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Research Article

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Gestational and Early Postnatal Exposure to a Mixture of Organophosphate Ester Flame Retardants Found in Canadian House Dust on Hindlimb Skeletal Development in Postnatal Day 4 Rats

Abstract

Background: Ever since organophosphate esters (OPEs) became the mainstream replacement for organobromine compounds in fire retardants (FRs), numerous studies have explored their potential as endocrine disruptors and developmental toxicants. The purpose of this study is to investigate the impact of gestational and early postnatal exposure of OPE mixtures on the ossification of hindlimb phalangeal in postnatal day 4 (PND4) rat pups, as the amount of OPEs within the diet mixture is relative to its composition in Canadian household dust.

Methods: Male and female adult Charles River Sprague-Dawley rats were exposed to OPE mixtures for 70 and 21 days, respectively. The OPE doses were determined to be 10x, 1,000x, and 30,000x the relative human exposure. The progenies were exposed to OPEs both gestationally (~21 days) and lactationally (4 days). At least 2 of each sex from each litter were sacrificed and processed at PND4 for skeletal staining using Alizarin red and Alcian blue. The samples were analyzed and compared against a reference sample to examine any abnormalities in ossification.

Results: At PND4, there is no significant effect of OPEs on the number of pups with abnormal ossification between the control and treatment groups. High doses of OPEs, at concentrations 30,000x of relative human exposures, showed a significant increase in the severity of delay of ossification at the middle phalanx of PND4 pups.

Limitations: Due to the limitation of small sample sizes (litter n=6-7) and a wide variance in data, there is no clear evidence on whether OPE exposure induces greater incidences of abnormal ossification in the digits of PND4 pups.

Conclusion: There is a delay in ossification from OPE exposure at the high dose (30,000x).

Introduction

Flame retardants (FRs) are ubiquitous within the environment; they are present in electronics, various textiles, and surface coatings of flammable household items, such as synthetic Christmas trees. A common type of organobromine compound is polybrominated diphenyl ether (PBDE) and these so-called brominated fire retardants (BFRs) dominated the market in the 1990s. However, BFRs were banned in both the European Union and United States in the early 2000s due to its proven threat to public health. Researchers have shown that BFRs induce liver toxicity and thyroid hormone dysfunction in rat models. (1) Furthermore, gestational studies have also found that exposure to BFRs in doses that approximate high human exposures (0.06 mg/kg/day) cause first-generation offspring (F1) phenotypes of fused digits and delayed ossification in the sternebrae in rats. (2) After BFRs were removed from the market, organophosphate esters (OPEs) have become the mainstream replacement chemical in fire retardants. OPEs are a class of organophosphorus chemicals that are added to flame retardants, which work by creating a charred phosphoric acid surface when encountered with a flame. The charred surface will then act like a solid shield between the fire and material, which protects the unburned portion form the fire. Since OPE flame retardants are additive flame retardants, the molecule is not chemically bonded to the polymer, which is why OPEs can be discharge to the environment, such as in house dust. (3)

However, are OPEs a responsible replacement for BFRs? Since then, many studies have investigated the effect of these chemicals on developmental toxicology in rat models. OPEs are reported to induce oxidative stress on mouse Leydig tumor cells *in vitro*, where all 7 OPEs tested (10 μM) significantly increased superoxide production. The same effect was not observed with BDEs, which is present in a majority of BFRs. (4) Previous studies used a murine limb culture model to investigate the effects on key transcription factors (TFs) in endochondral ossification, such as *Sox9*, *Runx2*, and *Sp7*. (5) These results concluded that when compared to the legacy chemical 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), exposure to triphenyl phosphate (TPhP), a common OPE, caused a significant decrease of TF mRNA expression, which can alter the formation of digits. Markers for endochondral ossification were investigated using fluorescent photography in murine limb bud culture, including COL10A1-mCherry and COL1A1-YFP, which are hypertrophic chondrocyte and osteoblast markers, respectively. When the limb buds were exposed to 3 μM of TPhP, expression of both markers were greatly suppressed and the limb buds showed abnormal ossification. (5) These data suggest that OPEs cause a delay during the transition from cartilage to bone in endochondral ossification during fetal development.

