A mixture of Persistent Organic Pollutants (POPs) and Azoxymethane (AOM) show potential synergistic effects on intestinal tumorigenesis in the A/J Min/+ mouse model

K.E.Aa Hansen a,*, S.M. Johanson a, C. Steppeler b, M. Sødring b,g, G.C. Østby c, H.F. Berntsen c,d, K.E. Zimmer c, M. Aleksandersen f, J.E. Paulsen b, E. Ropstad a

*Section for Experimental Biomedicine, Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Norway
bSection for Food Safety, Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Norway
cSection for Stationary Clinics, Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Norway
dDepartment of Administration, Laboratory Animal Unit, National Institute of Occupational Health, Norway
eSection for Biochemistry and Physiology, Department of Basic Sciences and Aquatic Medicine, Norwegian University of Life Sciences, Norway
fSection for Anatomy and Pathology, Department of Basic Sciences and Aquatic Medicine, Norwegian University of Life Sciences, Norway
gAnimalia, Norwegian Meat and Poultry Research Centre, Norway

A HIGHLIGHTS

• Can a mixture of POPs affect intestinal tumorigenesis in the A/J Min/+ mouse?
• Mice were exposed to POPs through the diet and received an injection of Azoxymethane.
• Results show an increased intestinal tumorigenesis in the A/J Min/+ mouse model.

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ABSTRACT

A multitude of cancer types, including breast, testicular, liver and colorectal cancer, have associations with exposure to Persistent Organic Pollutants (POPs). The present study aimed to investigate whether a mixture of POPs could affect intestinal tumorigenesis in the A/J Min/+ mouse, a model for human colorectal cancer (CRC). Pollutants were selected for their presence in Scandinavian food products and the mixture was designed based on defined human estimated daily intake levels. Mice were exposed through the diet, at control, low and high mixture concentrations, for 10 weeks. In a separate experiment, mice also received one subcutaneous injection of Azoxymethane (AOM) to explore whether this carcinogenic compound influenced the effect of the POPs. Intestinal tumorigenesis was examined by surface microscopy and histopathology. Moderate and dose-dependent increases in tumorigenesis were observed after dietary POP exposure. The AOM treatment alone stimulated the growth of colonic lesions, but did not increase the formation of new lesions. Combined AOM treatment and POP exposure demonstrated a synergistic effect on lesion formation in the colon, and to a lesser extent in the small intestine. This synergy was also evident by an increased number of malignant colonic tumors (carcinomas). In conclusion, the study shows that a mixture of POPs interacted synergistically with a known carcinogen (AOM), causing increased intestinal tumorigenesis in the A/J Min/+ mouse model.

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1. Introduction

Persistent Organic Pollutants (POPs) are man-made chemicals that are toxic to humans and wildlife, resistant to degradation and have the potential to bioaccumulate and biomagnify in living organisms (UNEP, 2015). The compounds have adverse health effects and have been associated with an increased risk of breast cancer (Hoyer et al., 2000; Cameron and Foster, 2009), testicular cancer (McGlynn et al., 2008; Giannandrea et al., 2011), liver cancer (Filgo et al., 2015), and colorectal cancer (Howsam et al., 2004; Song et al., 2014). The main route of non-occupational exposure to POPs in

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humans is through ingestion (Darnerud et al., 2006; Vester gren et al., 2012), which makes the GI tract the first organ of exposure. Traditional animal experiments only assess the impact of POPs using single compounds (Sethi et al., 2017) or compounds belonging to the same chemical group (Colter et al., 2018). However, carcinogenesis is a multistep process, so focus on individual compounds may prevent the discovery of potential synergism between multiple chemicals.

