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Research article

Effect of oral exposure to polycyclic aromatic hydrocarbons on goat's milk contamination

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Abstract – The impact of chronic exposure to polycyclic aromatic hydrocarbons (PAHs) on milk contamination was evaluated by oral administration of a mixture of fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo(k)fluorene, benzo(a)pyrene and benzo(g,h,i)perylene at 0.02 mg/kg to lactating goats for 28 days. We analysed PAHs and their major metabolites in milk by gas chromatography-mass spectrometry. The results evidence several major points: (1) benzo(k)fluorene, benzo(a)pyrene and benzo(g,h,i)perylene were not detected in the milk; (2) unexpectedly, the concentration of fluorene, phenanthrene, anthracene, fluoranthene, pyrene and chrysene did not change with time; (3) monohydroxylated PAH metabolites (-OH), namely 2-OH-fluorene, 3-OH-phenanthrene and 1-OH-pyrene were detected shortly after administration. The concentrations of 2-OH-fluorene and 3-OH-phenanthrene reached, respectively, maxima of 0.41 and 0.22 ng/mL during the first exposure week, whereas the concentration of 1-OH pyrene increased to reach a maximum of 0.97 ng/mL on day 14, then slightly decreased during the last two exposure weeks. Those findings suggest a lack of activation of a metabolism that could lead to an excretion of PAHs into milk under native forms. However, a slight increase in concentration could induce the metabolism, which should lead to an increase in the excretion of metabolites into the milk. In spite of the absence of a significant transfer of parent PAHs to milk, the appearance of metabolites in milk raises questions of their impact on human health.

Chronic exposure / PAHs / metabolites / milk contamination

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are potentially mutagenic compounds widely occurring in natural media such as soils, atmosphere, sediments and plants (Wild et al., 1992; Bryselbout et al., 2000; Juhasz et Naidu, 2000, Amellal et al., 2006). The potential carcinogenicity of PAHs is well known, and the ingestion of contaminated food is considered to be the main source of human exposure (IARC, 1983). Consumers might be exposed to PAHs by eating grilled or charred meats, contaminated cereals, flour, bread and vegetables. New rules established in 2005 set the maximum levels for benzo(a)pyrene intakes at 5000 ng/kg in fish and meat products and at 1000 ng/kg in children's foods. Currently, milk and dairy products represent more than 406 kg/eq/year and there is no legislation regulating PAHs and benzo(a)pyrene in particular. Several studies achieved in environmental conditions show that PAHs can be excreted in the milk of ruminants (Husain et al., 1997; Grova et al., 2000, 2002a; Feidt et al., 2005).

Studies to investigate the transfer of these pollutants in lactating ruminants have previously been carried out according to various modalities. West and Horton (1976) evaluated the transfer factor of ^{14}C benzo(a)pyrene at 0.01% in milk after chronic oral administration (1 mg/kg per day) in sheep. A similar study was performed in lactating goats with a single oral administration of ^{14}C-phenanthrene, ^{14}C-pyrene and ^{14}C benzo(a)pyrene at levels of 0.005 mg/kg. It showed transfer factors to the milk of 1.5% ,1.9% and 0.2%, respectively (Grova et al., 2002b). A recent study focused on the transfer and metabolism of phenanthrene in lactating goats allowed the detection and identification of the native compound and its metabolites in various compartments such as plasma, excretion products, and particularly in milk (Grova et al., 2005). In terms of food safety, knowledge of PAH metabolites' levels in food is of some interest, as they appear to be more reactive than parent compounds in terms of mutagenesis and carcinogenesis (Denison and Whitlock, 1995; Uno et al., 2001) or endocrine disruption (Fertuck et al., 2001; van de Wiele et al., 2005; van Lipzig et al., 2005). Unfortunately, these results do not correlate to the potential chronic exposure of animals by ingestion of contaminated ground and fodders (Crépineau et al., 2003). This study performed on transfer and metabolism of PAHs in lactating goats will contribute to the evaluation of the effect of chronic exposure to PAH mixture on milk contamination and give new insight into the form (parent or metabolised molecule) transferred to the milk.

