Environmental Pollution 313 (2022) 120140

Contents lists available at ScienceDirect

Environmental Pollution

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

BDE-209 and TCDD enhance metastatic characteristics of melanoma cells after chronic exposure☆

Benisio Ferreira Silva Filho [a,b], Francisco Filipak Neto [a,b], Micheli de Marchi [a,b], Erick Laurent Moggio [a,b], Izadora Volpato Rossi [b,c], Bruna Sabatke [c,d], Marcel Ivan Ramirez [b,c,d], Miguel Clodomiro dos Santos Lucena [e], Adriane Regina Todeschini [e], Ciro Alberto de Oliveira Ribeiro [a,b,*]

[a] Laboratório de Toxicologia Celular, Departamento de Biologia Celular, Universidade Federal Do Paraná, CEP 81.531-990, Curitiba, PR, Brazil
[b] Programa de Pós-Graduação Em Biologia Celular e Molecular, Universidade Federal Do Paraná, CEP 81.531-990, Curitiba, PR, Brazil
[c] Laboratório de Biologia Molecular e Sistemática de Tripanossomatideos, Instituto Carlos Chagas –Fiocruz PR, CEP 81.350-010, Curitiba, PR, Brazil
[d] Programa de Pós-Graduação Em Microbiologia, Parasitologia e Patologia, Universidade Federal Do Paraná, CEP 81.531-990, Curitiba, PR, Brazil
[e] Laboratório de Glicobiologia Estrutural e Funcional. Instituto Carlos Chagas Filho. Universidade Federal Do Rio de Janeiro, CEP 21.941-902, Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Keywords:
Metalloproteinases
Epithelial-mesenchymal transition
Microvesicles
Brominated compounds
Dioxin

ABSTRACT

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and BDE-209 (decabromodiphenyl ether) are persistent organic pollutants (POPs) produced by industrial activities and associated with several diseases. TCDD is a known human carcinogen, but few studies investigated about the effects of exposure to both compounds, i.e., whether BDE-209 and TCDD can render tumor cells more aggressive and metastatic. In the current study we investigated if the exposure of B16–F1 and B16–F10 melanoma murine cells to environmental relevant concentrations of TCDD and BDE-209 at 24 h and 15-day exposure modulates the expression of genes related to metastasis, making the cells more aggressive. Both pollutants did not affect cell viability but lead to increase of cell proliferation, including the upregulation of vimentin, MMP2, MMP9, MMP14 and PGK1 gene expression and downregulation of E-cadherin, TIMP2, TIMP3 and RECK, strongly suggesting changes in cell phenotypes defined as epithelial to mesenchymal transition (EMT) in BDE-209 and TCDD-exposed cells. Foremost, increased expression of metalloproteinases and decreased expression of their inhibitors made B16–F1 cells similar the more aggressive B16–F10 cell line. Also, the higher secretion of extracellular vesicles by cells after acute exposure to BDE-209 could be related with the phenotype changes. These results are a strong indication of the potential of BDE-209 and TCDD to modulate cell phenotype, leading to a more aggressive profile.

1. Introduction

Environmental pollution causes diseases to human population and according to the World Health Organization (WHO), air pollution accounts for approximately 4.2 million deaths per year owing to strokes, heart, and respiratory disease, or even lung cancer (WHO, 2020). Mohankumar et al. (2016) described the worst condition aggravated by proximity to industrial areas due to the production and deposition of hazardous compounds, establishing potential association of the chemicals in the environment and diseases (McGuinn et al., 2012). The persistent organic pollutants (POPs) are of particular concern to human health due to their resistance to degradation and widespread worldwide distribution by water, air, or biota (Nunes, 2018). According to European Union report (2021), POPs are a concern to human population because they are highly lipophilic and can bioaccumulate along chain food (European Union, 2021)

The 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a worldwide disperse pollutant produced as a subproduct of pesticide and bleaching cellulose industrial processes, as well as plastic and hospital waste incineration (Foster et al., 2011). The exposure can be by inhalation, skin contact, fat-rich foods (~90%), such as milk, cheese, meat and even breast milk (Sanabria, 2014). TCDD is toxic and can be associated with

☆ This paper has been recommended for acceptance by Dr Jiayin Dai.

* Corresponding author. Laboratório de Toxicologia Celular, Departamento de Biologia Celular, Universidade Federal do Paraná, Caixa Postal 19031, Bairro Jardim das Américas, Curitiba, PR, CEP 81.531-980, Brazil
E-mail address: ciro@ufpr.br (C.A. Oliveira Ribeiro).

https://doi.org/10.1016/j.envpol.2022.120140
Received 21 July 2022; Received in revised form 1 September 2022; Accepted 5 September 2022
Available online 10 September 2022

several diseases, such as hypertension, diabetes, liver damage, chloracne, cardiovascular disturbs, and cancer (Xu et al., 2018). Decabromodiphenyl ether (BDE-209) is the most abundant congener of polybrominated diphenyl ethers (PBDEs) family widely used as a flame retardant in the electronic products, plastic, and textile industries (Chen et al., 2012). Like TCDD, BDE-209 is highly lipophilic, bioaccumulating in food chain (Liu et al., 2017). Human exposure occurs mainly by food, particularly by contaminated fish, seafood, meat, milk, nuts or oils (Yu et al., 2015). According to Wu et al. (2007), the inhalation of domestic dust is also an important source of exposure, but studies revealed the presence of BDE-209 in umbilical cord blood and breast milk, leading to the exposure also during gestational and newborn period (Sjödin et al., 2008; Schecter et al., 2003; Zhi et al., 2018). According to Leonel et al. (2014) and Abdallah and Harrad (2014), PBDEs are cytotoxic, neurotoxic, and may cause reproductive toxicity disturbs and cancer.

Cancer is one of the leading public health problems in the world and one of the main causes of death before the age of 70 (INCA, 2020), while skin cancer is the most common type of cancer, with melanoma having the highest mortality rate (Mesquita et al., 2020). Many chemicals are carcinogenic, i.e., can cause cancer, but few is known about the phenotype changes in tumoral cells exposed to POPs in the case of pre-established disease. This is an issue since cancer patients can be chronically exposed to chemicals, such as TCDD and BDE-209, and the hypothesis that these chemicals can influence the progression of cancer or affect the outcome of the treatment have been recently investigated (Salgado et al., 2018; Marchi et al., 2021, Steil et al., 2022). Additionally, our group demonstrated that mice previously exposed to BDE-209 increased the spread of cells and formation of tumors, and decreased the disease treatment with dacarbazine, after B16–F10 cells inoculation (Brito et al., 2020).

