Toxicology Reports 5 (2018) 737–743

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

# Complex toxicity as disruption of adipocyte or osteoblast differentiation in human mesenchymal stem cells under the mixed condition of TBBPA and TCDD

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ARTICLE INFO

*Keywords:*
Tetrabromobisphenol A
2,3,7,8-tetrachlorodibenzo-*p*-dioxin
Human mesenchymal stem cell
Adipocyte differentiation
Osteoblast differentiation

ABSTRACT

People are frequently and unintentionally exposed to many chemical compounds, such as environmental pollutants and endocrine-disrupting chemicals (EDCs), in food and from the atmosphere. In particular, endocrine-disrupting TBBPA and dioxins are found in human breast milk and in the body. Conventional studies evaluate toxicity by administering a single substance to cells or animals, but evaluation of the toxicity of mixtures of these ingested compounds is essential for "true" toxicological assessment. We evaluated toxic effects *in vitro* using human mesenchymal stem cells (hMSCs). TBBPA increased the number of lipid droplets, and upregulated the expression of adipocyte-related mRNA, aP2 and LPL, through a PPARγ-dependent mechanism. TCDD suppressed lipid droplets and adipocyte-related mRNA levels. Adipocyte differentiation was stimulated by TBBPA and inhibited by TCDD in a dose-dependent manner. TBBPA did not influence osteoblast differentiation, but TCDD suppressed ALP staining and activity, calcium deposition, and osteoblast-related mRNA levels. In a mixture of TBBPA and TCDD, TBBPA inhibited TCDD suppression of adipocyte and osteoblast differentiation in a dose-dependent manner. Interestingly, we observed lipid droplets in TBBPA-treated cells differentiated into osteoblasts. These results suggest that TBBPA and TCDD disrupted differentiation into adipocytes and osteoblasts and contributes to a more complete toxicological understanding of exposure to these chemical substances.

## 1. Introduction

Society has developed many synthetic chemical compounds to increase our well-being and comfort. However, some of these chemical compounds pollute the environment, contaminate our food, and are absorbed directly into our body, which may affect our health by disrupting physiological functions such as endocrine systems. In particular, there are several known endocrine-disrupting chemicals (EDCs) including dioxins, diethylstilbestrol (DES), bisphenol A (BPA), tetrabromobisphenol A (TBBPA), dichlorodiphenyltrichloroethane (DDT), tributyltin (TBT), perfluorooctanoate (PFOA), phthalateas and polybrominated diphenylethers (PBDEs) [1–8]. We are frequently and unintentionally exposed to EDCs in food and from the atmosphere, which can be revealed through cohort studies. However, cohort studies involve a huge amount of time and expense, a large study population, and should control for a number of factors, such as race and age, which makes them unsuitable for evaluating mixtures of chemical substances. In addition, conventional studies evaluate toxicity by administering a single substance to cells or animals, but evaluation of a mixture of chemical compounds is needed for a more complete understanding or complex toxicological assessment. We evaluated toxic effects *in vitro* using stem cells to examine *in vivo* differentiation.

Human mesenchymal stem cells (hMSCs) are multipotent cells, which can be isolated from bone and adipose tissue [9,10]. MSC plasticity can be used for an *in vitro* model of differentiation because MSCs can differentiate into several tissue-forming cells such as bone, cartilage, fat, muscle, tendon, liver, kidney, heart, and even brain cells *in vitro*. In particular, the method of MSC differentiation into adipocytes and osteoblasts is well known [11–13]. An increase in adipose deposits in bone marrow decreases bone density and increases the risk of obesity [14,15]. Thus, there is close relationship of osteoporosis to obesity, which suggests that disruption of MSC differentiation into adipocytes

*Abbreviations:* ALP, alkaline phosphatase; aP2, adipocyte-specific protein 2; BFRs, brominated flame retardants; C/EBPα, CCAAT-enhancer-binding protein alpha; DOHaD, developmental origins of health and disease; EDCs, endocrine-disrupting chemicals; LPL, lipoprotein lipase; MSC, mesenchymal stem cell; PCDDs/DFs, polychlorinated dibenzo-*p*-dioxins and dibenzofurans; PPARγ, peroxisome proliferator activated receptor gamma; RUNX2, runt-related transcription factor 2; TBBPA, tetrabromobisphenol A; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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https://doi.org/10.1016/j.toxrep.2018.06.007
Received 26 March 2018; Received in revised form 4 May 2018; Accepted 11 June 2018
Available online 13 June 2018
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and osteoblasts by EDCs may exacerbate both osteoporosis and obesity.

