggests that some PAHs are carcinogenic and mutagenic in animals and most probably in humans [32]. BaP, which is the most studied and best known PAH, is widely used as a marker for total carcinogenic PAHs. However, in an evaluation by the EFSA [8] it was concluded that the sum of the concentrations of the four PAHs [(PAH4), (BaP), benz[a]anthracene (BaA), benzo[b]fluoranthene (BbF), and chrysene (Chr)] better reflects the levels of other genotoxic PAHs in food than BaP does alone. This reasoning led to a maximum level being established, not only for BaP but also for PAH4 in food [9]. According to the International Agency for Research on Cancer (WHO/IARC), three of the four components of PAH4 (BaA, BbF, and Chr) are all classified as 2 B, implying that they are possible human carcinogens. BaP is classified as a human carcinogen, class 1 [32]. For Phe

Abbreviations: BaA, benz[a]anthracene; BbF, benzo[b]fluoranthene; Chr, chrysene; BaP, benzo[a]pyrene; Flu, fluoranthene; fMNPCE, frequency of micronucleated polychromatic erythrocytes; i.p., intraperitoneal; p.o., per oral; Phe, phenanthrene; w, weeks.

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and Flu, the tumorigenic effects are not as clear as for PAH4, which resulted in the evaluation "not classifiable as to its carcinogenicity to humans", Group 3 [32].

Because we are always exposed to many compounds (either at the same time or sequentially), an interaction of toxicological significance may occur, which may be caused by different metabolic factors. Therefore, it can be assumed that the total effect of several DNA reactive compounds deviates from the sum of the single components. If data were available on the whole mixture of concern, the quantified carcinogenic effect would be calculated for the mixture. However, complete mixture data are often only available for some chemicals but not for all mixture components. With respect to PAHs, the tumorigenicity of both coal tar mixture and BaP in rodents has been studied [6]. In these studies it was demonstrated that mixtures resulted in different types of tumor compared with BaP alone. To what extent the role of, for instance, metabolism competition plays between different PAHs and different target organs for the PAHs is impossible to say. There are few epidemiological studies in which the association between dietary intake of PAHs and the risk for cancer has been evaluated. Consequently, any definitive conclusions cannot be made on such limited evidence [32]. Because of the difficulty in quantifying exposure in epidemiological studies, we must rely on animal studies with well-defined given doses. With reference to *in vitro* or *in vivo* studies, it is never possible to fully mimic the exposure to humans. Thus, instead of studying the effects of exposure to compounds sequentially, it seems more important to study the effects of different mixtures.

This study aimed to investigate the impact of Phe and Flu on the genotoxicity of PAH4. Different low doses of a mixture containing Phe and Flu together with PAH4 were analyzed for the frequency of micronucleated polychromatic erythrocytes (fMNPCEs) in two strains of mice (FVB and BALB/c mice). To evaluate a possible effect of the time needed for metabolism or uptake the injection of Phe and Flu was made not only at the same time as the injection of PAH4 but also 24 h before.

2. Material and methods

2.1. Animals

Experiments were performed in 6–8-week-old male FVB and BALB/c mice (20–30 g weight). The mice, bought from NOVA SCB-AB, Sollentuna, Sweden, were housed in a 12-h day and night cycle and permitted *ad libitum* consumption of food and water. For the experiment, blood samples of approximately 50 μL were taken from the orbital plexus in heparinized tubes under anesthesia with Fluothane (Zeneca, Göteborg, Sweden). The study was approved by the Uppsala Ethical Committee on Animal Experiments (application C322/12).

2.2. Chemicals

For exposure to mice, Flu (CAS 206-44-0) and Chr (CAS 218-01-9) were purchased from Fluka AG, Phe (CAS 85-01-8) from Schuchardt, München, benzo[a]anthracene (CAS 56-55-3) from Eastman Kodak Co, Rochester, N.Y., and BbF (CAS 205-99-2) and BaP (CAS 50-32-8) from Sigma–Aldrich. In the experiments the chemicals were processed into corn oil.

For the preparation of samples before analysis at the flow cytometer, Percoll from Pharmacia Biosystems, Uppsala, Sweden, PBS from Statens Veterinärmedicinska Anstalt, Sweden, and glutaraldehyde from TAAB Laboratories were used. The fluorescent dye Hoechst 33342 (HO342, a DNA dye) was purchased from Sigma–Aldrich, Sweden and the dye Thiazole Orange (TO, a RNA dye) from Molecular Probes, OR, USA.