Colorectal cancer (CRC) is the third most common cancer in humans worldwide and exposure to carcinogens through the diet is an essential risk factor (IARC, 2016). CRC develops as a result of intestinal epithelium from normal tissue, via benign neoplasms, into carcinomas (Kinzler and Vogelstein, 1996; Sancho et al., 2004). Up to 85% of CRC cases are considered sporadic and 1% are attributable to the hereditary CRC syndrome known as familial adenomatous polyposis (FAP) (Burt, 2000). Mutations in the tumor-suppressor gene adenomatous polyposis coli (APC) are responsible for FAP, and patients develop a vast number of adenomatous polyps in the intestine, which are likely to progress into malignant tumors (Kinzler and Vogelstein, 1996). In addition, dysfunctional APC alleles have been found in the majority of sporadic colorectal lesions (Fodde, 2002). Research on CRC caused by APC mutations is therefore highly relevant to human health.

The most widely used animal model for human CRC is the multiple intestinal neoplasia (Min/) mouse, on the other hand, also develops a large number of lesions in the colon, many of which progress to carcinomas over time (Sødring et al., 2016b). Therefore, the A/J Min/ mouse model more closely resembles CRC development in humans and was therefore chosen for the present study.

The A/J strain has been shown to be more susceptible to the induction of colorectal cancer by Azoxymethane (AOM) than its C57BL/6 genetic background (Moser et al., 1990), develops lesions primarily in the small intestine (Mollersen et al., 2004). The A/J Min/ mouse, on the other hand, also develops a large number of lesions in the colon, many of which progress to carcinomas over time (Sødring et al., 2016b). Therefore, the A/J Min/ mouse model more closely resembles CRC development in humans and was therefore chosen for the present study.

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2. Animals, materials and methods

2.1. Ethics statement

The study was performed at the Section for Experimental Biomedicine at The Norwegian University of Life Sciences in Oslo, Norway. The animal facility is licensed by the Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (https://www.aalac.org/). The animal experiment was approved by the unit’s animal ethics committee (Institutional Animal Care and Use Committee/IACUC) and the Food Safety Authority (application ID: FOTS 8127) and executed in compliance with the local and national regulations associated with laboratory animal experiments. The rodent and rabbit section of the facility is a Specific Pathogen Free (SPF) unit and follows a health monitoring program recommended by Federation of European Laboratory Animal Science Associations/FELASA (http://www.felasa.eu/). The care of the animals was carried out by two veterinary nurses with FELASA B certification and the study was performed by a veterinarian with FELASA C certification.

2.2. Chemicals and experimental diet

A thorough description of the design and preparation of the POP mixture can be found in Berntsen et al. (2017). A list of the individual compounds can be found in Table 1. In brief, compounds occurring in Scandinavian food products reported in studies prior to 2012 were selected for the POP mixture. Human estimated daily intake (hEDI) levels were derived and adjusted to a 25 g mouse consuming 3 g feed/day. However, due to the possibility of background exposure and interspecies differences in compound metabolism, concentrations were adjusted up to 5000× (low dose) and 100 000× (high dose) hEDI. All polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs) and other organochlorines were purchased from Chiron AS (Trondheim, Norway). All perfluorinated compounds (PFCs) and hexabromocyclododecane (HBCD) were obtained from Sigma-Aldrich (St. Louis, MO, USA), with the exception of perfluorohexane sulfonic acid (PFHxS) potassium salt which was purchased from Santa Cruz (Dallas, US). All chemicals were dissolved in an appropriate solvent and added to corn oil (Jasmin, fully refined, Yonca Gida San.A.S., Manisa, Turkey) intended for human consumption. Solvents were thoroughly evaporated under N₂-flow and the remaining oil was incorporated in AIN-93G mouse feed (TestDiets, St.Louis, MO) at the low and high mixture concentrations. The control diet contained only corn oil from which the solvent had been evaporated.