According to Tkach and Théry (2016), extracellular vesicles, constituted by exosomes (40–100 nm) and microvesicles (100 nm-1 μm) are actively released by cells and represent an important mechanism of cell communication at short and long distances. The molecules carried by these vesicles, such as enzymes and genetic material, can affect the cell physiology of neighboring cells in several ways (Théry et al., 2006). However, due to the wide range of molecules transported, it is not surprising that exosomes also play a key role in the pathogenesis of certain diseases, including cancer (Andaloussi et al., 2013).

In the current study, we investigated the *in vitro* effects of TCDD and BDE-209 on B16–F1 (low metastatic potential) and B16–F10 (high metastatic potential) after acute and chronic exposure through the expression of genes involved with metastasis spread (metalloproteinases and their inhibitors) and proteins that characterize the epithelial-mesenchymal transition (EMT) (E-cadherin and vimentin), and release of microvesicles.

2. Material and methods

2.1. Preparation of BDE-209 and TCDD

Stock solutions (1000X concentrated) of TCDD and BDE-209 (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and kept in sterile amber glass vials at −20 °C until use. Intermediary test concentrations (0.1 nM) were chosen based on data reported in human plasma (0.65 pg/g BDE-209, Frederiksen et al., 2010; 6.0 ppt TCDD in serum, Collins et al., 2006) and umbilical cord (1–10 ng/g of BDE-209, Leonetti et al., 2016). From these, a 10-fold lower (0.01 nM) and 10-fold-higher (1.0 nM) test concentration were established.

2.2. Cell culture

Melanoma murine B16–F1 and B16–F10 cells were obtained from the Cell Bank of Rio de Janeiro (Brazil) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Cultilab) supplemented with 1.5 g L^{-1} sodium bicarbonate, 5 mM HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid), 10% fetal bovine serum (Gibco) and 40 μg/mL gentamicin, at 37 °C and 5% CO_2. These culture conditions were maintained during the study (subculture and exposure). Subcultures were done after trypsinization (0.25% trypsin, 0.02% EDTA (ethylenediaminetetraacetic acid) in PBS (phosphate buffer saline), pH 7.2) for 5 min at 37 °C.

2.3. Exposure to contaminants

Cells were exposed to either BDE-209 or TCDD during 24 h (acute experiment) and 15 days (chronic experiment). For 24 h-experiment, the cells were seeded (6×10^3 cells/well) into 96-well microplates (Kasvi) and cultured for 24 h. Then, the culture medium was replaced by fresh medium containing the supplementation and either BDE-209 (0.01, 0.1 and 1.0 nM), TCDD (0.01, 0.1 and 1.0 nM), PBS (Control) or DMSO (0.02%, vehicle). After 24 h-exposure, cells were used for the assays. For 15-day experiment, the cells were seeded onto 75 cm^2 culture flasks (Kasvi, 1×10^5 cells/flask) and cultured for 24 h and then exposed to either BDE-209 or TCDD in culture medium with supplementation, with control and vehicle groups in parallel. Culture medium, containing either BDE-209 or TCDD, was replaced every 3 days until completing 15 days of exposure, and the cells were subcultured every 3–4 days, depending on cell confluence (~70%). On the 15th day of exposure, the cells were trypsinized, seeded (6×10^3 cells/well) into 96-well microplates (Kasvi), or used for the assays. All the experiments were carried out in triplicates and three independent experiments.

2.4. MTT assay

After exposure to pollutants, the culture medium was replaced by 200 μL of 0.5 mg mL^{-1} MTT (3-[4,5-dimethyl-2-yl]-2,5-diphenyl tetrazolium bromide). After incubation for 3 h at 37 °C and 5% CO_2, the cells were washed with phosphate buffer saline (PBS, pH 7.2) and the formazan crystals were solubilized with 100 μL DMSO for absorbance measurement at 550 nm (Reilly et al., 1998).

2.5. Neutral red assay

After exposure to pollutants, the culture medium was replaced by 200 μL of neutral red solution (40 μg/mL) were added. After incubation for 2 h at 37 °C and 5% CO_2, the cells were washed with a solution of formaldehyde (0.5%) and calcium chloride (0.1%) in water. The dye was extracted for 15 min with a solution of 50% ethanol and 1% acetic acid for absorbance measurement at 540 nm (Babich and Borenfreund, 1990).

2.6. Crystal violet assay

After exposure to pollutants, the cells were washed with PBS, fixed with methanol 70% for 30 min at -8 °C, stained with 100 μL of violet crystal (0.25 mg mL^{-1} in water) for 15 min at room temperature, and then washed with water. The dye was eluted using a 33% acetic acid solution, under constant agitation, for 30 min. The absorbance was measured at 570 nm (Gillies et al., 1986).

2.7. Clonogenic assay

After 15 day-exposure to pollutants, the cells were detached from culture flasks with cell scraper (Kasvi). Viability test (trypan blue) was performed, and the cells were counted in a Neubauer chamber. Then, the cells were seed into 24-well plates (250 cells per well) and cultured for 7 days at 37 °C and 5% CO_2 in the absence of BDE-209 and TCDD. Finally, the cells fixed with 70% methanol in PBS for 30 min and stained with 0.5% violet crystal for 15 min. Cell colonies were analyzed by the Image J software, to calculate the occupied space and thus identify if there was an increase in colonies (Munshi et al., 2005).

2.8. SDS-PAGE and western blotting

After culture and 15 day-exposure to either BDE-209 or TCDD, the cells were washed with PBS and homogenized in lysis buffer (150 mM NaCl, 30 mM Tris-HCl, pH 7.6; 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail, with protease inhibitors). Cell lysates were sonicated and centrifuged at 9000 *g* and 4 °C for 10 min. Supernatant was collected, protein concentration determined by Bradford method (1976), and 200 μl modified Laemli buffer was added. Samples were separated on SDS-polyacrylamide gels (8%) and were subsequently electroblotted to nitrocellulose membranes (Bio-Rad). The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 with 3% nonfat dry milk.

The blocked membranes were incubated overnight at 4 °C with primary antibodies against E-cadherin (1:3000, Cell Signaling Technology, USA), β-actin (1:3000, Sigma, USA) and Vimentin (1:2000 Cell Signaling Technology, USA). The blots were then washed, incubated with the appropriate secondary antibodies, developed using ECL (GE Healthcare), and exposed to Image Quant LAS 4000 (GE Healthcare). ImageJ software was used for densitometry analysis of immunoblots and measurements were normalized against β-actin.

2.9. RT-qPCR

2.9.1. Design of primers

Genes of interest, GenBank accession numbers and primers designed in Primer3web version 4.1.0 with their sequences confirmed in BLAST are shown in Table 1.

