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# Effects of novel brominated flame retardants on steroidogenesis in primary porcine testicular cells

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## HIGHLIGHTS

- Primary cells were used to investigate the effects of NBFRs on steroidogenesis.
- TBB exposure upregulated the pathway for aldosterone and cortisol biosysthesis.
- TBCO and TBPH exposure produced greater amounts of sex hormones.
- The study revealed subtle differences in the molecular targets of TBCO and TBPH.

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## ABSTRACT

Brominated flame retardants are chemicals with fire quenching properties which are extensively used in manufacturing. Historically, less regulated use of legacy brominated flame retardants (BFRs) for a number of years has resulted in ubiquitous contamination of the environment. As a result, some of the more persistent BFRs have been phased out and are being replaced by a next generation of brominated compounds for which there is little toxicological data. The study investigated effects of 2-ethylhexyl tetrabromobenzoate (TBB), 1,2,5,6-tetrabromocyclooctane (TBCO), and bis-(2-ethylhexyl) tetrabromophthalate (TBPH) on steroidogenesis in a porcine primary testicular cell model. TBB did not affect sex-steroid production in this cell model; rather the data suggest a flux towards synthesis of aldosterone and cortisol via up-regulation of *CYP21A2*. At the greatest concentrations of TBCO and TBPH tested greater production of sex hormones testosterone (T) and estradiol (E2) was observed. Effects were mediated by regulation of multiple molecular targets in the steroidogenesis pathway; *CYP11A* in the case of TBPH and *CYP17A1* in the case of TBCO. This investigation is the first of its kind to use a testicular mixed population cell model to investigate mechanism(s) of action of three chemically diverse compounds currently used in commercial fire retardants.



## 1. Introduction

Brominated flame retardants (BFRs) are chemicals added to commercial and end-user consumer products to improve their fire resistance. They are characterized by the presence of multiple bromine atoms covalently linked to carbon. Presence of the electronegative bromine atoms aid in resisting fire by quenching free radicals generated during combustion of organic chemicals, thereby mitigating propagation. BFRs are extensively used in fire retardant mixtures put into plastics, textiles, furniture, electrical components as well as in various industrial applications. Since these additives are not covalently linked to the matrix, they have the propensity to leach out of products. Legacy BFRs such as polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) have been produced in large quantities for four decades, and as a result are ubiquitous in the environment (de Wit, 2002; Harrad et al., 2009; Law et al., 2006; Sellström et al., 1998) biota (de Wit et al., 2006; Lam et al., 2009; Law et al., 2006; McDonald, 2002; Thomsen et al., 2003; Vorkamp et al., 2012; Weiss et al., 2004). The penta-, octa- and deca-versions of PBDEs, which were primarily used in early commercial fire-retardant mixtures have the tendency to bioaccumulate and biomagnify (Birnbaum and Staskal, 2004; Burreau et al., 2004; Law et al., 2006). In addition to being detected in urban industrial areas, these chemicals are of concern as they have been detected in polar regions, which suggest that they have a potential for long-range atmospheric transport

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(de Wit et al., 2006). In the 2009 Stockholm Convention penta- and octa-BDEs were listed as persistent, organic pollutants and have since been phased-out of production. Deca-BDE is slated to be phased out by the end of 2013.

As there are still statutory requirements for fire resistance in products such as textiles and electronics, and brominated organic compounds are the most efficient and cost-effective ways to impart such flame retardation properties, historically used BFRs are being replaced with brominated compounds which maintain the fire retarding properties of the technical mixtures (Covaci et al., 2011). 1,2,5,6-tetrabromocyclooctane (TBCO) is a component of Saytex BC-48 produced by Albermarle Corporation (Gauthier et al., 2009). 2-Ethylhexyl tetrabromobenzoate (TBB) and bis-(2-ethylhexyl) tetrabromophthalate (TBPH) are components of Firemaster® (Stapleton et al., 2008) and BZ-54 is synthesized by Chemtura corporation (Davis and Stapleton, 2009). This next generation of fire retarding chemicals is often synthesized by brominating backbones of chemicals used in other industrial processes. TBB is a brominated version of 2-ethylhexyl benzoate, a non-phthalate plasticizer used in paint mixtures and cosmetic products and TBPH is the brominated analog of diethylhexylphthalate, a commercially used plasticizer. While it is tempting to suggest that the brominated analogs mimic their non-brominated versions with respect to their physicochemical profiles, such extrapolations are not warranted without empirical evidence. Bromination alters physical and chemical properties of parent compounds. These new compounds require independent studies investigating their presence, distribution, degradation, absorption, accumulation, toxicities, and critical mechanism(s) of action.

There is currently, little, publically available information on these newer chemicals. TBPH was detected in fatty tissue of porpoises and dolphins in South China Sea (Lam et al., 2009) and demonstrated a tendency to bio accumulate in fathead minnows (Bearr et al., 2010). TBB and TBPH have been shown to cause damage to DNA in hepatic tissue of fish (Bearr et al., 2010) and have been detected in air samples from the North American Great Lakes region at 10-fold greater concentrations in urban areas compared to remote areas (Ma et al., 2012). In humans, dust was the major route of exposure to BFRs (Lorber, 2008; Wilford et al., 2005) and though studies have reported the presence of TBB, TBCO and TBPH in house dust (Dodson et al., 2012; Stapleton et al., 2008), the bioavailability of these chemicals and implications for health of humans due to these exposures are unclear. Attempts at identifying the metabolic products of TBB and TBPH have been made using an *in vitro* model (Roberts et al., 2012), although toxicities of the metabolites are not known. A correlation between concentrations of TBPH in house dust and concentrations of triiodothyronine (T3) in blood plasma of humans has been reported (Johnson et al., 2013). Recent investigations using the components of FM 550 have demonstrated endocrine disruptive effects caused by TBB and TBPH in rat tissue (Patisaul et al., 2013), whereas *in vitro* studies with TBB, TBCO, and TBPH have demonstrated ER- and AR-mediated antagonistic effects and elevated steroidogenesis of sex hormones (Saunders et al., 2013). Based on these preliminary reports, we investigated the effects of TBB, TBCO, and TBPH on steroidogenesis in a primary neo-natal porcine testicular cell model and assessed the molecular effects of the NBFRs on key targets in the steroid biosynthesis pathway.