2.3. Study design

In Experiment 1, 66 mice were used and each litter was randomly divided into 3 exposure groups (control, low and high POP diet) at weaning and exposed for 10 weeks (Fig. 1). In Experiment 2, 21 mice were exposed to the mixture of POPs in the same way, but in addition, these mice were also given one subcutaneous injection of 8.5 mg/kg AOM (Sigma-Aldrich, St. Louis, MO, USA) during their second week after birth. After 10 weeks of POP exposure, all mice were sacrificed and sampled. Because of high offspring mortality after the AOM injection, the breeding of mice for Experiment 2 was terminated for animal welfare reasons prior to completion of breeding the individuals for the study. This resulted in a lower number of animals compared to Experiment 1.

2.4. Animal model

The A/J Min/ mouse model was established by backcrossing the Min/ trait onto the genetic background of the A/J strain for > 12 generations (Sødring et al., 2016b). In the present study, a total of 87 A/J Min/ mice were used. The animals were bred in-house. Female A/J Min/+ mice were mated with male A/J Min/+ mice and their A/J Min/+ offspring were used in the present study. The pups were marked with ear punches and genotyped at weaning, as
Abbreviations: PCBs (polychlorinated biphenyls); OCPs (organochlorine pesticides); BFRs (brominated flame retardants); PFAAs (perfluorinated acids).

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average EDI a</th>
<th>Daily intake</th>
<th>EDI b 25 g</th>
<th>Feed measured c</th>
<th>EDI b 25 g mouse</th>
<th>EDI b 25 g mouse</th>
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<tr>
<td></td>
<td>70 kg person</td>
<td>ng/kg/day</td>
<td>mouse pg/</td>
<td>5000 × ng/g</td>
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<td></td>
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<td>feed</td>
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<td>trans-</td>
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<tr>
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<tr>
<td>PFDA</td>
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<tr>
<td>PFUnDA</td>
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<td>0.096</td>
<td>2.4</td>
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<td>240</td>
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<tr>
<td>∑PFAAs</td>
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<td>28.6</td>
<td>142</td>
<td>2858</td>
<td>17.7</td>
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</table>

Abbreviations: PCBs (polychlorinated biphenyls); OCPs (organochlorine pesticides); BFRs (brominated flame retardants); PFAAs (perfluorinated acids).

a Average EDI (Estimated daily intake) values of POPs for a 70 kg human e based on a literature review of Scandinavian EDI values (Berntsen et al., 2017).
b EDI values for a 25 g mouse corresponding to human EDI values.
c EDI values for a 25 g mouse corresponding to human EDI values × 5000.
d EDI values for a 25 g mouse corresponding to human EDI values × 100,000.
e Measured concentrations of the various compounds in the 5000 × feed.
f Measured concentrations of the various compounds in the 100,000 × feed.
g EDI values for a 25 g mouse consuming 3 g of the 5000 × feed/day e based on concentrations measured in the feed of the current project.
h EDI values for a 25 g mouse consuming 3 g of the 5000 × feed/day e based on concentrations measured in the feed of the current project.

Previously described in Sødring et al. (2015).

2.5. Housing and husbandry

During mating, animals were housed in groups in open type III cages (Tecniplast, Buguggiate, Italy). During exposure and AOM injection animals were housed in closed type III PVC cages (Allentown Inc, USA) for health and safety reasons. All cages contained standard aspen bedding, cellulose nesting material and red polycarbonate houses (Tecniplast, Buguggiate, Italy). The animals were given their assigned feed, and tap water in standard drinking bottles (Tecniplast, Buguggiate, Italy), ad libitum. The animal room was on a 12:12 light–dark cycle, with a room temperature of 21 ± 2 °C with 20 air changes per hour and 45 ± 5% relative humidity. The cages, bedding, nesting material and water bottles were changed once a week.