Conventional PCR was carried out to evaluate the synthesized primers, amplicon quality, and whether they corresponded to the base pair size of each amplicon predicted by *in silico* tests (Gene Runner version 6.5.52). Finally, the performance was evaluated by qPCR with SYBRgreen. The ideal concentrations of primers and cDNA, 6 pmol and 5 μM, respectively, were used. Beta-actin, GAPDH and beta-2-microglobulin were tested as endogenous reference genes but the last two genes were affected by exposure to BDE-209 and TCDD (data not shown). Then, beta-actin gene, which was not affected by the compounds, was used as the reference gene since its expression was not affected by exposure to the compounds.

Table 1
Primers designed and used for qPCR.

Gene	GenBank	Primers	Amplicon (pb)
MMP9	NM_013599	F - 5′ TGACAAGAAGTGGGGTTTCTG 3′ R - 5′ GGCACGCTGGAATGATCTA 3′	101
MMP2	NM_008610	F - 5′ TGACCGGGATAAGAAGTATGG 3′ R - 5′ ACTTGTTGCCCAGGAAAGTG 3′	110
MMP14	NM_008608	F - 5′ TATGGGCCCAACATCTGTG 3′ R - 5′ TCCATCACTTGGTTATTCCTCA 3′	113
PGK1	NM_008828	F - 5′ GTGCCAAATGGAACACAGAG 3′ R - 5′ AACATTGCTGAGAGCATCCA 3′	113
TIMP1	NM_001044384	F - 5′ GCGTACTCTGAGCCCTGCT 3′ R - 5′ TCACTCTCCAGTTTGCAAGG 3′	111
TIMP2	NM_011594	F - 5′ AACAGGCGTTTTGCAATG 3′ R - 5′ GAATCCTCTTGATGGGGTTG 3′	105
TIMP3	NM_011595	F - 5′ ACCTCACCTCCTTCCCATC 3′ R - 5′ CCAGTGCTAAATTTGCAAGAAA 3′	100
RECK	NM_016678	F - 5′ ATAGGCTGCAAACCCATCAT 3′ R - 5′ TGTCACCTTAGCAATAGTGTCCA 3′	108
BETA ACTIN	NM_007393.5	F - 5′ CCACCATGTACCCAGGCATT 3′ R - 5′ GAGTACTTGCGCTCAGGAGG 3′	106

2.9.2. Total RNA extraction and cDNA synthesis

Total RNA extraction from samples was performed using the Purelink RNA mini-Kit (Applied Biosystems - Thermo Fisher), according to the manufacture's protocol. High-quality total RNA was obtained, confirmed by quantification in spectrophotometer using A260/A280 absorption ratio, which presented an average ratio of 1.818, indicative of RNA purity. Then the material was converted to total cDNA using Superscript IV kit (Applied Biosystems - Thermo Fisher) following the manufacturer's instructions.

2.9.3. qPCR

The analysis was performed by real-time PCR (qPCR) using murine β-actin as a reference gene thus establishing comparisons in expression levels between the studied genes of each analyzed sample. The reactions were carried out in a Step one fast system thermocycler (Applied Biosystems), using SYBRgreen PCR Master Mix for each reaction, and thermocycling as recommended by the manufacturer, according to the kit utilized (40 cycles – denaturation at 94 °C followed by annealing at 60 °C).

2.10. Extracellular vesicles (EVs) isolation

Culture medium from confluent cells cultured in 75 cm^2 bottles was collected after 24 h exposure to pollutants, and EVs were isolated by differential centrifugation methodology. Initially, the cell supernatant was centrifuged at 800 *g* for 10 min to remove cells, at 4,000 *g* for 30 min, 4 °C to eliminate cell debris, and finally at 11,000 *g* for 120 min to pellet the extracellular vesicles.

2.11. Characterization and visualization of extracellular vesicles

2.11.1. Extracellular vesicles visualization by transmission electron microscopy

To investigate the cell ultrastructure, B16–F1 cells were cultured and exposed to BDE-209, at a concentration of 1 nM, in 75 cm^2 flasks for 24 h, mechanically removed and centrifuged in 2 mL tubes. The pellet was chemically preserved in a fixative solution (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylic acid (CaCo) buffer - pH 7.2–7.4) for 1 h at room temperature. Then, the pellets were washed with 0.1 M CaCo buffer and post-fixed with 1% osmium tetroxide (Electron Microscopy Science®) in 0.1 M CaCo buffer solution (pH 7.2–7.4) for 1 h, washed in 0.1 M CaCo and dehydrated in an increasing ethanol series (Merck®), propylene oxide (Electron Microscopy Science®) and embedded in PolyEmbed 812 DER736 resin (Electron Microscopy Science®). Ultrathin sections (70 nm) were obtained using an Ultramicrotome Leica and contrasted with uranyl acetate (5%) (Electron Microscopy Science®) for 20 min and lead citrate (Electron Microscopy Science®) for 5 min. The sections were analyzed in a JEOL TEM 1200 EXII at Center for Electronic Microscopy at Federal University of Paraná.

2.11.2. Extracellular vesicles characterization by nanoparticles tracking analysis

For nanoparticle tracking analysis (NTA, Nanosight, Malvern, U.K.), each pellet was suspended in PBS (1:50) and analyzed with a Nanosight LM10 (Malvern™, U.K.), with readings performed in triplicate during 60 s videos at 10 frames per second at room temperature, with the following parameters: camera shutter −8, camera gain −12, detection threshold −8. The mean size and the concentration of particles resulting from the replicates were presented.

2.12. Statistical analysis

Three independent experiments were performed for all the endpoints. The data were evaluated using Graph Pad Prisma 5 software (©1995–2017 GraphPad Software, Inc. All rights reserved demo version). Either One-way ANOVA followed by Dunnett's post-test (data with normal distribution), or Kruskal-Wallis test followed by Dunn's post (data without normal distribution) were performed when applicable. A value of $p < 0.05$ was assumed as statistically significant.

3. Results

3.1. Cell viability and colony formation

First, for evaluating the capacity of BDE-209 and TCDD to affect B16–F1 cell viability, three methods were applied: crystal violet, neutral red and MTT. In general, the compounds were not toxic to B16–F1 cell line (Fig. 1). Indeed, the number of attached cells, determined by crystal violet assay, even increased at the highest concentration of BDE-209 (1.0 nM, 25%) at 24 h-exposure and the intermediate concentration of TCDD (0.1 nM, 215%) at 15 day-exposure (Fig. 1A–B).

For B16–F10 cells, 24 h-exposure to BDE-209 and TCDD did not affect cell viability (Fig. 2A, C, 2E), but effects were observed at 15 day-exposure. The number of attached cells (crystal violet assay) increased after exposure to BDE-209 (48%) and TCDD (41%), at intermediate concentration (0.1 nM), as well as the dehydrogenase-dependent metabolic conversion of MTT to formazan (MTT assay) for the highest concentration of BDE-209 (1 nM, 85%) and the intermediate concentration of TCDD (0.1 nM, 60%).