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2. MATERIALS AND METHODS

2.1. Animals

Three Alpine goats (50 ±5 kg; second and third lactations, second month post-partum) from the herd of the "Domaine Expérimental de la Bouzule" (Champenoux, France) were used for this experiment as a model of the lactating ruminant. They received a seven-day adaptation period within individual boxes. Ambient temperature was close to 22 °C and natural light conditions prevailed. The animals were mechanically milked twice a day, and the average milk production during the seven days before the experiment was 2.5 ±0.8 L/day. The goats were fed with meadow hay, water and mineral salt ad libitum and received a concentrate ration twice a day (at 8 am and 5 pm) consisting of dehydrated fodder beet (1100 g), crushing maize (400 g), soya meal (200 g) and dehydrated alfalfa (800 g). The diet composition was established to meet the maintenance and milk production (about 3 L/day) needs of the lactating ruminants (INRA, 1988). Measurements of the production (mL), protein level (g/L) and fat level (g/L) were performed every week in order to verify the quality of the milk.

2.2. Experimental design

The animal protocol was in accordance with the general guidelines of the Council of European Communities (1986, No. 86/609/CEE) and the French Animal Care Guidelines. A control sample of milk was collected from each goat three days before starting the daily administration of PAHs. After morning milking, each animal received 2 mL of vegetal oil (ISIO 4, Lesieur, Neuilly-sur-Seine, France) containing 1 mg of each PAH (0.02 mg/kg of body weight) for 28 consecutive days. PAHs were directly administered into the mouth of the animal with a syringe. The syringe was then flushed twice with 2 mL of vegetal oil and the contaminated oil was given to the animals. The total of 6 mL oil ingested by each animal is not expected to affect rumen activity (Murphy and Morgan, 1983). The PAH mixture was composed of 1 mg of the following compounds: Fluorene (Fluo), Phenanthrene (Phe), Anthracene (Ant), Fluoranthene (Fluot), Pyrene (Pyr), Chrysene (Chrys), Benzo(k)Fluoranthene (B(k)F), Benzo(a)pyrene (B(a)P) and Benzo(g,h,i)perylene (B(g,h,i)Per). Milk samples were collected on days 7, 14, 21 and 28 of the trial. Both evening and the following morning milking were pooled, weighed and stored at –20 °C. The levels of PAHs in water and ration samples were quantified by gas chromatography coupled to mass spectrometry in the single ion monitoring mode. The target PAHs in the diet (Fluo 1.8 ng/g DM, Phe 9.3 ng/g DM, Pyr 0.9 ng/g DM and B[a]P not detected) and water (Fluo and B[a]P not detected, Phe 24 ng/L, Pyr 16 ng/L) were analysed and the concentrations revealed a total daily supply of less than 1% of the intake.

2.3. Milk analyses

The analytical procedure for the extraction and purification of PAHs and their metabolites has been previously described (Grova et al., 2005; Lutz et al., 2006). Milk concentrations of the following PAHs: Fluo, Phe, Ant, Fluot, Pyr, Chrys, B(k)F, B(a)P and B(g,h,i)Per (purchased from Sigma Aldrich, St Quentin Fallavier, France) and major monohydroxylated metabolites: 2-hydroxyfluorene (2-OH Fluo), 3-hydroxyphenanthrene (3-OH Phe) and 1-hydroxypyrene (1-OH Pyr) from Chiron (Trondheim, Norway), were determined as follows. The 3-OH B(a)P is considered as the most abundant metabolite of B(a)P, which is the reference of PAH toxicity. The 1-OH Pyr was selected as the usual indicator of environmental contamination (Jacob and Seidel, 2002). The 3-OH Phe is known to be the major metabolite excreted in goat's milk (Grova et al., 2005). Moreover, the choice of the 2-OH Fluo enables us to consider a decreasing number of benzene cycles.