2.6. Sample collection and identification of intestinal lesions

The A/J Min/+ offspring were sacrificed at 13 weeks of age. They were anesthetized with isoflurane gas (Isoflurane Baxter, San Juan, Puerto Rico), bled by cardiac puncture and euthanized by cervical dislocation. The small intestine and colon were collected, fixed and dyed as previously described in Sødring et al. (2016a). Briefly, the intestines were tanned with PBS, fixed flat, and stored in 10% neutral buffered formalin for at least 24 h, before being stained with 0.2% methylene blue dissolved in formalin. The liver was collected and weighed. All tumors that were found (one in the liver, one from the...
forelimb, one sub-mandibular and one from the abdomen) were also collected and fixed in 10% neutral buffered formalin. The blood, cecum, spleen and retroperitoneal adipose tissue were collected and stored for analysis in another project. For surface microscopy and transillumination of the intestines, an inverted light microscope (CKX41, Olympus Inc., Hamburg, Germany) with a digital color camera (DP25, Olympus) was used. In the colon, lesions were detected as either flat aberrant crypt foci (flat ACF; <30 crypts) or tumors (>30 crypts covering more than approximately 0.4 mm²) as explained by Sødring et al. (2015).

2.7. Histology

After scoring, the intestines were prepared using the Swiss roll technique as described earlier by Sødring et al. (2016b). The Swiss rolls were embedded in paraffin and 3 μm thick histological sections were cut and stained with haematoxylin eosin (HE) and periodic acid Schiff (PAS). All Swiss rolls were sectioned at three crypts covering more than approximately 0.4 mm² as preneoplastic lesions (hyperplastic and dysplastic). Tumors with distinct invasive growth were classified as adenomas, whereas tumors confined to the mucosa without infiltrative growth were classified as adenomas. Tumors that were found outside the intestine were also embedded in paraffin, sectioned and stained with HE and PAS, and examined in the microscope.

Female A/J +/+ X Male A/J Min/+  

Experiment 1  
POPs  
Exposure group: Control. 10 Female A/J Min/+ 10 Male A/J Min/+  
Exposure group: Low. 10 Female A/J Min/+ 15 Male A/J Min/+  
Exposure group: High. 11 Female A/J Min/+ 10 Male A/J Min/+  

Experiment 2  
POPs + AOM injection  
Exposure group: Control. 6 Female A/J Min/+ 2 Male A/J Min/+  
Exposure group: Low. 2 Female A/J Min/+ 4 Male A/J Min/+  
Exposure group: High. 3 Female A/J Min/+ 4 Male A/J Min/+  

Fig. 1. Study design of the two experiments, including exposure groups (control, low and high), breeding of A/J Min/+ mice and the number of animals (females and males) in each group. In both experiments, A/J Min/+ mice were exposed to a mixture of POPs through feed for 10 weeks. In addition, mice in Experiment 2 received one subcutaneous injection of AOM (8.5 mg/kg) during the second week after birth.

2.8. Statistical analyses

Statistical analyses were performed using JMP Pro 13° (SAS, Cary, NC, USA). Least squares analyses were used to analyze data on body measures. Experiment 1 and 2 were analyzed separately by the following model:

\[ Y_{ijpmn} = \mu + G_i + E_j + e_{ij} \]

where:

- \( Y_{ijpmn} \) = observation of either body weight, relative liver weight, relative colon length or relative small intestine length.
- \( \mu \) = overall mean of body weight, relative liver weight, relative colon length and relative small intestine length.
- \( G_i \) = effect of sex, \( i = 1 \) (Male) or 2 (Female).
- \( E_j \) = effect of exposure group, \( j = 1 \) (control), \( j = 2 \) (low), \( j = 3 \) (high).
- \( e_{ij} \) = error term.

Measures of histological changes and visually scored lesions did not meet the assumption of normality. Log transformation provided an improved, but not satisfactory, fit to the normal distribution. Initially least squares analyses were performed on log-transformed data with sex and exposure group as explanatory variables. Some sex differences were noticed, but few interactions were found between the exposure group and the sex of the animal. Thus, exposure effects were not dependent on the sex. In the final analyses, univariate non-parametric tests were used. Differences between exposure groups and the control were assessed using Steel’s test, which controls for the overall experiment wise error rate (Type I). Differences between sexes were investigated using the Wilcoxon two-sample test. The level of significance was set to 5%. Size and location distribution figures were produced using Excel 2013°.