2.3. Design of the study

In this study different low doses of six PAHs on the frequency of micronucleated polychromatic erythrocytes, fMNPCE in peripheral mouse blood using the flow cytometer-based micronucleus assay *in vivo* were analyzed. The PAHs analyzed were; phenanthrene (Phe), fluoranthene (Flu), benzo[a]pyrene (BaP), benz[a]anthracence (BaA), benzo[b]fluoranthene (BbF), and chrysene (Chr). The four PAHs (PAH4; BaP, BaA, BbF, and Chr) were always injected as a mixture. Phe and Flu were injected and analyzed sequentially together in a mixture and also in a mixture with PAH4. The main goal of the study was to clarify whether the two common PAHs (Phe and Flu) influence the genotoxicity of PAH4. The study was divided into five experiments: Experiments I–V (see Table 1).

On all occasions, the four compounds in PAH4 (BaP, BaA, BbF, and Chr) were in a mixture with corn oil and given in doses of 10 mg/kg b.w. of each of BaP, BaA, and BbF and 2 mg/kg b.w. of Chr. The lower dose of Chr was applied because of the low solubility in corn oil. The doses of Phe and Flu varied (see the text below and Table 1). On all occasions when Phe and Flu were used, the doses are given as a number in brackets after Phe and Flu: for example, "Phe (20) +Flu (20)", indicating that the doses were 20 mg/kg b.w.

2.3.1. Experiment I

In Exp. I, we wanted to determine whether Phe and Flu, separately, are genotoxic (Table 1). Male FVB mice were given three doses of Phe and Flu i.p. in sequential order. One group constituted the control group (corn oil, five mice); three groups of mice were exposed to different doses of Phe (30, four mice, 60, four mice, and 120, five mice) mg/kg b.w.; and three groups were exposed to Flu (30, four mice, 60, four mice, and 120, five mice) mg/kg/ b.w. Three acrylamide-exposed mice constituted the positive control group. In this experiment 34 mice were divided into eight groups.

2.3.2. Experiment II

The aim of this experiment was to clarify whether mice exposed to (a) a mixture of Phe and Flu (Phe (20) +Flu (20)) had any impact on the fMNPCE and (b) if three low-dose mixtures of Phe and Flu given at the same time as PAH4 would change the genotoxicity of PAH4 (Table 1). The experiment included six groups of FVB mice: control group (corn oil, five mice), Phe (20) +Flu (20) (four mice), PAH4 (five mice), Phe (5) +Flu (5) in a mixture with PAH4 (four mice), Phe (10) +Flu(10) in a mixture with PAH4 (four mice), and Phe (20) +Flu (20) (five mice) in a mixture with PAH4. In total, 27 mice were used.

2.3.3. Experiments III–V

The aim of the experiments was partly to verify the results from Exp. II, *i.e.*, whether mixtures of Phe and Flu influenced the genotoxicity of PAH4. In Exp. III, we exposed the four groups of 6-week-old FVB mice to either corn oil (four mice), Phe (10)+Flu(10) (four mice), PAH4 (four mice), and Phe and Flu simultaneously with PAH4 (four mice). Sixteen mice divided into four groups were used. In Exp. IV and V, Phe and Flu were injected 24 h before PAH4. In exp. IV, FVB 8-week-old male mice were exposed to Phe (10) + Flu (10) (five mice) or Phe (30) + Flu (30) (six mice) per oral (p.o.) before PAH4. Five mice constituted the PAH4 exposed group and four the control group. Exp. V was designed in almost the same way as Exp. IV. However, in exp. V, two mice made up the control group and two the positive control group. There were six mice in each of the three PAH-exposed groups. To see whether the choice of mouse strain has any impact on the results, we used 7-week-old male BALB/c mice in Exp. V.

Table 1
The design of the experiments. In the boxes the groups of FVB or BalbC mice in the different experiments are shown: for example, in Exp. I, eight groups (X) were studied. The doses of Phe and Flu are presented in brackets. The doses of PAH4, a mixture of BaP, BaA, BbF, and Chr, were always the same in the different experiments, *i.e.*, 10 + 10 + 10 + 2 mg/kg b.w. of BaP, BaA, BbF, and Chr, respectively. On all occasions, the chemicals were administrated i.p., except when Phe and Flu were administrated 24 h before PAH4, at which time it was given p.o.