Prior to extraction, 10 mL of milk fortified with several internal standards (d10-Phe, d10-Pyr and d12-perylene, Interchim, Montluçon, France) was adjusted to pH 5.7 with 100 µL of glacial acetic. Deconjugation was performed with 3750 units of purified *Helix pomatia* juice (Biosepra, Villeneuve-la-Garenne, France) and milk samples were incubated for 16 hours at 37 °C to induce hydrolysis of glucuronide and sulphate conjugates of hydroxymetabolites. Samples were then shaken with 20 mL cyclohexane/ethyl acetate (50:50, v/v; SDS, Peypin, France) for 30 minutes. After centrifugation (15 min at 1000 *g*) the supernatant was evaporated. Residues were dissolved in 3 mL cyclohexane and applied onto an Envi-Chrom P SPE (Styrene-divinylbenzene copolymer resin, Envi Chrom P: 0.5 g) column previously conditioned with water, methanol and cyclohexane (SDS, Peypin, France). After rinsing with 3 mL cyclohexane, PAHs were eluted with 12 mL cyclohexane/ethyl acetate (50:50, v/v). After evaporation to dryness, 2 mL cyclohexane and 2 mL methanol/water (80:20, v/v) were added, mixed for 30 seconds, and the cyclohexane phase was extracted after centrifugation (1000 *g*, 5 min). The methanol/water phase was washed again with 2 mL cyclohexane, centrifuged (1000 *g*, 5 min) and the cyclohexane layer was added to the first cyclohexane phase. At this point, the methanol layer containing hydroxylated metabolites was set aside for later analysis. The supernatant containing the PAHs in cyclohexane was evaporated; saponification was achieved with 5 mL 10% KOH for 80 minutes at 90 °C to prevent any fatty residues from being present in the final product. 3 mL water and 5 mL cyclohexane were then added and the mixture was shaken prior to centrifugation (1000 *g*, 5 min). The supernatant was harvested, an external standard (d12-chrysene) was added, and the cyclohexane was evaporated. The residue was dissolved in 20 µL toluene. The previously separated methanol fraction, as described above, was evaporated and extracted with 4 mL water/ethyl acetate (50:50; v/v). After vortexing and centrifugation (1000 *g*, 5 min), the supernatant was evaporated, supplemented with an external standard (1-OH chrysene, Interchim, Montluçon, France), and hydroxymetabolites were derivatised with 20 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, Fluka, Buchs, Switzerland).

2.4. Chromatography conditions

The quadrupole MS (HP-5973) system used was coupled to a HP-6890 gas chromatograph, both from Hewlett-Packard (Palto Alto, CA, USA). The split/splitless injector was maintained at 250 °C, time of splitless mode was set at 1.5 minutes, and the injected volume was 2 µL. The column used for the separation of phenanthrene and its metabolites was an OV-1

(Ohio-Valley) (30 m × 0.25 mm I.D., film thickness 0.25 µm). The GC parameters used on the column were 120 °C (2 min), 10 °C min^{-1} until 150 °C (2 min), 5 °C min^{-1} until 250 °C (0 min), then 15 °C min^{-1} until 300 °C (5 min). Helium (N55) was used as carrier gas at 1 mL/min flow. The electronic beam energy on the spectrometer was set at 70 eV in the electron impact (EI) mode. The detection limit of each compound in the milk was based on a signal to noise ratio of 3/1. The detection limits were 0.3 ng/mL for Fluo; 0.2 ng/mL for Phe, Ant, Fluot, Pyr, Chrys, B(k)F, B(a)P and B(g,h,i)P; 0.05 ng/mL for 2-OH Fluo and 3-OH Phe; 0.04 ng/mL for 1-OH Pyr and 3-OH B(a)P.

2.5. Statistical analysis

The concentrations of PAHs and their metabolites in milk, as well as their excreted quantities were analysed statistically using the SAS mixed procedure (version 9.1, SAS Institute, Cary, NC) with the repeated time option (Littell et al., 1996). The model took account of the sampling day as a fixed factor. The goats were considered as replicates. The co-variance structure between the different sampling times was defined as being auto-regressive after checking with Akaike and Schwarz-Baysan criteria (1996). The values at a given exposure time were compared in the form of least square means using Tukey's t-test, and significance was declared at a threshold of 0.05.

3. RESULTS AND DISCUSSION

The milk characteristics and body weight of the animals were not changed by PAH treatment (Tab. I). This study allows us to establish that chronic administration of PAHs, under available forms, did not lead to a significant increase in PAHs in goat's milk. Indeed, during the 4 exposure weeks, the levels of native PAHs in milk did not show any significant difference compared with control milk (Tab. II).