3. Results

3.1. Effects on body weight, liver weight and intestinal length

The high mixture concentration of POPs significantly decreased the terminal body weight of both the mice who only were exposed to POPs (Experiment 1) and also the mice that were injected with AOM (Experiment 2), compared to the control group (Table 2). In addition, there was a significant increase in liver weight, relative to body weight, in the high group of both experiments. Colon length, relative to body weight, was not affected by AOM or POPs. However, the length of the small intestine was significantly increased by the high concentration of POPs after the AOM injection (Experiment 2). Notably, AOM alone did not change any of the parameters measured.

3.2. Scoring of intestinal lesions

The effects of dietary exposure to the mixture of POPs on intestinal tumorigenesis was examined in mice by scoring of intestinal lesions (Table 3). High levels of POPs (Experiment 1) significantly increased the number of flat ACF in colon, when compared to the control group. Although not significant, a trend was observed towards increased flat ACF load in the high and the low groups (\( p = 0.051 \) and \( p = 0.058 \), respectively). The low mixture concentration increased the number of colonic tumors, compared to the control group. However, this was not evident after exposure to the high mixture concentration (\( p = 0.096 \)). No other parameters measured in the small intestine and colon were affected by dietary POPs alone.
In combination with the AOM injection (Experiment 2), POPs significantly increased the number of flat ACF, flat ACF load, number of tumors, average tumor size and tumor load in the colon of mice in the high group (Table 3). In addition, the number of tumors in the small intestine was significantly higher in the high group compared to the controls. No significant changes were observed after exposure to the low mixture concentration of POPs in Experiment 2. However, there were clear trends towards increases in several parameters, including a 7-fold increase in the colonic tumor load.

### 3.3. Size distribution and location of intestinal lesions

To demonstrate the distribution of size, lesions were divided into five different size categories. Fig. 2a presents the number of lesions per size category for each exposure group in both experiments and clearly illustrates the shift towards larger lesions observed in AOM treated animals. The AOM treatment alone did not appear to give any new lesions, but instead stimulated the growth of the colonic lesions. Notably, the increase in the number of lesions provoked by POPs seemed to be more pronounced in experiments and clearly illustrates the shift towards larger lesions observed in AOM treated animals. The AOM treatment alone did not appear to give any new lesions, but instead stimulated the growth of the colonic lesions. Notably, the increase in the number of lesions provoked by POPs seemed to be more pronounced in
AOM treated animals than in untreated animals, particularly in the colon. This implies a synergistic effect of AOM and POPs. In the small intestine, AOM alone did not induce any apparent changes, but a moderate synergistic effect on tumor formation seemed to occur between AOM and the high level of POPs.

Location of lesions along the intestine (Fig. 2b) shows an increased number of lesions in the middle and caudal parts of the colon and caudally in the small intestine, in both experiments. In addition, the figure illustrates how dietary exposure to POPs enhances the number of lesions in both experiments, represented by more lesions in the high and low groups compared to the control group.

3.4. Histopathology

Histology from tumors collected from non-intestinal tissue showed no metastases originating from the intestinal lesions. Instead, they were either hyperplastic lesions or metastases from the local tissue.

In Experiment 1, lesions were found in the intestines of animals from all exposed groups. The total number of lesions in the small intestine was higher than that of the colon (Table 4). No significant differences were observed between the control group and the exposed groups. Preneoplastic changes and adenosomas were the most frequent lesions, and only a few animals had carcinomas. Fig. 3 illustrates the types of lesions in the colon in Experiments 1 and 2.