In the clonogenic assay, TCDD and BDE-209 did not affect the total area occupied by cell colonies for both B16–F1 and B16–F10 cells.

3.2. Gene expression

The gene expression was analyzed in terms of general profile for both cell lines after 15 day-exposure with the measure of TIMP2, TIMP3, RECK, MMP2, MMP14 and PGK1 genes that allow evaluate the status of the cancer cell. For B16–F1 cells, exposure to BDE-209 lead to a decrease of TIMP2 (0.1 and 1.0 nM) and RECK (1.0 nM), and slight increase of MMP2 and MMP14 (1.0 nM) genes. Differently, TCDD caused increases of MMP2 and PGK1 genes (0.01, 0.1 and 1.0 nM), and decreases of TIMP2 (0.01, 0.1 and 1.0 nM), TIMP3 (0.1 and 1.0 nM) and RECK (1.0 nM) genes. For B16–F10 cells, the gene expression profile was generally not affected by BDE-209 and TCDD for almost all genes, except for an apparent increase of PGK1 (BDE-209: 0.1 and 1.0 nM; TCDD: 1.0 nM) (Fig. 3).

Comparison of both cell lines revealed that the more metastatic cells (B16–F10) have a much lower expression of inhibitors than metalloproteinases and PGK1, and the expression of the genes were generally not affected by BDE-209 and TCDD. Conversely, the less metastatic cells (B16–F1) had no such striking difference of metalloproteinase/inhibitors levels, but the expression of the genes were more prone to be affected by BDE-209 and TCDD, toward increased expression of metalloproteinases and decreased expression of the inhibitors. Regardless of

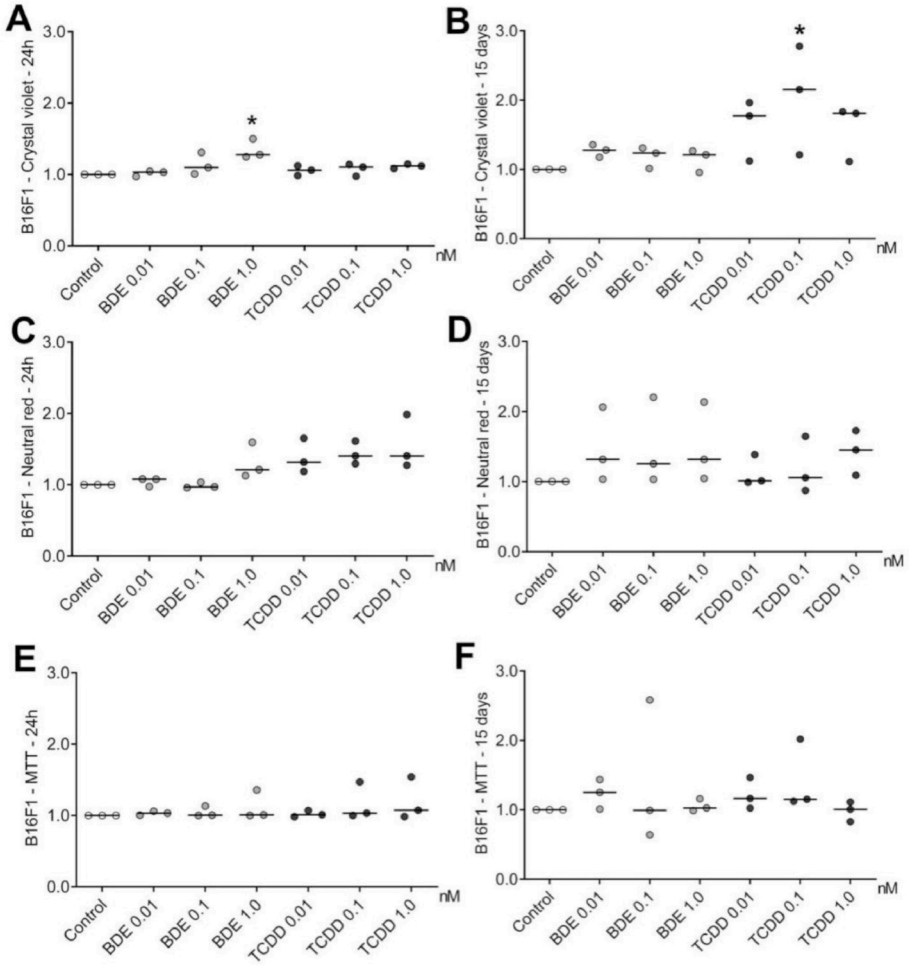

Fig. 1. Cell viability in B16–F1 cells exposed to BDE-209 and TCDD for 24 h and 15 days. Crystal violet, neutral red and MTT assays. Median (horizontal bar) and independent experiments (dots). Kruskal-Wallis test followed by Dunn's post hoc test. *$p < 0.05$. N = 3.