Barker et al. proposed the developmental origins of health and disease (DOHaD) hypothesis, which suggests that environmental factors, such as nutrition and exposure to chemicals during pregnancy and postnatal development, influence adolescent and adult health, and the risk of various diseases [16]. Moreover, there is high sensitivity to EDCs at critical developmental points such as the embryonic and neonatal periods [17]. The effect of EDCs seems to be higher in the fetus and in infants than in adults. In addition, we previously observed contamination of EDCs, such as TBBPA and dioxins, in human breast milk and in the body; the average concentration of TBBPA in a Japanese mother's breast milk was 1.9 ng/g lipid, and polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/DFs: 17 congeners) and coplanar polychlorinated biphenyls (Co-PCBs: 14 congeners) were 3.6 and 3.5 pg TEQ/g lipid, respectively [18–21]. The contamination levels in human milk have been widely studied to provide a good indicator of overall human exposure, especially for infants. Thus, the exposure of EDCs such as TBBPA and TCDD in the fetus and infants cause the failure to thrive.

In 2015, 18,000 tons of TBBPA were used in Japan [22], and TBBPA pollutes the environment during manufacture, use, and disposal. TBBPA bioaccumulates in the food chain [23,24], and is considered an EDC because it activates peroxisome proliferator activated receptor (PPAR) γ in mice [25,26]. In addition, TBBPA facilitates adipocyte differentiation of 3T3-L1 cells via PPARγ activation [18]. On the other hand, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a persistent organic pollutant generated from waste incinerators as an undesired by-product. TCDD contaminates the body because of a long half-life and bioaccumulates in the food chain [27]. Exposure of pregnant rats to TCDD results in altered bone geometry, mineral density, and mechanical strength in the offspring [28]. TCDD interferes with osteoblast and osteoclast differentiation in bone marrow stem cells [29], inhibits adipogenesis, and attenuates insulin-induced glucose uptake in 3T3-L1 cells [30].

The unintentional intake of BFRs and dioxins via food and breast milk, and the toxic effects of individual molecules have been studied, but the effects of mixtures of these toxicant have not been previously examined. Here, we investigated the toxic effects of mixtures of TBBPA and TCDD on adipocyte and osteoblast differentiation in hMSCs.

## 2. Material and methods

### 2.1. Chemicals

TBBPA and TCDD were purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Chemicals were dissolved in dimethyl sulfoxide (DMSO) to a final concentration in culture medium of 0.1% v/v. StemBeads fibroblast growth factor 2 (FGF2) was purchased from Stem Culture (Rensselaer, NY). All other reagents were the highest quality commercially available and obtained from Sigma-Aldrich (St. Louis, MO) and Nacalai Tesque (Kyoto, Japan).

### 2.2. Cell cultures

After Institutional Review Board approval, human MSC (MSC-R14) isolated from bone marrow of ilium was provided by RIKEN BRC (Ibaraki, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin and 3 ng/mL StemBeads FGF2 at 37 °C [31]. The two chemicals and DMSO were uesd at a concentration not showing cytotocity determined by a tetrazolium-based colorimetric assay, the WST-8 kit (Nacalai Tesque), according to the manufacturer's protocol (data not shown).

### 2.3. Adipocyte differentiation and oil red O staining

hMSCs were seeded on a 24-well plate. Two days after confluence (designated as Day 0), cells were treated with adipocyte differentiation medium containing 1 µM dexamethasone, 0.5 mM 3-isobuthyl-1-methylxanthine (IBMX), 200 µM indomethacin and 10 µg/mL insulin with and without TBBPA and/or TCDD. After three days (Day 3), the media was replaced and maintained with and without TBBPA and/or TCDD containing 10 µg/mL insulin alone to Day 21.