In Experiment 2, the mice fed the high concentration of POPs had significantly more colonic lesions of all types compared to the control group (Table 4). A trend was also evident towards increases in the number of small intestinal preneoplastic lesions ($p = 0.067$). Interestingly, this increase of lesions appeared to be due to the synergistic effect between AOM and the high level of POPs, as suggested above.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Colon</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preneoplastic</td>
<td>Adenoma</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>POPs</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>0.84 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>1.05 ± 0.30</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>POPs + AOM</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>2.00 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>5.57 ± 1.11</td>
</tr>
</tbody>
</table>

Table 4
Histopathological examination of lesions (preneoplastic, adenoma or carcinoma) in colon and small intestine (SI) of A/J Min/+ mice from Experiment 1 (POPs exposure) and Experiment 2 (POPs exposure + AOM injection). Results are presented as mean ($±SE$). Differences between exposed groups (low and high) and control were assessed with Steel’s test and indicated in bold when significant ($p < 0.05$). Trends with $p < 0.07$ are denoted *.

Fig. 3. Histological lesions observed in colon. a. Preneoplastic lesions (dysplasia and hyperplasia) are present in the luminal part of crypts (arrows) of a mouse of the low exposure group of Experiment 1. b. A carcinoma (arrow) in the mucosa infiltrates Muscularis Muscosa and Submucosa. Mouse of the low exposure group of Experiment 1. c. A small focus with preneoplastic crypt lesions in a mouse of the low exposure group of Experiment 2. d. Mucosal adenoma (ad) in a mouse from the high exposure group of Experiment 2.

4. Discussion

In the present study, we investigated whether a mixture of POPs could affect intestinal tumorigenesis in the A/J Min/+ mouse model. In a separate experiment, we also investigated whether a sub-carcinogenic exposure of AOM could influence the effect of POPs. We found that POPs alone increased the intestinal tumorigenesis moderately and in a dose-dependent manner. Comparing the two experiments, AOM alone did not seem to increase the formation of new lesions, or have a deleterious effect on the mice. However, the growth of colonic lesions was stimulated by AOM treatment. A strong synergistic effect was apparent between POPs and AOM on the formation of colonic lesions, and to a lesser extent...
on lesions in the small intestine. Interestingly, this synergy was also associated with a significant increase of malignant tumors (carcinomas) in the colon.

4.1. Effects of POPs on body weight and liver weight

The concentration of each compound in the high dose was generally below the No Observed Adverse Effect Level (NOAEL), where such a level was available (Bernsten et al., 2017). Although we did not observe any clinical signs in the animals during the present study, we did observe apparent adverse effects at the end of the study, indicated by reduced body weight and increased relative liver weight in both experiments. These effects were seemingly unrelated to AOM treatment and may have been caused by additive or synergistic effects between individual POPs in the mixture. In another experiment using the same mixture but a different mouse strain (129/C57BL/6F0), there was no significant effect of the high POPs feed on body weight (Hudecova et al., 2018). This suggests there are mouse strain differences in sensitivity to POPs.

Aberrant Apc expression as a consequence of the germline mutation in Apc has been shown to affect the ability of the liver to metabolize xenobiotics (Benhamouche et al., 2006), and may lead to degrees of pollutant tolerance. In addition, the large number of intestinal lesions in the mice exposed to the high dose of POPs in our experiment may have contributed to a lower absorption rate of nutrients, which could have reduced the body weight of mice in this group.

Our findings of increased relative liver weights is in line with other studies where animals have been exposed to perfluorinated compounds (Seacat et al., 2003; Tan et al., 2013). These chemicals have been thoroughly investigated for hepatotoxicity, because of their high affinity to serum proteins and subsequent accumulation in the liver (Jones et al., 2003).

The highest concentration of POPs in our study is relatively large, but the low mixture concentration could potentially be considered more relevant for humans when taking life-long exposure and slow pollutant metabolism into account (Martignoni et al., 2006; Hudecova et al., 2018).

