The effects of four organophosphate ester flame retardants and BDE-47 on endochondral ossification in a murine *ex vivo* limb bud culture model

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Introduction:

Flame retardants (FRs) are added to a plethora of consumer products, from couches to computers, to slow fire propagation. In past decades, the most popular FRs in North America have been the brominated flame retardants (BFRs), particularly, polybrominated diphenyl ethers (PBDEs). By the mid-2000s, restrictions on the use of PBDEs were put in place due to their persistence, bioaccumulative properties and toxicity. Following this global phase-out, the use of alternative flame retardants, such as the organophosphate esters (OPEs), has increased [1]. Indeed, the levels of OPEs detected in the environment now often surpass those of PBDEs [2]. However, we know relatively little about the safety of these replacement chemicals.

Endochondral ossification is the process by which most bones in the vertebrate skeleton are formed. A complex signalling network drives its normal progression from the initial cartilage anlagen formation (chondrogenesis) through to bone matrix deposition and mineralization (osteogenesis). An external insult, such as a chemical exposure, may cause dysregulation, resulting in adverse effects on bone quality during skeletal development. PBDE exposures have been associated with the suppression of osteogenesis. Previously, our lab showed that *in utero* exposure to an environmentally relevant PBDE mixture increased the incidence of ossification anomalies in gestational day 20 Sprague-Dawley rat fetuses and postnatal day 4 pups [3-4]. Little is known about whether OPE exposures affect bone formation. The goal of this study was to test the hypothesis that various OPEs have an impact on endochondral ossification in the developing limb and to compare their potential effects to those of a predominant PBDE congener, brominated diphenyl ether-47 (BDE-47).

Materials and methods:

The CD1 transgenic reporter mice used for this study express fluorescently tagged markers of the three major stages of endochondral ossification: Collagen type 2A1-enhanced cyan fluorescent protein (COL2A1-ECFP, chondrogenesis), COL10A1-mCherry (early osteogenesis), and COL1A1-yellow fluorescent protein (COL1A1-YFP, late osteogenesis). Limb buds were collected from gestational day 13 embryos and cultured, as previously described [5]. The treatments were: vehicle (dimethyl sulfoxide); brominated diphenyl ether-47 (BDE-47), at 10 or 50 μM; triphenyl phosphate (TPHP), tricresyl phosphate (TMPP), isopropylated triphenyl phosphate (IPPP), or tert-butylphenyl diphenyl phosphate (BPDP), at 1, 3 or 10 μM. Limbs (n = 8-10 culture bottles, 6–8 limbs/bottle) were cultured for 6 days with a medium change on day 3. On days 1, 3 and 6 of culture, photos were taken using a Leica M165 Fluorescent Stereo Microscope. The extent of cartilage anlagen formation was quantified using a morphological scoring system [5]. Scores were analyzed using Mann-Whitney U tests.

Results and discussion:

Observation of the expression of the fluorescent markers of chondrogenesis and endochondral ossification in limb buds revealed that BDE-47 had no notable effect at the concentrations tested. In contrast, the expression of early and late osteogenesis markers, COL10A1-mCherry and COL1A1-YFP, respectively, was reduced by all four OPEs at 3 μM, the second lowest concentration tested. The expression of these markers was largely abolished in the 10 μM treatment groups. These data demonstrate that exposure to these OPEs impedes the progression of endochondral ossification during skeletal development. Interestingly, low concentrations of TPHP or BPDP induced premature

COL10A1-mCherry expression in the digits, in the absence of any accompanying effects on COL1A1-YFP expression; the meaning of this unexpected observation remains to be determined.

Morphological scoring revealed that treatment with 50 μM BDE-47 produced limbs that developed on average three fewer phalangeal cartilage condensations across all digits, compared to controls. Thus, BDE-47 has a significant impact on cartilage anlagen formation in the limb. In contrast, concentrations as low as 1 μM of all four OPEs had significant effects on endochondral ossification. TPHP had the steepest concentration-response curve, producing the smallest effect of all four OPEs at 1 μM and the largest at 10 μM. At 1 μM, BPDP produced the largest effect of all four OPEs.

In summary, all four OPE flame retardants tested (TPHP, TMPP, IPPP, and BPDP) had detrimental effects on endochondral ossification in limb bud cultures. Moreover, the impact of these OPEs on limb bud development was greater than that of BDE-47. Thus, in the context of bone health, at least some OPEs may be more toxic than their brominated predecessors and warrant further study.

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