This study focuses on the gestational and early lactational effect of OPEs on ossification of the hindlimbs of F1 pups. It is predicted that, as the concentration of OPEs exposure increases, there will be a greater delay in bone formation in endochondral ossification in the digits. The amount of OPEs of each treatment diet is designed to be relative to the concentration

and chemical composition humans are expected to be exposed to in Canadian house dust in mg/kg/day (Table 3). The three treatment groups are 30x, 1,000x, and 30,000x of the relative human exposure concentration. Pups are sacrificed at postnatal day 4 (PND4) for skeletal staining, as delay of ossification is evident at the middle phalanx in the hind paw during this stage. The first sign of ossification at the middle phalanx occurs at gestational day (GD) 19+, where birth of a rat fetus normally occurs on GD 20/21. Between PND0 and PND7, the middle phalanx presents a clearly defined wide band of ossification. (6) Thus, by PND4, any effect from OPEs on bone development will be evident with abnormal ossification of the middle phalanx. During endochondral ossification, mesenchymal cells are first committed to becoming cartilage cells and then condensed into compact nodules before differentiating into chondrocytes. These chondrocytes then rapidly proliferate and become hypertrophic chondrocytes, which concludes the final stages of chondrocyte differentiation before cartilage is replaced by bone. Endocardial ossification occurs in the vertebral column, the pelvis, and the limbs. (7) The combination of Alcian blue and Alizarin red dyes are optimal for staining endochondral ossifications, as they stain for cartilage and bone, respectively. Alcian blue is a cationic dye that binds to the high concentration of glycosaminoglycans (GAGs) and glycoproteins within the cartilage, while Alizarin red acts as an anionic dye that binds calcium in bones. (8) With the use of these two dyes, the stages of endochondral ossification can be clearly distinguished.

The principle results show a significant increase in the incidences of delayed digit formation in pups from the highest dose treatment group (30,000x). This abnormality in digits is more severe as the dose increases across the treatment groups. In addition, the delay of skeletal ossification is rather global. These results imply that OPEs might not be a responsible replacement for PBDE and the search for finding a better market replacement continues.

Materials and Methods

Animals and treatment

Adult Charles River Sprague-Dawley rats (St. Constant, Quebec, Canada) were separated into four groups and each animal was acclimated to either a control, low, medium, or high-dose OPE mixture treatment diet (0x, 30x, 1,000x, and 30,000x the approximate daily exposure to OPEs in Canadian house dust in mg/kg/day). The dose was approximated relative to body mass and food consumption (Table 1). Since the chemical mixture has never been tested on animals before, a broad range of doses were chosen, with the lowest dose being relative to human exposure and the highest dose being that where toxicity is expected. 30x the human exposure level is still relevant, as it is calculated relative to human exposure via dust and there are many other avenues through which humans can be exposed to OPEs.

Treatment group	Low-dose (30x)	Medium-dose (1,000x)	High-dose (30,000x)
OPEs diet mixture dose/kg of bodyweight/day	48.06 µg	1,602 µg	48,060 µg

Table 1. Dose approximation relative to body mass and food consumption.

Female rats were treated with the diet mixture for 30 days, which covers four days of the menstrual cycle, and 21 days of gestation and lactational exposure. Male rats were treated for 70 days to cover an entire cycle of spermatogenesis and epididymal transit. A male and female rat of the same treatment group were then mated when the female was in proestrus. If the female rats were confirmed to be pregnant by the presence of a vaginal plug the next day, this would be considered as gestation day 0 (GD0) and the birth of F1 pups was marked as postnatal day 0 (PD0). Two pups of each sex were sacrificed at PND4 and fixed in 95% ethanol (EtOH) until further processing.

Skeletal processing and staining

The following protocol was adapted from previous guidelines used in the Barbara Hales lab (unpublished) and the methods have been described by Rigueur and Lyons. (8) The hindlimb sections were submerged in 70°C water using forceps for five minutes to soften the tissue. The skin was incised vertically, proximally to distally, using micro-scissors along the lumbar vertebrae first (Fig. 2A) and was peeled towards the tail. Two cuts were then made from the tarsus to the phalanges on both sides of the feet (Fig. 2B, 2C). This skin was also peeled off, but the skin casing on the tip of the digits was spared. Another two additional incisions were made on the caudal and ventral side of the tail, and approximately 0.25 mm of skin casing was left at the distal end to prevent damage. Any residual organs, fat, skin, and muscle were carefully removed. The muscles were perforated using a 30 1/2 gauge needle to create channels and the skin casing on the digits was loosened with the needle tip. Then the skeleton was fixed in 95% EtOH for at least 12-24 hours before staining.