The concentrations of PAHs ranged from 0.1 to 4 ng/mL. Among the 9 PAHs distributed by the oral route, 3 were not detected: the B(k)F, the B(a)P and the B(g,h,i)Per. These three compounds present a high molecular weight (252, 252 and 276 g/mol, respectively) and more than 4 ring cycles. Previous studies carried out on cow's milk showed that of the 16 PAHs evaluated, only 9 of them were detected and all of them presented less than 5 ring cycles (Grova et al., 2000, 2002a). We previously reported that after a single administration of three ^{14}C-labelled PAHs to lactating goats, the B(a)P showed the lowest transfer in terms of radioactivity, probably under metabolite forms (Grova et al., 2002b). All these results revealed that after a single dose (Grova et al., 2002b) or after chronic oral administration (in controlled conditions or in breeding animals; Grova et al., 2000), the PAHs' molecular weight and the number of ring cycles appear to be determining factors for PAHs' transfer ability into the milk. The studies carried out using in vitro models with caco2 cells (Buesen et al., 2003; Cavret et al., 2005; Cavret and Feidt, 2005) highlighted the fact that the low bioavailability and the high ability of B(a)P to be biotransformed induce a low transfer through the gastro-intestinal barrier, contrary to Phe or Pyr. The B(k)F and the B(g,h,i)P, which presented similar chemical and physical properties to B(a)P, should also be less absorbed than the lighter compounds.

This experiment of chronic exposure in controlled conditions allows us to clearly demonstrate the absence of transfer of heavy compounds into the milk. An interesting observation in terms of food safety was that the highest and most toxic compounds were not transferred into the milk (5 ring cycles) during a chronic oral administration at low levels. The light compounds (< 5 ring cycles) were found in both control and contaminated samples in a range between 0.1 and 4 ng/mL.

Table I. Average milk production and quality of the three lactating goats.

	Goat 1	Goat 2	Goat 3
Production (mL)	2807 ± 165	3208 ± 270	1650 ± 160
Fat level (g/L)	44.2	31.4	30.8
Protein level (g/L)	33.9	26.8	37.4

Table II. Concentration of polycyclic aromatic hydrocarbons (PAHs) in milk during 28 days of chronic oral administration of PAHs at 0.02 mg/kg to lactating goats. Fluo: Fluorene, Phe: Phenanthrene, Ant: Anthracene, Fluot: Fluoranthene, Pyr: Pyrene, Chrys: Chrysene, B(k)F: Benzo(k)Fluoranthene, B(a)P: Benzo(a)pyrene, B(g,h,i)Per: Benzo(g,h,i)perylene, NS: Not Significant, ND: Not Detected, SD: standard deviation, M: mean, RMSE : Root Mean Square Error.

Concentration ng/mL milk (n = 3)	Day 0		Day 7		Day 14		Day 21		Day 28		Time Effect	RMSE
	M	SD	M	SD	M	SD	M	SD	M	SD		
Fluo	0.79	0.01	0.86	0.27	1.26	0.76	1.03	0.41	0.77	0.23	NS	0.16
Phe	1.08	0.03	1.08	0.02	1.85	0.35	1.28	0.25	1.44	0.10	NS	0.16
Ant	0.27	0.06	0.28	0.14	0.65	0.39	0.30	0.16	0.50	0.14	NS	0.03
Fluot	0.70	0.39	0.64	0.41	0.81	0.40	0.77	0.47	0.69	0.49	NS	0.01
Pyr	0.61	0.28	0.54	0.24	0.78	0.38	0.68	0.27	0.69	0.10	NS	0.02
Chrys	0.79	0.24	1.11	0.38	1.28	0.93	1.37	1.12	1.00	0.15	NS	0.16
B(k)F	ND		ND		ND		ND		ND		–	–
B(a)P	ND		ND		ND		ND		ND		–	–
B(g,h,i)Per	ND		ND		ND		ND		ND		–	–

Table III. Concentration of monohydroxylated polycyclic aromatic hydrocarbons (PAHs) in goat's milk during the exposure period. 2-OH Fluo: 2-OH Fluorene, 3-OH Phe: 3-OH Phenanthrene, 1-OH Pyr: 1-OH Pyrene, 3-OH BaP: 3-OH BenzoaPyrene, ND: not detected, SD: standard deviation, M: mean, RMSE: root mean square error. * $P < 0.05$. ** $P < 0.01$ difference statistically significant versus control group (Tukey's t-test).