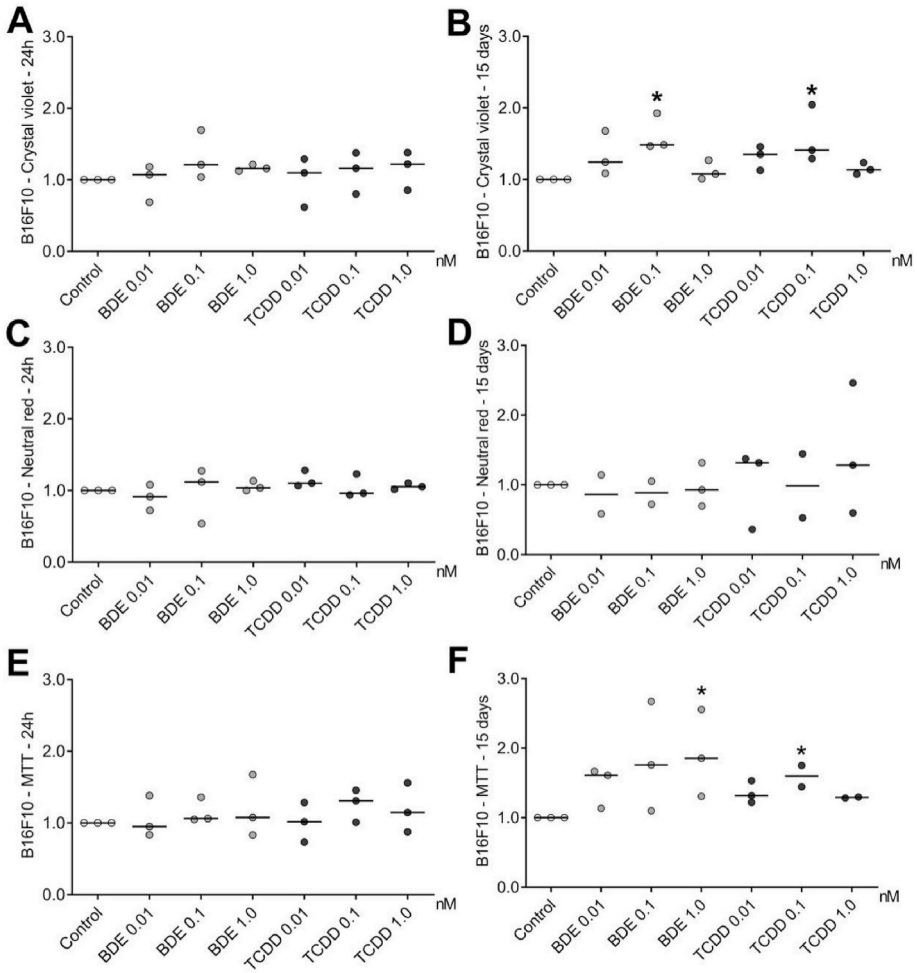

Fig. 2. Cell viability in B16–F10 cells exposed to BDE-209 and TCDD for 24 h and 15 days. Crystal violet, neutral red and MTT assays. Median (horizontal bar) and independent experiments (dots). Kruskal-Wallis test followed by Dunn's post hoc test when applicable. N = 3.

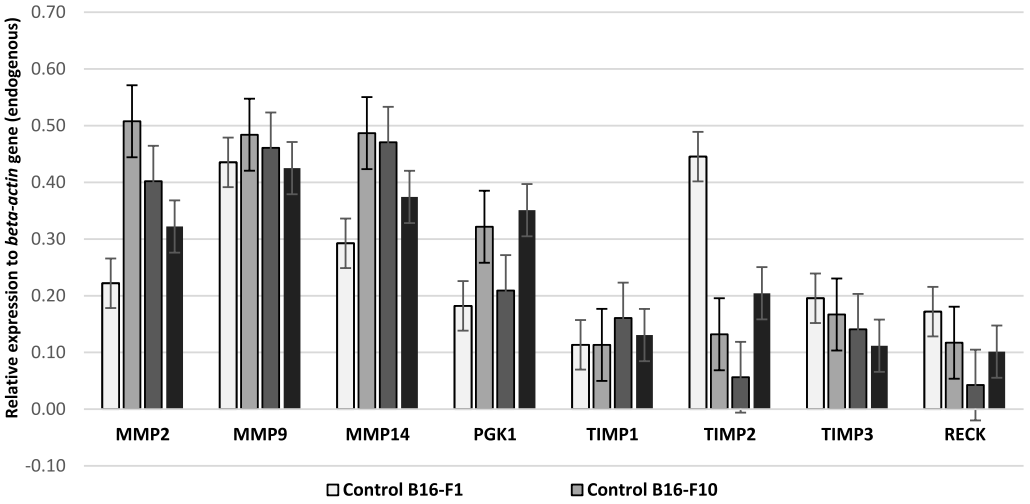

Fig. 3. Relative gene expression of B16–F1 and B16–F10 cells after 15 day-exposure to BDE-209 and TCDD. The expression profile of each gene for both control and exposed groups is presented relative to the endogenous β-actin. Mean ± SD. *p < 0.05. Three independent experiments were performed.

concentration and cell line, the difference in gene expression between the groups was very small (Fig. 3).

3.3. Effects of BDE-209 and TCDD in E-cadherin and vimentin expression

Since metalloproteinases play a role in the epithelial-mesenchymal transition (EMT), here we analyzed the impact of BDE-209 and TCDD treatment in the expression of E-cadherin and vimentin. Exposure to the

compounds led to increase of vimentin and decrease of E-cadherin expression in both B16–F1 and B16–F10 cells (Fig. 4).

3.4. Characterization and visualization of microvesicles

The mean size of EVs derived from control cells and BDE-209 exposed cells were similar (266.10 nm and 286.90 nm, respectively), but there was an increase in the mean size of EVs that received DMSO vehicle (360.10 nm) (Fig. 5A). Interestingly, the exposure of B16–F1 cells to BDE-209 resulted in a higher release of EVs (855.0×10^7 particles/mL) when compared to EVs from the control and vehicle-exposed cells (226.50×10^7 particles/mL and 88.50×10^7 particles/mL, respectively, Fig. 5B).

Although the average size of EVs did not reveal difference between the control cell and the treatment with BDE-209, the analysis of the size distribution profile for EVs shows significant differences in the peaks present in the samples. The size distribution profile of EVs derived from control and DMSO-treated cells was similar (Fig. 5C and D), however, after exposure to BDE-209, a widespread peak of EVs between 200 and 500 nm is evident (Fig. 5E).

The ultrastructural results showed a heterogeneous population of B16–F1 cells, with cells presenting either normal, poor, or high contrasted cytoplasm (Fig. 6A, B and C). In general, these cells have euchromatin-rich rounded nucleus, with one or more nucleoli (Fig. 6B, C and E). Some cells are poor in cytoplasmic organelles (Fig. 6E), but others have many mitochondria (Fig. 6G), developed Golgi apparatus, smooth endoplasmic reticulum (Fig. 6F and H) and glycogen deposits (Fig. 6).

Cells exposed to BDE-209 (0.1 nM) for 24 h were like the control cells (Fig. 6J and K), except for the presence of extracellular vesicles (microvesicles and exosomes). These vesicles were found in large number around cells that present a more electron-dense cytoplasm (Fig. 6K and L). Fig. 6J shows a cell in the final stage of mitosis and yet these vesicles were found in large numbers. These extracellular vesicles present different shapes and sizes; they are usually rounded (Fig. 6K, L and M, Fig. 5D) and may be released from cells with a more electron-dense cytoplasm, called here 'donor cells' (Fig. 6K and L), disperse through extracellular space (Fig. 6C) and fuse with the plasma membrane of less electron-dense cytoplasm cells (Fig. 6M), called here as 'target cells'.

4. Discussion

The risk of exposure to POPs are still underestimated to human population, despite of organ toxicity and carcinogenic potential have been widely recognized by governmental agencies such as Environmental Protection Agency (EPA-USA) and International Agency for Research on Cancer (IARC). However, few studies have investigated the consequences of BDE-209 and TCDD exposure to the malignancy of tumor cells or prognosis of the disease (Brito et al., 2020, Marchi et al., 2021). The current study revealed that BDE-209 and TCDD can change B16–F1 cells phenotype by affecting the expression of molecules favoring metastatic events, even under environmental relevant concentrations. Additionally, cells acutely exposed to BDE-209 release extracellular vesicles that may be involved in cell communication. These findings are of great importance in cancer progression and treatment, but still poorly investigated.