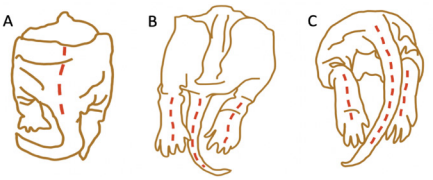

Figure 1. Sites of skin incision for skeletal preparation on the hindlegs of PND4 pup. (A) Dorsal view. (B) Ventral view. (C) Posterior view.

The staining solution contained 1.5 mL of Alcian blue, 1 mL of Alizarin red, 1 mL of glacial acetic acid, and 16.5 mL of 70% EtOH for a 20 mL vial. The skeletons were incubated at 37°C for 32 hours and washed with distilled water three times to remove the leftover dye. The stained skeletons were then left in 0.5% potassium hydroxide (KOH) for approximately five days at room temperature or until the soft tissues were mostly clear.

After the soft tissues were cleared, the skeletons were then washed three times with distilled water and placed in 2:1:1 solution (Table 2) overnight for the final stage of the clearing. For long-term storage, the skeletons were store in 1:1 storage solution (Table 2) until analysis.

1:1 solution	2:1:1 solution
1 part 70 % EtOH 1 part glycine	2 parts 70 % EtOH 1 part glycine 1 part benzyl alcohol

Table 2. Ratio of components in 1:1 and 2:1:1 solution.

Skeletal examination

Each pup was examined by comparing the ossification levels of all parts of the hindlimbs against reference samples (Fig. 2). The different ossification levels were categorized as normal, faint, or no ossification (Fig. 3A, 3B, and 3C respectively). All examinations of the pups were done by observation in the vial. In some cases, if the skeleton disintegrated during the washing or processing steps, it was poured out onto an agarose gel to be sorted and examined instead.

Data analysis

Proportions calculated relative to treatment groups (n=fetus) were analyzed using a 1-tail Fisher's exact test. Proportions calculated relative to litter size (n=litter) or paw number (n=paws) were assessed using a 1-tailed Mann-Whitney U test in GraphPad Prism 8.

Results

Middle phalanx ossification morphology

Representative samples on the degree of ossification at the middle phalanx were photographed as references. Alizarin red and Alcian blue stains for bone and cartilage, respectively. Blue-tinted areas on the paw represent cartilage and dark purple areas represent stained bones, which are considered to be positive stains by Alizarin red. An adequately developed middle phalanx at PND4 presented a thick band of ossified center that was stained positive by Alizarin red (Fig. 2A). In contrast, the ossification center at the middle phalanx can also be entirely absent (Fig. 2B), or faintly ossified (Fig. 2C).

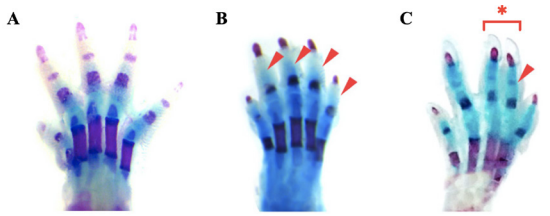

Figure 2. Effects of gestational and early postnatal exposure to OPE mixtures on the hind paw phalangeal ossification and formation. (A) Sample of normal ossification of the PND4 pup hind paw. (B) No ossification of the middle phalanx at digits 2,3,4, and 5. (C) A case of faint ossification of the middle phalanx at digits 2,3, and 4, as indicated by the *, with no ossification at digit 5.

Exposure to OPE mixtures on hind paw phalangeal ossification and formation

To determine the effect of gestational and early postnatal exposure to OPE mixtures on hind paw ossification, the proportion of affected pups with delayed ossification was calculated relative to the litter size (n=litter) or to the treatment group (n=pups). Affected pups included both individuals with either faint or no ossification of the middle phalanx in any digit. It is important to normalize proportions of abnormalities within litters, as the dams rather than the pups were acclimated to different concentrations of OPE diet mixtures.