Treatment →	corn oil	Flu	Phe	Phe + Flu	PAH4	Phe + Flu injected at the same time as PAH4	Phe + Flu injected 1 day prior to PAH4	pos. cont
Experiment I (FVB, 8 w)	*X*	*X*(30 mg/kg,bw)	*X*(30 mg/kg,bw)					
		X(60 mg/kg,bw)	*X*(60 mg/kg,bw)					*X*
		X(120 mg/kg,bw)	*X*(120 mg/kg,bw)					
Experiment II (FVB, 8 w)	*X*					*X*(5 + 5 mg/kg,bw)		
				X(20 + 20 mg/kg,bw)	*X*	*X*(10 + 10 mg/kg,bw)		
						X(20 + 20 mg/kg,bw)		
Experiment III (FVB, 6 w)	*X*			*X*(10 + 10 mg/kg,bw)	*X*	*X*(10 + 10 mg/kg,bw)		
Experiment IV (FVB, 8 w)	*X*				*X*		*X*(10 + 10 mg/kg,bw)	
							X(30 + 30 mg/kg,bw)	
Experiment V (BalbC, 7 w)	*X*				*X*		*X*(10 + 10 mg/kg,bw)	*X*
							X(30 + 30 mg/kg,bw)	

2.4. Micronucleus assay

Under light anesthesia, blood samples were collected from the orbital plexus of each animal 46 h after the last injection. The choice of sampling time is based on the knowledge that the time between the appearance of polychromatic erythrocytes (PCEs) in bone marrow and peripheral blood is about 20 h [2,5,17]. From each animal, blood was drawn and three parallel aliquots of 5 μl blood were layered for purification on a 65% Percoll gradient and centrifuged as described by [12].

The procedures used for fixation, fluorochrome staining, and flow cytometry have been described elsewhere [11,12,1]. Briefly, after centrifugation, the purified cells in the pellet were fixed in a solution of glutaraldehyde and stored one day at 4 °C. On the day of analysis, the fixative was discarded and the remaining cell pellet was stained in 1 ml buffer for 45 min at 37 °C. The staining buffer was made by mixing 500 μl HO342 and 80 μl TO with 100 ml PBS.

2.5. Enumeration of micronucleated erythrocytes

The stained samples were analyzed on a dual laser FACStar Plus flow cytometer (Beckton Dickinson, Sunnyvale, CA, USA) equipped with an argon ion laser operating at both multiline UV and 488 nm. The setting and equipment of the flow cytometer have been described elsewhere [13].

2.6. Calculation of PCE and MNPCE frequency

Regions of interest were defined to evaluate normochromatic erythrocytes (NCEs), PCEs, and micronucleated PCEs (MNPCEs). The relative frequency of PCE, PCE/(PCE + NCE), and MNPCE, MNPCE/(MNPCE + PCE) were calculated. A mean of about 100,000 PCE was analyzed from each mouse. The calculation procedure has been previously described [2]. Outliers were not removed from the analysis.

2.7. Statistical methods

Student's *t* tests (two-tailed) were applied for all statistical analyses of the results. In this study, the mean value of fMNPCE (‰) of the control group was compared with the mean value for mice exposed to PAH4 in each of Exp. II–V. To determine whether mice exposed to the mixture of PAH4 and Phe + Flu were significantly different from the PAH4-exposed mice the same type of *t*-test was used. In Exp. I, the mean value of fMNPCE (‰) for the control group was compared with the mean values given for each of the Phe- and Flu-exposed animals.

3. Results

Before the analysis on the influence of Phe and Flu on PAH4, we tested the dose response relationship of Phe and Flu sequentially (Experiment I). Although one of the mean fMNPCEs was significantly different from the control group, there was no significant dose-related change in fMNPCE with increasing dose (Fig. 1 and Table 2). Further, the proportion of PCE among all erythrocytes (%PCE) did not show any cytotoxicity (data not shown).