Concentration ng/mL milk (n = 3)	Day 0		Day 7		Day 14		Day 21		Day 28		Time Effect	RMSE
	M	SD	M	SD	M	SD	M	SD	M	SD		
2-OH Fluo	0.00	0.00	0.41	0.25	0.51	0.34	0.67	0.34	0.47	0.21	*	0.057
3-OH Phe	0.00	0.00	0.22	0.09	0.37	0.17	0.30	0.12	0.13	0.03	**	0.009
1-OH Pyr	0.00	0.00	0.86	0.53	0.97	0.27	0.53	0.41	0.32	0.15	*	0.048
3-OH BaP	ND		ND		ND		ND		ND		–	–

However, the statistical analysis ($P > 0.05$) did not reveal an increase in these molecules during the 4 weeks of exposure (Tab. II). This result is in accordance with Lutz et al. (2006), who indicated that a chronic administration of contaminated soil by cow's ruminal canula did not increase the level of PAHs in milk. Lactating ruminants exposed daily to PAHs via the ingestion of contaminated matrices (at an environmental level of 0.02 mg/kg/day) did not present a significant increase in PAH concentration in milk.

Of the 4 monohydroxy-metabolites investigated, only 3 of them were detected: the 2-OH Fluo, the 3-OH Phe and the 1-OH Pyr (Tab. III). Three different profiles were observed: firstly, the concentrations of 2-OH Fluo and 3-OH Phe seemed to plateau out at day 7 (0.41 ± 0.25 and 0.22 ± 0.09 ng/mL, respectively). The highest daily excreted quantities were 1582 ng (± 330) and 930 ng (± 360), respectively, for 2-OH Fluo and 3-OH Phe. Secondly, the variance analysis revealed an increase in 1-OH Pyr during the first 2 weeks of exposure ($P < 0.05$, 0.97 ± 0.27 ng/mL on day 14, corresponding to 2382 ± 141 ng excreted), followed by a slight decrease over the two last weeks. Finally, this study did not allow the detection of the presence of 3-OH B(a)P in milk before or after the treatment (Tab. III).

The difference between the three compounds may be explained by the reduced number of metabolites for Pyr compared with the numerous metabolites of Phe and Fluo. Moreover, the absence of 3-OH B(a)P could be partially explained by the very poor absorption of B(a)P and its strong metabolism at the enterocyte levels (Cavret and Feidt, 2005). The same concentration profiles of metabolites in milk were also described in cow's milk after chronic exposure to contaminated soil (Lutz et al., 2006).

Contrary to the native compounds, target monohydroxylated metabolites were not detected in control samples. However, the 2-OH Fluo, the 3-OH Phe and the 1-OH Pyr were present in the milk at day seven, and their levels increased significantly compared with controls ($P < 0.05$) during the rest of the exposure period (Tab. III). The absence of metabolites in control milk may suggest a lack of activation of the metabolism at an environmental level, which could lead to an excretion of native forms of PAHs into milk. According to Grova et al. (2005), this experiment highlighted a metabolism induction in goats chronically exposed to a slight increase in PAH concentration (0.02 mg/kg/day), revealed by the presence of metabolites in milk. This metabolism induction contributes to the increase in excretion of metabolites into urine and milk (e.g., 40% of the radioactivity associated with Pyr was excreted via the urine, Grova et al., 2002b) and partially explains the significant increase in metabolite levels observed during the experiment.

The impact of metabolites in milk on human health is not currently understood; little information is available. Analytical analysis showed that 100% of metabolites present in milk are under conjugated forms (Grova et al., 2005). These sulphate and glucuronide groups increase the molecular weight and the solubility of PAH metabolites. This increase in solubility could change their availability and their absorption in the intestinal tract, where they could again be metabolised under reactive forms.

4. CONCLUSION

The daily oral exposure of lactating goats to a mixture of 9 PAHs for 28 days resulted in a significant increase in monohydroxylated metabolites in milk. The constant level of native PAH forms and the significant appearance of metabolites confirm that in terms of risk assessment, exposure of lactating ruminants to PAHs remains problematic. Considering that their toxicity and bioavailability are not known, it is surprising that the proven documented transfer of metabolites is largely ignored. The authors believe that it would be useful to characterise these two parameters in order to evaluate the potential risk of transfer within the food chain.

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