Once the proportions were calculated relative to litter size (Fig. 3A), all treatment groups showed a large variance in the data, including the controls. All groups, except the low-dose group, had at least one litter with an abnormal ossification proportion of one, and all groups had at least one litter with an abnormal ossification proportion of zero. The data did not indicate any significant differences between the three treatment groups and the controls. Similarly, with the proportion calculated relative to the pups (Fig. 3B), the bars represent the proportion of pups that have delayed ossification of the middle phalanx in each group, and again, the data did not support a significant difference between the three treatment groups and the controls.

Effect of exposure to OPE mixtures on the ossification of the second and fifth digits in the hind-paws

In rats, the pattern of ossification of the phalanx follows the pattern of development in the metacarpals, which is in the order of 3,4,2,5. (6) Thus, reduced ossification at the middle phalanx of the second and fifth digits is a strong indication of delayed bone development.

The proportion of affected pups with delayed ossification was calculated as the number of individuals with no ossification in digits 2 and 5 relative to litter or pups. When the proportion was calculated relative to litter, the controls lost most of its variance in data compared to Fig. 3A. Only the medium and high-dose groups had litters with a proportion above 0.4, whereas in Fig. 3A, three out of four groups had a data range from 0 to 1. When the proportion was calculated relative to the number of pups (Fig. 3D), there was no significant difference between the low, medium, and high-dose groups when compared to the controls.

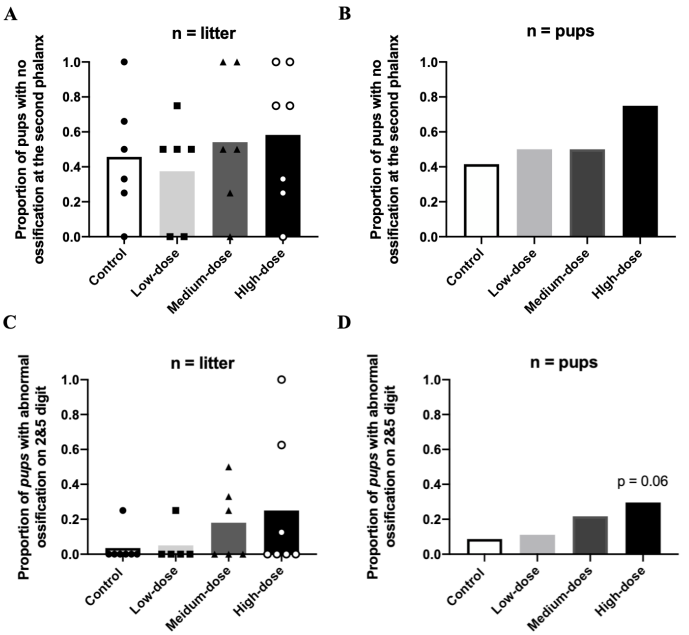

Figure 3. Proportions of pups with abnormal ossification per litter or per treatment group. (A) Proportion of pups showing abnormal ossification at the middle phalanx relative to the total number of pups in a litter (n=7,6,6, and 7 for control, low, medium, and high-doses, respectively). The bars represent the mean value. (B) Proportion of pups showing abnormal ossification at the middle phalanx relative to the total number of pups examined per treatment group (n=23,18,23, and 27 for control, low, medium, and high-doses, respectively). (C) Proportion of pups showing no ossification at the middle phalanx in both the second and fifth digit relative to the total number of pups examined in its litter (n=7,6,6, and 7 for control, low, medium, and high-doses, respectively). Bars represent the mean value of proportions. (D) Proportion of pups with no ossification of the middle phalanx in both the second and fifth digit is calculated relative to the total number of pups examined per treatment group (n=23,18,23, and 27 for control, low, medium, and high-doses, respectively).