4.1. Low concentrations of BDE-209 and TCDD were non-toxic to B16 cells

Cell viability assays were performed after acute (24 h) and chronic exposure (15 day) to BDE-209 and TCDD to investigate changes, such as alterations in the number of attached cells (crystal violet assay), cell dehydrogenases activity (MTT assay) and integrity of the endolysosomal system (neutral red assay). In this sense, when analyzed together, these

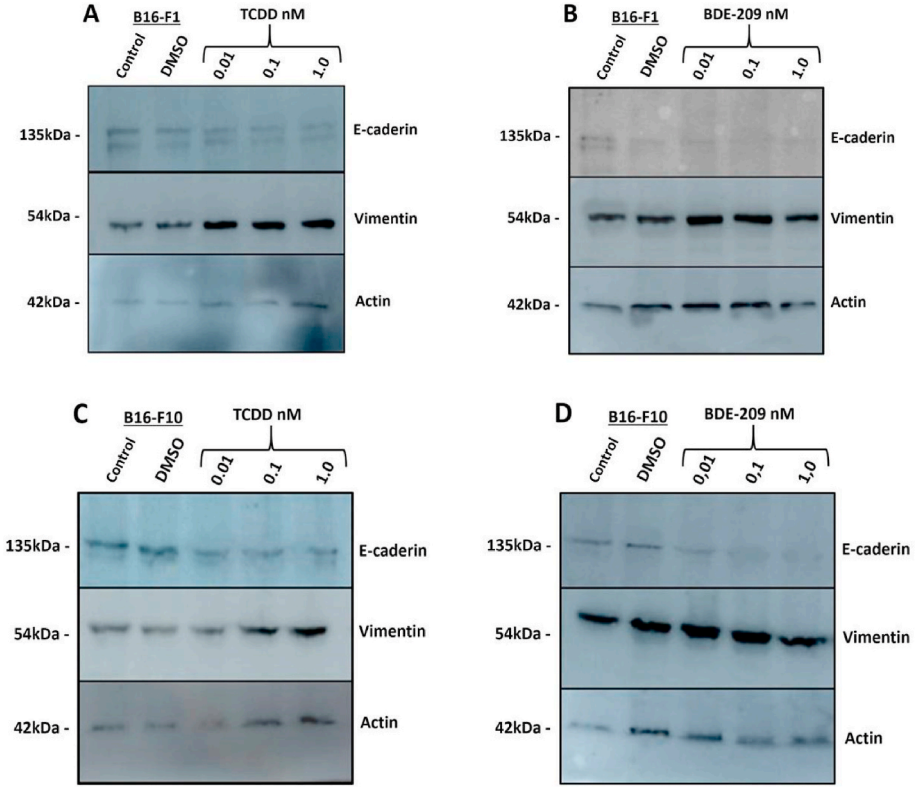

Fig. 4. Western blotting for E-cadherin and vimentin in B16–F1 (A and B) and B16–F10 (C and D) cells after 15 day-exposure to BDE-209 and TCDD respectively. Actin was used as a reference protein. The images are representative of 3 experiments.

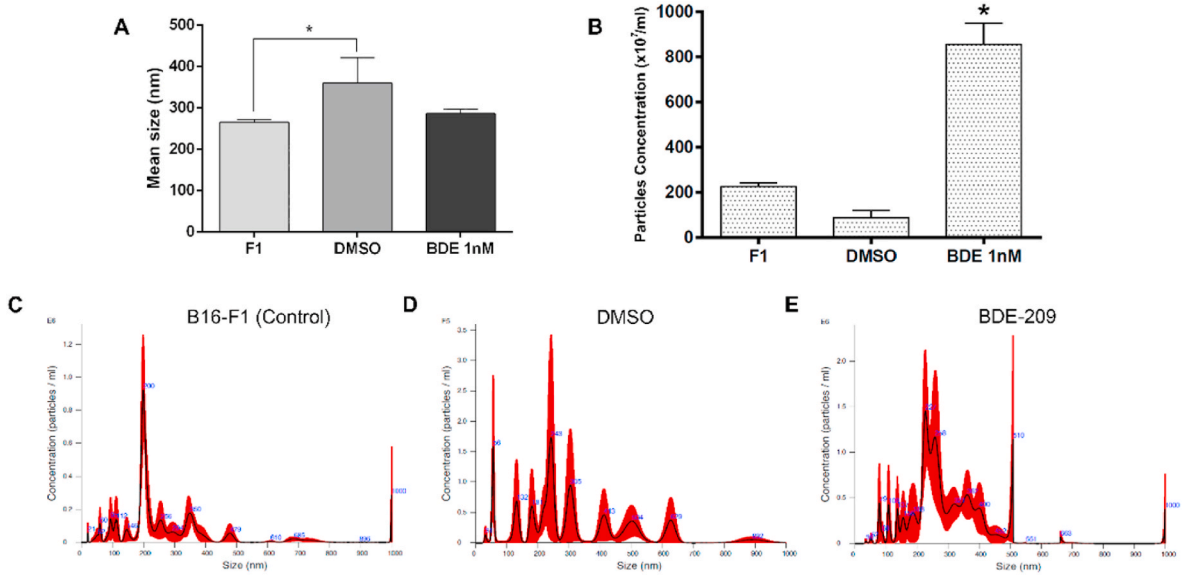

Fig. 5. Characterization of microvesicles released from B16–F1 cells after acute exposure to BDE-209. (A) Mean size and (B) concentration of particles (x107/mL). Distribution of size and concentration of EVs derived from the (C) Control cells, (D) DMSO treated cells and (E) BDE-209 exposed cells. *p < 0.01.

assays provide valuable data regarding cell viability/cytotoxicity. Environmental relevant concentrations of BDE-209 and TCDD were not toxic to B16–F1 and B16–F10 cells, as no significant alterations in cell viability were observed on the mentioned assay. However, increases of the cell number (crystal violet) and of metabolic conversion of MTT (MTT assay) indicate that the both BDE-209 and TCDD may induce cell proliferation, decrease cell death rate or both, particularly after 15 day-exposure.