The occurrence of high mortality in offspring after neonatal AOM treatment (Experiment 2) was surprising, as the dosage used has not previously been associated with increased mortality. It is therefore unclear whether the lethality observed was caused by an abnormally high sensitivity to AOM, either alone or in combination with stress. However, we can conclude that the event was not caused by dietary POPs, since the AOM injection was given prior to weaning.

4.2. Effects of POPs on intestinal lesions

The process of cancer is divided into three phases; initiation, promotion and progression (Farber and Cameron, 1980). Depending on their mode of action, compounds may interfere with the molecular processes within each of these phases, and ultimately affect the carcinogenic process. Initiation is the irreversible heritable change in DNA, while promotion is the non-genotoxic advantages of mutated cell growth (Ludwig and Robertson, 2013). In the present study, the high mixture concentration of POPs initiated the formation of new lesions in the colon of AJ Min/+ mice, which was reflected by a significantly larger intestinal area covered by flat ACF. The low concentration of POPs did not affect the number of newly formed lesions, but promoted intestinal tumorigenesis by resulting in more colonic tumors of a larger diameter (>30 crypts). This initiating and promoting effect was not visible in the small intestine.

Previous studies have reported that some POPs affect both carcinogenic initiation and promotion in vivo. Liver tumorigenesis was initiated by a mixture of PCBs (Kanechlor 500) in mice (Ito et al., 1973). The same study also showed a promotional effect of the PCBs when administered together with hexachlorobenzene (HCB, α or β). Developmental exposure (in utero and via lactation) to dieldrin initiated the formation of mammary, ovarian and liver tumors in a transgenic mouse model for mammary tumorigenesis (Cameron and Foster, 2009). In utero exposure to perfluorooctanoic acid (PFOA) induced hepatocellular adenomas in CD-1 mice (Filgo et al., 2015). However, PFOA and PFOS did not increase the formation of intestinal lesions (Ngo et al., 2014). HCB was shown to promote mammary, liver and lung tumorigenesis in xenograft mouse models, without having initiating effects (Pontillo et al., 2013). The organochlorine metabolite p,p'-DDE has been shown to promote CRC growth in mice injected with a suspension of the human colorectal adenocarcinoma cell line DLD1 (Song et al., 2014). The study also demonstrated that the CRC promotion by p,p'-DDT was achieved through the Wnt/β-catenin signaling pathway mediated by oxidative stress. p,p'-DDT elevated the production of reactive oxygen species (ROS). This inhibited enzymes, and reduced antioxidative systems and damaged the ACF to tumors in intestinal cells. Subsequently, there was an accumulation of β-catenin and the consecutive expression of target genes, which induced the proliferation of colorectal cancer cells and thus promoted CRC growth. The study also demonstrated that an increased production of ROS could affect colorectal carcinogenesis by interacting with specific pathways or by damaging DNA.

Furthermore, the metabolic activation of compounds may create products or intermediates that can interfere directly with DNA. PCBs have been shown to form highly reactive products and by-products that have the ability to mutate DNA, as reviewed by Ludewig and Robertson (2013). PBDEs are structurally similar to PCBs and have been shown to induce ROS formation, leading to chromosomal breakage (Ji et al., 2011). POPs may therefore have the ability to affect DNA and to increase tumorigenesis by inducing mutations in oncogenes or tumor suppressor genes such as KRAS, p53 and APC. Changes in these genes are necessary for the development of colorectal cancer (Fodde, 2002). It has also been shown that most intestinal lesions in the Min/+ mouse have lost their remaining functioning Apc allele (Luongo et al., 1994). In the present study, mutations in Apc might have caused the formation of new lesions and enhanced the growth from flat ACF to tumors in the AJ Min/+ mice. However, this remains to be investigated.