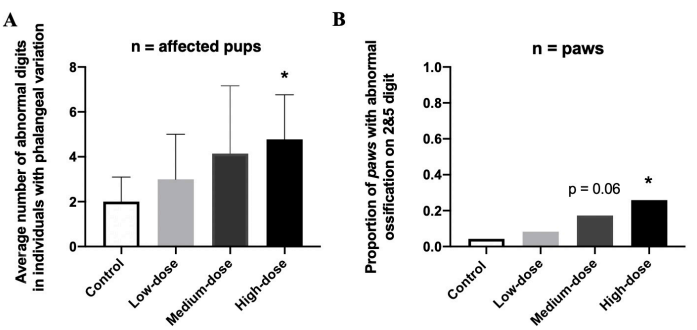

Figure 4. Number of abnormal digits per affected individual and proportion of affected paws with abnormal ossification on digits 2 and 5 in different treatment groups. (A) Average number of digits with abnormal ossification in the middle phalanx in affected PND4 pups. The error bars represent standard deviation (n=6,3,7, and 9 for control, low, medium, and high-doses, respectively). The * indicates $p<0.05$ when compared to the controls. (B) Proportion of paws showing no ossification at the middle phalanx in both the second and fifth digits. The bars represent the proportion of paws affected relative to the total number of paws examined (n=46,36,46, and 54 for controls, low, medium, and high-doses respectively). No error bars are present, as there is only one data point for proportion per group. The * indicates $p=0.009$, $p<0.05$ when compared with the controls.

High-dose treatment group causes severer delay in endochondral ossification

Within the affected individuals, the number of digits with no ossification at the middle phalanx was varied. The average number of digits affected per pup in the subgroup of affected pups was calculated to determine the dose-dependent effect of OPEs on the middle phalanx (Fig. 4A). The average number of affected digits increased as the dose of OPE mixture increased. Despite the large error bars, a significant increase was observed in the high-dose group when compared against the controls ($p=0.0114$, $p<0.05$, Mann-Whitney test). Although there is an upward trend of severity as the dose increases, the dose-dependent relationship was not significant in a 1-way ANOVA test (Fig. 4).

The proportion of pups with no ossification at the middle phalanx of digits 2 and 5 was calculated again, this time relative to the number of paws (Fig. 4B). There was a significant increase in the high-dose treatment group when compared against the controls ($p=0.009$). Although both the low and medium-dose groups had a higher proportion of affected pups than the controls, the differences were not significant. The proportions were also calculated within both sexes, however, no statistical significance was found.

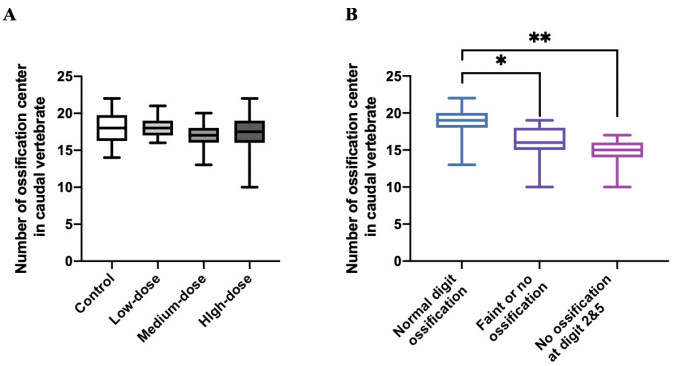

Figure 6. Correlation between middle phalanx ossification and caudal vertebrate length (* $p<0.0001$. ** $p<0.0001$). (A) Box and whisker graph of caudal vertebrae length in the control and the three treatment groups. The error bars are maximum to minimum (n=23,18,23, and 27, respectively). (B) Box and whisker graph of three different subgroups: normal digit ossification, faint or no ossification, and no ossification at digits 2 and 5. Medians are 19, 16, and 15, respectively. The error bars are maximum to minimum (n=42, 44, 15, respectively).

Abnormal ossification at the middle phalanx correlates with lower caudal vertebrate length

Other than quantifying the delay in ossification at the middle phalanx in the digits, the number of ossified centers in the caudal vertebrae was also measured to examine the over-all extent of ossification in the PND4 pups. A sample of long caudal vertebrate has around 21 ossification centers (Fig. 5A); in contrast, an abnormally short caudal vertebrae has only 14 ossification centers at PND4 (Fig. 5B). A normal tail length at PND4 has around 20 ossification centers according to past experimental results (unpublished). A single case of abnormal caudal vertebrate ossification was also observed from the high-dose group (Fig. 5C).