In general, the concentrations used in current study are not toxic to cells. Zhi et al. (2018) utilized higher concentrations of BDE-209 (5, 15, 25, 50 and 100 nM) for 24 h, and observed an increase of cell viability in HeLa, MCF-7/ADR, OVCAR-3 and CHO cells, while Gao et al. (2016) described an increase of cell proliferation in primary epithelial cells isolated from human fetal palatal shelves (hFPECs) exposed to TCDD (0.01, 0.1, 0.5 or 1 nM) after 5 days. Also, the authors reported an advance of G1 to S phase, as well as an increase in cell volume that initiates the G2/M phase. Differently than observed in the present study, BDE-209-related cell viability decrease had been reported in human neuroblastoma SH-SY5Y at higher test concentrations (1, 2, 4, 8 μg/mL, 24 h-exposure, MTT assay; He et al., 2008), HepG2 hepatocarcinoma (0.5 and 5 nM, 24 and 48 h-exposure, cell death assay; Pereira et al., 2017), and adrenal gland PC-12 cells (6.25, 12.5, 25, 50 and 100 μM, 48 h-exposure, MTT and ROS production; Liu et al., 2017). However, Kim et al., (2009) reported increased response in MTT for rat insulinoma INS-1 cells exposed to TCDD (100 nM, 6 h) and Zhou et al., (2017) reported an attenuation of this activity after exposure to TCDD lower concentrations (0.01–10 nM, for 24 h). The present results are in accordance with the literature as the tested concentrations are lower and environmental relevant. This is an important finding as the aim of the current study is not the evaluation of cytotoxicity but identify relevant cell physiology changes in order to investigate the role of these pollutants on cancer prognosis.

4.2. BDE-209 and TCDD did not affect the clonogenic capacity of B16 cells, but may increase the expression of invasion-related genes

Comparatively, the gene expression profile of non-exposed B16–F1 and B16–F10 cells were clearly different, which is a key aspect for melanoma spread: increased expression of MMPs and decrease expression of their inhibitors, favoring the tumor cell invasiveness and metastasis (Hofmann et al., 2000), by degrading extracellular matrix molecules to migrate and invade new sites (Roy et al., 2009). However, the exposure to BDE-209 and TCDD led to a well-defined expression profile with increased MMPs and PGK1 and decreased inhibitors and RECK in B16–F1 cells, similar to found in high metastatic B16–F10 cells. In particular, the decrease of the expression of TIMP2 gene after exposure to BDE-209 deserves attention, because TIMP2 reduces the invasive potential of tumor cells by inhibiting metalloproteinases, such as MMP2 (Neidhart et al., 2021). Here, BDE-209 exposure also led to increase of MMP2 (and MMP14) expression.

MMP14 is necessary to promote metastasis, and its inhibitors may be effective in preventing the spread of melanoma. Shaverdashvili et al., (2014) used an orthotopic murine melanoma model to demonstrate that MMP14 is necessary for metastasis; in the absence of MMP14 the metastasis process did not occur. It was also reported in that study that MMP2 is activated by MMP14 (Shaverdashvili et al. 2014). This relationship is observed in all exposures - MMP2 and MMP14 increased proportionally in BDE-209 and TCDD-exposed groups compared to the control in both B16 cell lines. The protein expressed by RECK gene is involved in the modulation of MMP2 and MMP14 (Dong et al., 2010), which reinforces that BDE-209 can alter gene expression profile toward metalloproteinases activity. Other important aspect is that BDE-209 affected gene expression profile at similar level or even worse than TCDD, a well-recognized compound by its toxicity and tumor induction and promotion potential (Fujisawa et al., 2018).

B16–F10 cells are more aggressive compared to B16–F1 due to their higher proliferation rate and greater metastatic potential, besides being genetically less stable genetically than B16–F1 (Overwijk and Restifo, 2001). Therefore, the expression of MMPs and PGK1 genes is greater in relation to inhibitors, and it may explain why these POPs did not affect gene profile in B16–F10 at the same level it had done in B16–F1 cells.

4.3. BDE-209 and TCD may promote epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype. These include enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis (Das et al., 2019). One of the EMT characteristics is the decrease of E-cadherin and increased of vimentin expression (Craene and Berx, 2013). E-cadherin is a calcium-dependent

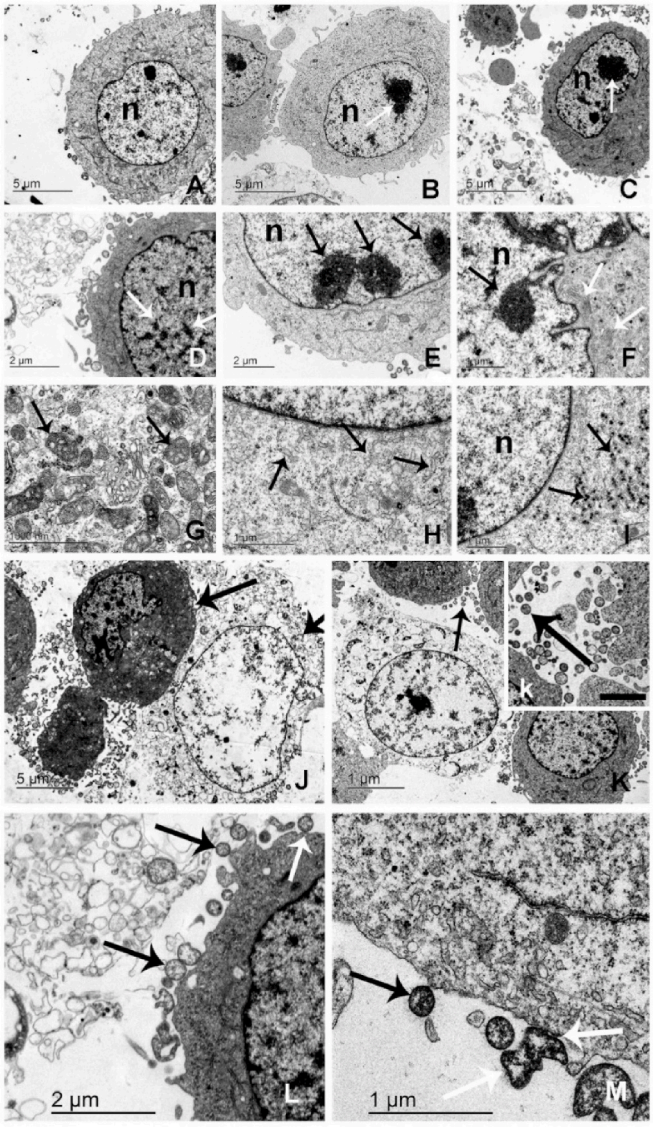

Fig. 6. Ultrastructure of murine melanoma cells (B16–F1) after acute exposure to BDE-209. A, B and C show three distinct cells from control group. Note the differences between the nuclei (n) and the cytoplasm contrast. White arrows indicate the single nucleolus. D. Note the presence of heterochromatin "spots" without the typical deposition around the nuclear envelope. E. Multiple nucleoli are observed. F. Note the atypical nuclear morphology (n), heterochromatin (black arrow) and a developed Golgi apparatus (white arrows). G. The arrows indicate the large number of mitochondria, the presence of smooth reticulum (H) and glycogen deposits (I). J. The arrows indicate two distinct cells after BDE-209 exposure. K. Extracellular vesicles are related to the dark cells (arrow). Observe in detail the high diversity of vesicles (arrow). Scale = 0.5 μm. L. The arrows show the vesicles emerging from the 'donor cell'. White arrow indicates the moment of vesicle exclusion from the cell. M. Observe the vesicles (black arrows) near the cell surface and fusion (white arrow).