4.3. Effects of AOM and POPs on intestinal lesions

AOM is converted to methylyazoxymethanol (MAM) by cytochrome P450 enzymes (CYP450) located in both the liver and the intestines (Sohn et al., 2001). This highly reactive metabolite causes DNA mutations that are thought to initiate colorectal carcinogenesis (Takahashi and Wakahayashi, 2004). Different strains of mice vary in their susceptibility to AOM-induced CRC, and the AJ strain is known to be highly sensitive (Rosenberg et al., 2009). In addition, Min/+ mice exposed to AOM during their first two weeks of life have been shown to be particularly susceptible to induced and spontaneous intestinal carcinogenesis (Paulsen et al., 2003).

In the present study, neonatal mice in Experiment 2 were given one injection of AOM. This treatment did not seem to initiate the formation of new colonic lesions. Instead, it promoted the growth of already existing lesions, as evident from the increased number of tumors and colonic lesions of the larger size classes in mice from the control group. Combined exposure to AOM and POPs both
initiated and promoted colorectal carcinogenesis and resulted in a severe lesion burden, especially in mice exposed to the high mixture concentration of POPs. This large effect on tumorigenesis, compared to the relatively moderate initiation and promotion by POPs alone, indicates a synergistic effect between AOM and POPs. The high group exhibited the most extreme outcomes, which could be explained by the relatively high concentration of pollutants. However, the numerical differences from the control group demonstrate that the low mixture concentration also displayed initiating and promoting effects in the colon, as shown by a 7-fold increase in colonic tumor load. As with AOM, POPs are metabolized by CYP450 (Docea et al., 2017) and CYP450 has been shown to be a strong biomarker for the presence of POPs in animal tissue (Bachman et al., 2015). This similarity could be the origin of the synergistic effect observed between AOM and POPs, but this remains to be investigated. Previous studies in mice (Swiss and Balb/c mice only exposed to POPs (Experiment 1). However, carcinomas scoring was not evident from the histopathological examination of the two different methods.

4.4. Histopathology

In the present study, the histopathological characterization of intestinal lesions differentiated between preneoplastic lesions, adenomas and carcinomas. The preneoplastic lesions included both hyperplastic and dysplastic cells. Dysplasia is a known hallmark of malignant potential and is closely related to APC mutations (Jen et al., 1994). Moderate to severe dysplasia has previously been shown in flat ACF from both traditional (C57BL/6) and AOM Min/+ mice (Paulsen and Alexander, 2001; Paulsen et al., 2006; Sødring et al., 2016a). In addition, flat ACF have been shown to be reliable surface biomarkers of Apc-driven colorectal carcinogenesis (Sødring et al., 2016a).

The initiating and promoting effect observed by intestinal scoring was not evident from the histopathological examination of mice only exposed to POPs (Experiment 1). However, carcinomas were observed in both the colon and small intestine, which could be an indication of the promotional effect explained above. In mice from Experiment 2, AOM and the high mixture concentration increased all types of lesion, which further confirms the synergistic effect observed between AOM and POPs. Interestingly, one injection of AOM alone did not cause the formation of colonic lesions. Instead, it induced the formation of preneoplastic lesions and adenomas, suggesting that AOM did not act as a tumor promoter in the AOM Min/+ mice after only one injection.

Because of the difference in method of analysis between intestinal scoring and histopathology, identical results could not be expected. However, similar trends were observed and both methods showed the same synergistic effect between AOM and POPs. It is important to emphasize that the histopathological examination was conducted on only three slides from each intestine. Thus, this method only investigated a small part of the area in question, compared to the scoring of the whole surface of the intestine, which could explain the discrepancy in findings between the two different methods.

5. Conclusion

The present study indicates that a mixture of POPs designed on the basis of human exposure, together with an injection of AOM, increased intestinal tumorigenesis in AOM Min/+ mice. Furthermore, a synergistic effect was observed between POP exposure and one injection of AOM. The results emphasize the importance of anticipating synergies when assessing the carcinogenic potential of compound mixtures.

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