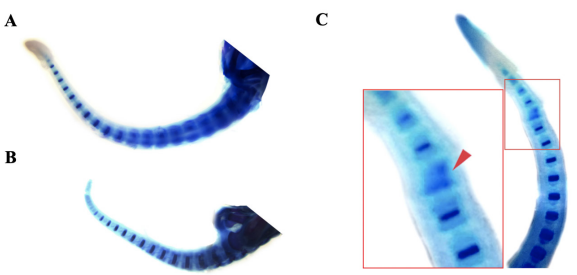

Figure 5. Images of stained caudal vertebrates comparing length and the only case of kinked tail. (A) Normal length tail with around 20 caudal vertebrae. (B) An unusually short tail of around 14 vertebrae; part of the caudal vertebrae above the pubic bone is not shown. (C) Kinked tail and abnormal ossification of the caudal vertebrae.

To investigate the relationship between caudal vertebrae length and the effect of exposure to OPE mixtures, the length was compared across the treatment groups and also between individuals with either normal or abnormally ossified digits. There was no significant difference in the average length of the caudal vertebrae between the three treatment groups and control (Fig. 6A). However, the average caudal vertebrae length in the two subgroups of individuals with affected digits was significantly lower when compared against those with normal digit ossification (*$p<0.0001$, **$p<0.0001$, Fig. 8B). No other abnormalities were observed other than in the digits and caudal vertebrae.

Log transformation of data and Pearson's correlation coefficient analysis

Log_{10} transformation was applied to the x-axis of Fig. 3B, 3C, and 4B to extract a linear correlation between the two variables: proportion and dose. Although the Pearson's R for Fig. 3D and 4B were above 0.83, the p-values were both >0.1, which is not statistically significant as p must be <0.05. Low statistical significance might be due to small sample size. Other

Discussion

In normal hind paw ossification of rats, the middle phalanx's primary ossification center appears by GD19+ in all digits except the thumb (digit 1), which lacks a middle phalanx. (6) Any middle phalanx that has less ossification than the reference sample (Fig. 2A) is considered to have delays in bone development. With these criteria, the proportion calculated in Fig. 3A and Fig. 3B for individuals with delayed ossification included pups with either faint or no ossification of the middle phalanx.

However, the data showed a large variance in all groups (Fig. 3A). This could be caused by the broad definition of "delayed ossification" that was used when calculating the proportion. Pups with faint ossification could be categorized as displaying "normal ossification" since there are natural differences in the speed of normal digit development. Large variances in data could mask out potential statistical significances. With the vast range of proportions in the controls, there was no consistent baseline data that can be used to compare with other treatment groups. To reduce the variance in data, the criteria for "delayed ossification" was refined to include only the individuals with no ossification at the middle phalanx in any digit. However, similarly to Fig. 3A and Fig. 3B, there are variances in the data with no significant difference between the groups (graph and calculation not shown). The criteria was further refined to include only the individuals with no ossification of digits 2 and 5. Since the order of rat digit development is 3,4,2, and 5, any lack of ossification at the last two digits by PND4 is a strong indication of a severe delay in the development of bones.

When the proportions of individuals with no ossification at digits 2 and 5 were calculated relative to the litter (Fig. 3C), the data was less "noisy" than Fig. 3A. All treatment groups had reduced variance, especially in the controls. A clear distinction was observed between the data points of the control and high-dose group, despite the difference not being statistically significant. When calculating the proportions relative to the pups (Fig. 3D), there was no statistical significance between the treatment groups and the controls.

The number of pups examined per treatment group were 23, 18, 23, and 27 for the control, low, medium, and high-dose groups respectively. Due to the small litter size of F0 dams and the limited time for this project, only 6 litters per treatment group were examined. To increase the sample size for data analysis, the proportion of digits 2 and 5 was calculated per paw (Fig. 4B), which increased the sample size to 46, 36, 46, and 54, respectively. By normalizing the proportion relative to the number of paws, it accounted for the severity of the effect of OPEs, which was proven to be significant in Fig. 4A. In conclusion, the number of pups that have delayed ossification in each treatment group is similar, yet abnormal ossification was present in the high-dose group.