In Exp. II we hypothesized that Phe and Flu will change the genotoxicity of other PAHs (here PAH4). To test this hypothesis FVB male mice were exposed i.p. to three low doses of Phe and Flu at the same time as PAH4. PAH4 alone induced a significant increase in the fMNPCE compared with corn oil ($p = 0.013$) (Fig. 2, 3 and Table 2). The fMNPCE for Phe (20) +Flu (20) when not in a mixture with PAH4 was not significantly different when compared with the solvent, *i.e.*, corn oil. The fMNPCE for the three mixtures of Phe, Flu, and PAH4 when all six PAHs were mixed with each other (Phe (5)+ Flu (5) + PAH4, Phe (10) + Flu (10) + PAH4, and Phe (20)+ Flu (20) + PAH4) was not significantly different from the fMNPCE for mice treated exclusively with PAH4 (Fig. 2 and Table 2).

As a next step (Exp. III), we repeated some of the analyses conducted in Exp. II. Instead of using 8-week-old FVB mice as in Exp. II, we used FVB mice aged 6 weeks. The age of the mice did not materially change the results in Exp. II, *i.e.*, Phe and Flu in a mixture with PAH4, Phe (10) + Flu (10) + PAH4 did not significantly change the mean fMNPCE in mice exclusively exposed to PAH4 ($p > 0.05$). In comparison with fMNPCE for the mice given corn oil, fMNPCE for the PAH4-exposed mice and those given Phe and Flu together with PAH4 showed a genotoxic effect ($p \leq 0.05$, Fig. 3 and Table 2). However, the difference between the control group and the PAH4-exposed mice reached borderline significance ($p = 0.054$, two-tailed *t*-test).

The results in Exp. IV and V did not show any genotoxic effect of Phe and Flu in a mixture exposed p.o. 24 h before PAH4 as compared with mice injected with only PAH4 (all ps > 0.05, Fig. 3 and Table 2).

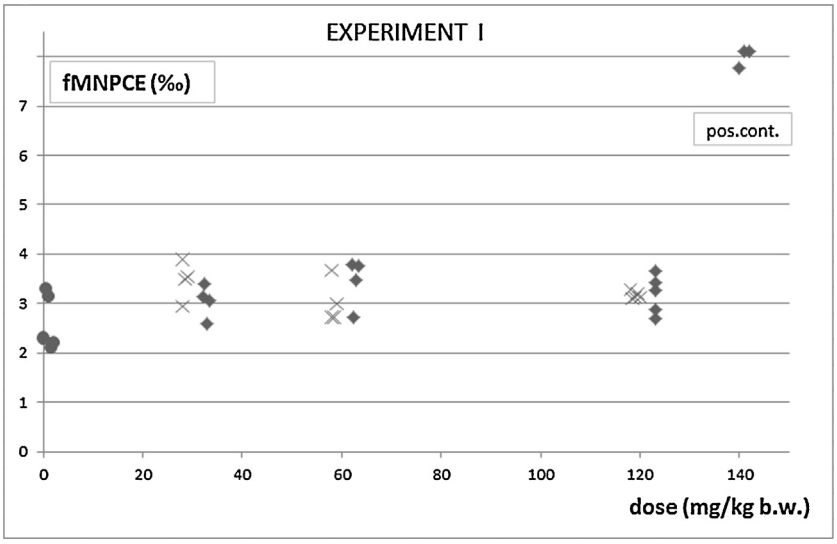

Fig. 1. EXPERIMENT I: The dose response relationship of fluoranthene, Flu, and phenantrene, Phe, treated FVB male mice.
The fMNPCE (‰) in peripheral blood at 46 h after i.p. injections of different doses of Flu or Phe. In total, 34 FVB mice were used. One group constituted the control group (five mice); three groups were exposed to different doses of Phe: 30 (four mice), 60 (four mice), and 120 (five mice) mg/kg b.w.; and three groups were exposed to Flu: 30 (four mice), 60 (four mice), and 120 (five mice) mg/kg/ b.w. Except for the lowest dose group of Phe (30 mg/kg, b.w., $p = 0.04$), none of the PAH-treated groups was significantly different from the control group, *i.e.* all ps > 0.05. Three acrylamide-exposed mice, 120 mg/kg/b.w., constituted the positive control group (here $p < 0.001$). Cytotoxicity, expressed as the proportion of PCE among all erythrocytes, did not change with higher doses (data not shown). The mean fMNPCEs (‰) for the different groups are listed in Table 2.
♦ = Flu, × = Phe.

In Exp. IV and V, both FVB and BALB/c mice were used. The fMNPCEs in the control mice were significantly lower than those in the PAH4-exposed mice.