## 2. Materials and methods

### 2.1. Isolation and propagation of porcine testicular cells

Testes were collected from neonatal piglets (1to2 weeks of age) and placed in Dulbecco Phosphate buffer solution (Lonza Walkersville Inc., Walkersville, MD) containing 10 μg/ml penicillin and 100 U/ml streptomycin. Isolation, purification, and culturing were done as previously described (Bernier et al., 1983; Lejeune et al., 1998). Briefly, the testes were decapsulated, minced, and digested with 0.5 mg/ml of collagenase solution. Tissue was collected following 90 min digestion and filtered through 0.4 μm nylon filter. The cell suspension was centrifuged, and the cell pellet was washed in DMEM medium. Resuspended cells were layered on a percoll® gradient (21, 26, 34, and 60%) and centrifuged at 800 × *g* for 20 min. The heterogeneous population of cells containing Leydig, Sertoli, and interstitial cells was isolated from the 34% layer of the percoll gradient and resuspended in DMEM -F12 medium (1:1) containing antibiotics, 10% Nuserum, and ITS premix. Viable cells were enumerated by use of the trypan blue exclusion method. Cytochemical staining for 3β-HSD activity was carried out by exposing the cells to a solution containing 0.2 mg/ml nitrotetrazolium blue 0.12 mg/ml Dehydroepiandrosterone and 1 mg/ml of beta NAD in PBS (0.05 M, pH 7.4) (Sigma Aldrich, St Louis, MO). 60–70% of the isolated cells were confirmed to be Leydig cells as they stained positive for 3β-HSD enzyme activity (Mendelson et al., 1975), S1. $3 \times 10^5$ cells were seeded in each well of a 24-well plate and incubated for 72 h at 34 °C and 5% $CO_2$. Following 72 h of incubation, the medium was replaced with one containing the test chemicals TBB (Wellington Laboratories, ON, Canada), TBPH (Waterstone Technology, IN, United States), and TBCO (Specs, Delft, Netherlands) or DMSO solvent control. The system was cultured under the control of porcine LH (Lutropin-V, Bioniche, ON, Canada) at a concentration of 1 ng/ml (Lejeune et al., 1998).

### 2.2. Viability of cells

Metabolically active cells in the heterogeneous population were determined by use of WST-1 reagent (Roche Applied Science, Indianapolis, IN) according to manufacturer's protocol. Briefly, $3 \times 10^4$ cells were exposed to the test chemicals for 48 h. The percent viable cells following exposure to chemicals was estimated by comparing absorbance at 440 (A440) of cells exposed to NBFRs with that of the solvent control.

### 2.3. Quantification of hormones

Conditioned medium was collected after exposure of the heterogeneous cells to the test chemicals TBB, TBCO, and TBPH for 48 h. Hormones (T and E2) were extracted by organic solvent liquid–liquid extraction as described (Chang et al., 2010). In brief, 300 μl of media was extracted using 2.5 ml of hexane and Ethylacetate (1:1). The organic fraction was collected following centrifugation at 400 g for 5 min. Following a second extraction, organic fractions were pooled and dried under a stream of nitrogen. Dried organic content was reconstituted with 150 μl of appropriate buffer (supplied with the ELISA kit). T and E2 quantification was done by the use of an ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's recommendations.

### 2.4. Expression of genes

Primary cells exposed to test chemicals were harvested simultaneously with conditioned media. RNA was isolated by use of Trizol (Life Technologies Corporation, Carlsbad, CA) and quantified by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Welmington, DE, USA). cDNA was synthesized from 2.5 μg of each RNA sample by the use of a QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON) according to the manufacturer's recommendations. Relative quantification of transcript abundance was carried out using SYBR green by the ΔΔCt method. Expression of target genes was reported relative to the expression of housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). The thermal cycle profile was: denaturization for 15 s at 95 °C followed by annealing and extension for 1 min at 60 °C for a total of 40 PCR cycles. In each case, product homogeneity was confirmed by a melt curve analysis. The sequence of primers used for the amplification of target genes has been published previously (Lervik et al., 2011).

### 2.5. Statistical analyses

Statistical analyses were done by the use of IBM SPSS Statistics 20. For each of the data sets, Shapiro–Wilk's test was used to test for normality of data (significance > 0.05), and Levene's test was applied to ensure homogeneity of variances (significance > 0.05). Differences between treatment conditions and the solvent control were evaluated by a one-way ANOVA followed by a Dunnet's post hoc test. *P*-values less than 0.05 were considered significant.

## 3. Results

### 3.1. Determination of toxicity of NBFRs

Exposure of neo-natal porcine testicular cells to the three NBFRs revealed the highest testable concentration that did not cause cellular toxicity (Fig. 1). TBB was not cytotoxic to primary cells at concentrations from $5 \times 10^{-1}$ to $5 \times 10^{-5}$ mg TBB/L. TBPH caused 15% lesser viability relative to unexposed cells ($P = 0.0029$) at only