Differentiated cells were stained with oil red O to detect lipid droplets in adipocytes. After washing twice with phosphate buffered saline (PBS), cells were fixed with 4% paraformaldehyde at room temperature, and then stained with 3.3 mg/mL oil red O in 60% isopropanol for one hour. Cells were washed with PBS, and observed under an IX71 microscope (Olympus, Tokyo, Japan). Stained oil red O was eluted with isopropanol and the absorbance was measured at a wavelength of 550 nm using a SPECTRA FLUOR (TECAN, Männedorf, Switzerland) for quantitative analysis and normalized to the cell protein contents, determined using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

### 2.4. Osteoblast differentiation and alizarin red S staining

At Day 0, cells were treated with osteoblast differentiation medium containing 0.1 µM dexamethasone, 10 mM β-glycerophosphate and 50 µg/mL L-ascorbic acid with and without TBBPA and/or TCDD, and maintained for 21 days.

Differentiated cells were examined by alizarin red S staining for the presence of calcium deposits. Briefly, cells were fixed with ice cold 70% ethanol, rinsed with distilled water, and then stained with 40 mM alizarin red S dissolved in distilled water (pH 4.2; adjusted with 10% ammonium hydroxide) for 5 min. Cells were washed with distilled water, and observed under an IX71 microscope. After imaging, the dye was eluted with 10% acetic acid, and the absorbance was measured at 450 nm using a TriStar LB 941 microplate reader (Berthold, Bad Wildbad, Germany) and normalized to the cell protein contents, determined using a BCA protein assay kit.

### 2.5. ALP activity assay and cell matrix ALP staining

Cells were washed with PBS and then lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, and 1% TrironX-100). ALP activity was determined colorimetrically by incubating protein lysates with the substrate *p*-nitrophenyl phosphate in a 96-well plate at 37 °C for 60 min. The reaction was stopped by adding 0.2 M NaOH. Absorbance was measured at 415 nm using a TriStar LB 941 microplate reader, and normalized against the corresponding protein concentrations determined with a BCA protein assay kit using bovine serum albumin as a standard.

For ALP staining, cells were washed with PBS, and fixed with 4% paraformaldehyde. Cells were stained with a mixture of 0.1 mg/mL naphthol AS-MX phosphate, 0.6 mg/mL fast-blue BB salt, 2 mM $MgCl_2$, 5 µL/mL *N,N*-dimethylformamide, and 100 mM Tris-HCl (pH 8.8) buffer at 37 °C for 20 min. When the cells turned blue, they were washed and visualized using an IX71 microscope.

### 2.6. Adipocyte and osteoblast mRNA level quantification by real-time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA from hMSCs was extracted with ISOGEN (Nippongene, Toyama, Japan) and reverse transcribed with PrimeScript RT Master Mix (TaKaRa, Kyoto, Japan). PCR reactions were performed using a KAPA SYBR FAST Universal qPCR kit (Kapa Biosystems, Boston, USA) and assayed using a Thermal Cycler Dice (TaKaRa). The oligonucleotides used for RT-qPCR included β-actin (forward: 5′-AGATCAAGATC ATTGCTCCTCCTG-3′, reverse: 5′-CAAGAAAGGGTGTAACGCAACT AAG-3′), aP2 (forward: 5′-AGGAAAGTCAAGAGCACCATA-3′, reverse: 5′-CACCAGTTTATCATCCTCTCG-3′), LPL (forward: 5′-AGAGGACTTGG AGATGTGGA-3′, reverse: 5′-TCATAGCCCAGATTGTTGC-3′), PPARγ (forward: 5′-GCGATTCCTTCACTGATAC-3′, reverse: 5′-CTTCCATTACG GAGAGATCC-3′), C/EBPα (forward: 5′-TGGACAAGAACAGCAACGA

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