In all five experiments no significant differences were noted between the frequencies of PCE for the different groups of mice (data not shown).

4. Discussion

Most of the studies on the genotoxicity of PAHs have focused on different compounds individually rather than analyzing the genotoxic effect of different PAHs in a mixture. The aim of this study was therefore to elucidate whether the common PAHs in food (*i.e.*, Phe and Flu in a mixture) interfere with the genotoxic effect of PAH4, which is important because Phe and Flu are not fixed with a maximum level in food and often occur at high levels.

The PAHs included in PAH4, BaP, BaA, BbF, and Chr are all chemically inert and need to be metabolized *via* the P450 system before they bind to DNA and thereby increase the risk of DNA breaks. The binding of BaP to CYP 450 enzymes was first described by Miller [19]. Today, there are a large number of articles describing the interaction between different PAHs and the P450 system. Several enzymes (*e.g.*, CYP1A1, CYP1A2, and CYP1B1) have been shown to play an important role in the metabolic activation of possible carcinogenic PAHs. Depending on what is being studied (*e.g.*, level of mRNA, protein, dose), the results do not always point in the same direction, making interpretation difficult [22–24,26,30,33]. In several studies Phe and Flu have been found to integrate with the same

Table 2
The mean fMNPCE in peripheral blood and the estimated standard deviation of the groups of mice included in the five experiments. Figures brackets show the administered dose (mg/ kg, b.w.) of the actual compound: for example, 4.38 ± 0.4 (5 + 5) means that the fMNPCEs was 4.38‰ ± 0.4 and the dose of Phe and Flu was 5 mg/kg, b.w. The administered dose of PAH4 was always 10 + 10 + 10 + 2 mg/kg b.w. for BaP, BaA, BbF, and Chr, respectively. Outliers were never excluded.

Treatment →	corn oil	Flu	Phe	Phe + Flu	PAH4	Phe + Flu injected at the same time as PAH4	Phe + Flu injected 1 day prior to PAH4	pos. cont
Experiment I mean fMNPCE (‰)	2.6 ± 0.6	3.05 ± 0.4 (30) 3.43 ± 0.6 (60) 3.17 ± 0.4 (120)	3.47 ± 0.4 (30) 3.02 ± 0.5 (60) 3.17 ± 0.4 (120)					8.12 ± 0.1
Experiment II mean fMNPCE (‰)	3.67 ± 0.3			3.68 ± 0.2 (20 + 20)	4.34 ± 0.3	4.38 ± 0.4 (5+5) 4.29 ± 0.1 (10 + 10) 4.92 ± 0.6 (20 + 20)		
Experiment III mean fMNPCE (‰)	2.7 ± 0.3			3.42 ± 0.1 (10 + 10)	3.37 ± 0.5	3.53 ± 0.4 (10 + 10)		
Experiment IV mean fMNPCE (‰)	3.3 ± 0.2				3.85 ± 0.4		3.80 ± 0.5 (10 + 10) 3.60 ± 0.3 (30 + 30)	
Experiment V mean fMNPCE (‰))	2.7 ± 0.2				4.38 ± 0.9		3.55 ± 0.9 (10 + 10) 3.9 ± 0.4 (30+30)	7.8 ± 0.3