The caudal vertebrae length is correlated with the extent of abnormal ossification at the digits (Fig. 6B). Pups with abnormal digit ossification in the middle phalanx were more likely to have a shorter tail than pups with normal ossification. This data suggests that the delay in ossification of the pups is a global effect rather than location specific. A single case of kinked tail with abnormal caudal vertebrate ossification was observed in the high-dose group (Fig. 5C). At the distal end of the caudal vertebrae, the third last caudal vertebra has an abnormal pattern of ossification: it lacks a single thick band of primary ossification center. The staining pattern is either due to the dispersion of Alizarin red or a dark stain of Alcian blue. The kink in the tail could also be an indication of neural tubal defects during fetal development, but further analysis is required to determine the exact cause of this special case of malformation. Previous studies found that OPEs such as TPhP, t-butylphenyl diphenyl phosphate (BPDP), isopropylated triphenyl phosphate (IPPP), and tricresyl phosphate (TCrP) showed adverse effects, especially on bone ossification. (5, 9) These chemicals constitute approximately 20% of the OPE mixture. Many OPEs found in house dust have not been tested regarding its effect on endochondral ossification and their effects on bone formation are still unknown.

In conclusion, there is no clear effect of exposure to OPE mixtures on hind paw development due to limitations, such as a small sample size. However, a high-dose exposure to OPEs induces a significant delay in the ossification of the middle phalanx in the hind paw. The fact that only the high-dose treatment group was statically significant could either be due to the nature of OPEs, whereby a very high dose is required to affect skeletal development, or variations in the data that masked other statistical significances. Further experiments with a larger sample size could minimize the variances in data to confirm why only the high-dose treatment groups were significant when compared to the control. Since only the hindlimb section was analyzed due to time constraints in this study, fore-limb sections could also be examined for a more extensive and complete analysis.

The ultimate goal of finding a responsible replacement is to replace the toxic legacy chemical with one that is harmless or at least, less toxic. However, the OPEs used in fire retardants that filled the market gap after BFRs were banned are not entirely safe in terms of their effects on endocrine systems. (5) Experimentally, the no-observed-adverse-effect-level (NOAEL) for variations in cervical vertebrae and phalanges of PBDEs is 0.75 mg/kg, and the NOAEL for phalangeal abnormal ossification of OPEs was 1602 mg/kg in this experiment. (1) Since this experiment has a lot of shortcomings regarding small sample size and large variance, it is difficult to fairly compare to BFR's NOAEL, which has been studied more extensively. Based on this study, there is no concrete evidence on whether OPEs are a responsible replacement for PBDEs in fire-retardants, as the data only suggests that OPEs significantly induce abnormal ossification at an extremely high concentration, which may not be particularly relevant to human exposures.

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% in Mixture	Symbol	CAS RN	Name	Supplier	Purity (%)
0.11%	TXP	25155-23-1	Trixylyl phosphate	ACROS	-
0.19%	BPDP	56803-37-3	t-Butylphenyl Diphenyl Phosphate	Scientific Polymer Products	-
0.48%	CDPP	26444-49-5	Cresyl diphenyl phosphate	Alfa Aesar	94
6.74%	IPPP	68937-41-7	Isopropylated Triphenyl Phosphate	NTP, NIEHS, US	97
4.27%	IDDPhP	29761-21-5	Isodecyl diphenyl phosphate	Scientific Polymer Products	-
0.79%	TnBP	126-73-8	Tri n-Butyl phosphate	Sigma	99
7.31%	TCPP	13674-84-5	tris(chloropropyl) phosphate	AKScientific	90
2.48%	TCEP	115-96-8	tri2-chloroethyl	Sigma	97
59.07%	TBOEP	78-51-3	Tributoxyphosphate	Sigma	94
1.18%	EHDPhP	1241-94-7	Ethylhexydiphenylphosphate	AKScientific	95
3.88%	TPhP	115-86-6	Triphenylphosphate	Sigma	99
5.06%	TDiCPP	13674-87-8	Tris(dichloroisopropyl) phosphate	J&K Scientific	95
8.44%	TCrP	1330-78-5	Tricresyl phosphate	Alfa Aesar	-

Table 3. OPEs mixture composition and general information of chemicals.

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