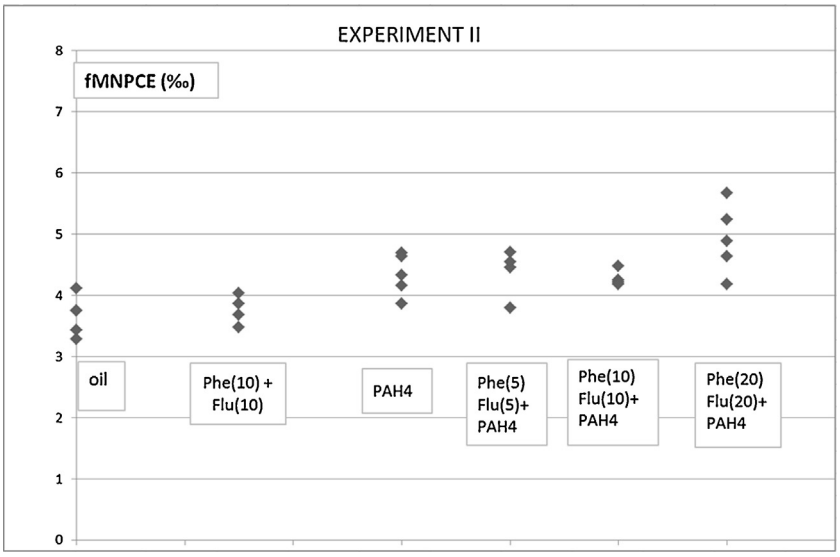

Fig. 2. EXPERIMENT II: The frequency of micronucleated polychromatic erythrocytes fMNPCE (‰) in peripheral blood in FVB mice at 46 h after i.p. injections of different doses of Flu and Phe alone and in a mixture (given at the same time) with PAH4. These treatments are presented on the *x*-axis. The doses of Phe and Flu are given as a number in brackets: for example, Phe (20) +Flu (20) reflects that the doses were 20 mg/kg b.w. for each of the two PAHs. Phe (10) +Flu (10) was not significantly different from the control level ($p > 0.05$). fMNPCEs for PAH4 alone and in a mixture were significantly different from the control level ($p \leq 0.05$). None of the fMNPCEs for mice given the mixture of all six PAHs was significantly different from mice exposed to PAH4 alone. The four compounds in PAH4 (BaP, BaA, BbF, and Chr) were in a mixture with corn oil and given in doses of 10 mg/kg b.w. of each of BaP, BaA, and BbF and 2 mg/kg b.w. of Chr. The mean fMNPCE (‰) for the treatment groups are given in Table 2.

metabolic system as PAH4 [25,29,22,16]; for instance, Phe and Flu have demonstrated both inhibiting and activating effects on the P450 metabolizing capacity. This observation was the reason why we hypothesized that a mixture of all these six compounds might influence the genotoxicity of PAH4.

In all five experiments we have chosen to study a possible genotoxic effect in a low-dose region. For many reasons, we found this design more biological plausible: for example, there is no saturation of the metabolic capacity, which may occur at high doses. These low-dose studies are meaningful when using the sensitive flow cytometer-based micronucleus test, with a laser operating at two wavelengths. Rather than analyzing about 2000 young erythrocytes (PCEs) from each mouse, a mean of about 100 000 PCEs per mouse was analyzed in this study. In a recently published study on the dose response of BaP in the dose region 0 to 32 mg/kg b.w. the trajectory of the dose response curve was linear [3]. This result indicates that in this low-dose interval there is no measurable decrease of the

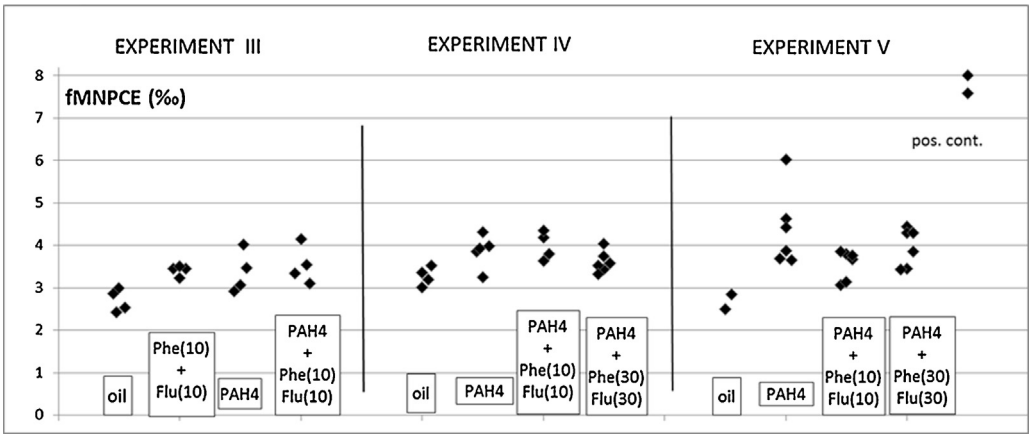

Fig. 3. EXPERIMENT III: The frequency of micronucleated polychromatic erythrocytes fMNPCE (‰) in peripheral blood in 6-week-old FVB mice at 46 h after i.p. injection of different doses of Flu and Phe alone and in a mixture (given at the same time) with PAH4. fMNPCEs in the control group (corn oil) were significantly lower than in all the other groups ($p \leq 0.05$). Phe and Flu in a mixture with PAH4 had no significant effect on the fMNPCEs in PAH4-exposed mice. The mean fMNPCEs (‰) for the treatment groups are summarized in Table 2. The four compounds in PAH4 (BaP, BaA, BbF, and Chr) were in a mixture with corn oil and given in doses of 10 mg/kg b.w. of each of BaP, BaA, and BbF and 2 mg/kg b.w. of Chr.

EXPERIMENT IV: The fMNPCEs (‰) in peripheral blood in FVB mice at 46 h after i.p. injection with corn oil or PAH4. Twenty-four hours before the injection of PAH4, Flu and Phe were administered p.o. in two of the groups. fMNPCEs in the control group were significantly lower compared with the levels in PAH4-exposed mice as well as in mice given PAH4 + Phe(10) +Flu(10), $p < 0.05$. The mean fMNPCEs (‰) for the different groups are shown in Table 2. The four compounds in PAH4 (BaP, BaA, BbF, and Chr) were in a mixture with corn oil and given in doses of 10 mg/kg b.w. of each of BaP, BaA, and BbF and 2 mg/kg b.w. of Chr.

EXPERIMENT V: The frequency of micronucleated polychromatic erythrocytes fMNPCE (‰) in peripheral blood in BALB/c mice at 46 h after i.p. injection of corn oil and PAH4. Twenty-four hours before the injection of PAH4, Flu and Phe were given p.o. in two of the four groups. fMNPCEs in the control group were significantly lower than in the other groups ($p < 0.05$). The mean fMNPCEs (‰) in the groups are presented in Table 2. The four compounds in PAH4 (BaP, BaA, BbF, and Chr) were in a mixture with corn oil and given in doses of 10 mg/kg b.w. of each of BaP, BaA, and BbF and 2 mg/kg b.w. of Chr.

pos.cont. = positive control group, given 50 mg/kg b.w. of BaP.

metabolizing capacity of BaP. It is plausible that other genotoxic PAHs (such as PAH4) produce a similar result.

Before studying the possible impact of Phe and Flu on the genotoxicity of PAH4, Phe and Flu were analyzed separately as well as together. It was important to clarify this distinction in case Phe and Flu affected the fMNPCE of PAH4. The results did not reveal any dose-related effect on fMNPCE and are in accordance with some other *in vivo* MN tests [7,20,27]. Considering the genotoxicity of Phe and Flu *in vitro*, there are no unequivocal conclusions [31]. One explanation for the different results may be that metabolic systems in *in vitro* systems differ from those in *in vivo* systems. [20] demonstrated that metabolites of Flu were much lower in an *in vitro* system using Chinese hamster cells than in an *in vivo* system using mice.

However, our results show that different low doses of Phe and Flu in a mixture did not significantly change the fMNPCE in mice exposed to PAH4, *i.e.*, they might not be efficient in triggering genotoxic metabolic pathways, at least at the dose levels used in this study. However, it is important to bear in mind that because of that we only tested the genotoxic effect of a mixture of chemicals, it is impossible to in detail explain the reason behind an obtained result—metabolism is only one factor among several possible factors. Even the exposure of two inbred strains (Balb C and FVB) with a possibly different metabolizing capacity did not show any significant change in the fMNPCEs. Our results indicate that the age of mice and the time of injecting Phe and Flu in correlation with PAH4 did not influence the genotoxicity of PAH4.

To our knowledge there are no other studies in the literature with the same compounds as those used in the present study. There are, however, some published reports about possible effects *in vitro* when Phe or Flu alone is in a mixture with a genotoxic PAH. For example, BaP in a mixture with Phe induced increased levels of DNA adducts in HepG2 cells [26]. Furthermore, Flu and BaP were shown to act additively for the induction of sister chromatid exchange in HepG2 cells [29]. Finally, [33] demonstrated a twofold increase of certain PAH-DNA adducts when liver cells were treated with Flu together with BaP and [28] concluded that Flu inhibited apoptosis induced by dibenzo(a,l)pyrene. All these authors suggested that this was probably due to an interference with the metabolic activation by Phe or Flu.

Our results suggest no additional effect by Phe and Flu on the genotoxic activity of PAH4 *in vivo*. Although there are an unlimited number of possible combinations of different PAHs to be studied, the two we chose are of special interest because they are ubiquitous pollutants in certain foodstuffs.

Conflict of interest

The authors declare that there are no conflicts of interests.

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