protein responsible for cell-cell adhesion, playing an essential role in epithelial cell behavior, tissue formation and cancer suppression (van Roy and Berx, 2008); while vimentin is generally not present in normal epithelial cells *in vivo*, however, it can be expressed in epithelial cells *in vitro*, as well as in tumor cells of epithelial origin (Robinson and Han, 2006). In the current study, the studied POPs-exposed B16 cells presented increased vimentin expression and decrease E-cadherin expression.

MMPs seem to play important roles, as the members of this family have various implications in the complex pathogenesis of EMT (Scheau et al., 2019). Considering that BDE-209 and TCDD affected gene expression profile of metalloproteases and their inhibitors, as well as the expression of proteins involved in EMT, BDE-209 and TCDD-exposure can make B16–F1 cells more aggressive and similar to B16–F10 cells.

Although the mechanisms involved in the EMT induced by the chronic exposure of melanoma cells to TCDD and BDE-209 were not investigated here, aryl hydrocarbon receptor (AHR) may be involved on the effect observed. TCDD and BDE-209 activate the AHR, a ligand-activated transcription factor (Alonso et al., 2008; Veldhoen et al., 2008; Murray et al., 2014; Otarola et al., 2018). AHR has been reported to exert either positive or negative effects on EMT depending on cell type (Zhu et al., 2020; Moretti et al., 2020). Recently, it was demonstrated that he AHR mediate EMT in thyroid carcinoma (Moretti et al., 2020) and lung cells mediated by D-kynurenine (Duan et al., 2018). There is also evidence that AhR signaling pathway modulates the expression of genes involved in extracellular matrix remodeling, specifically MMPs (Hillegass et al., 2006). MMPs can promote metastasis by inducing EMT (Przybylo and Radisky, 2007) and have been associated with EMT in cancer progression through different mechanisms. First, high levels of MMPs in the tumor microenvironment can directly induce EMT in epithelial cells, and cancer cells that endure EMT can produce more MMPs, enabling cell invasion and metastasis. Second, EMT can generate activated stromal-like cells that drive cancer progression via further MMP production (Przybylo and Radisky, 2007). Overall, the expression of AHR in B16 cells and its role in TCDD and BDE-209 induced EMT must be further analyzed.

4.4. Microvesicles released after exposure to BDE-209 revealed a sophisticated mechanism of communication among B16–F1 cells

The association of microvesicles with the increase in the metastatic capacity of murine melanoma B16 cell lines was first described by Honn et al. (1986), but the studies on the role of these vesicles in tumor progression are still poorly exploited. The present study showed that B16–F1 cells, with low metastatic potential, exhibit a more metastatic phenotype by the expression of E-cadherin, vimentin and genes related to MMPs and TIMPs. Additionally, these cells largely increase the microvesicles release 24 h after exposure to BDE-209. These findings lead to the hypothesis that the presence of microvesicles could be an important cell communication mechanism among B16–F1 cells, interfering with the development and tumor progression.

Comparatively, tumor cells release a much larger number of microvesicles than normal cells, which are in general involved with initiation, growth, progression, and resistance of tumors to chemotherapy. We recently demonstrated exposure to BDE-209 led to an upregulation of the ABCB1 and ABCC4 genes in B16–F1 cells after acute exposure, followed by an increased protein activity after chronic exposure. These proteins are described as ATP-binding cassette (ABC) transporters in plasma membrane and are involved with chemoresistance (Marchi et al., 2021). According to Tkach and Théry (2016), tumor exosomes can intrinsically modify the mobility and invasiveness of the tumor's own cells, and it would explain the phenotypic changes observed by the expression of metastatic genes in the present study, after exposure to BDE-209. Brito et al. (2020) reported greater spread of tumor nodules in mice exposed to BDE-209 and injected with B16–F10 cells. It is possible, therefore, that the large number of microvesicles released by B16–F1 cells may be one of the possible explanations to understand the effect of BDE-209 in promoting phenotype changes and tumor development.

Overall, the current study is corroborated by Brito et al. (2020), who reported an increase of metastases incidence in the brain, liver, kidney, and gonads of mice previously exposed to BDE-209 and inoculation of B16–F10 cells. Additionally, the present results raise evidence that melanoma B16 cells exposed to BDE-209 and TCDD acquired a profile of gene and protein expression that may enhance their metastatic potential. BDE-209 has potential to modulate tumor cell phenotype that is similar or even higher than that of TCDD. The consequences of these

results to the progression of melanoma in humans is difficult to predict based only in *in vitro* data, but the study of Brito et al. (2020) may shed some light on this question, as the authors reported an increase metastasis in non-target organs in mice previously exposed to BDE-209, respectively. At least, these new approaches open additional opportunities of investigation on the role of organic pollutants as potential carcinogenic agents and.

5. Conclusion

Our data show that the pollutants TCDD and BDE-209 modulated the phenotype of tumor cells in the short and long-term exposures, which may have a deleterious impact on cancer prognosis. Acute exposure to BDE-209 and TCDD at environmental relevant concentrations (0.01, 0.1 and 1 nM) did not compromise the viability of B16–F1 and B16–F10 cells, but the downregulation of E-cadherin and upregulation of vimentin gene expression may indicate epithelial-mesenchymal transition. Up regulation of MMP2, MMP9, MMP14 and PGK1 genes and downregulation of TIMP2, TIMP3 and RECK genes in B16–F1 cells exposed to BDE-209 led to a profile very similar to that of B16–F10 cells, the more metastatic cell sub-line. Additionally, the release of extracellular vesicles from cells exposed to BDE-209 as a mechanism of cell communication may cause phenotype changes in B16–F1 cells, but further studies are necessary to confirm this hypothesis. As they are taken up not only by tumor cells, but also by healthy cells, extracellular vesicles may play an important role in tumor growth and invasion, tumor-associated angiogenesis, immunologic remodeling and pathogenesis. Together, these data alert the impact of environmental pollution on tumor aggressiveness and cancer prognosis.

Credit statements

Benisio Ferreira Silva Filho: Participated with the ideas, formulation of overarching research goals, development or design of methodology. Made the formal analysis and application of statistical, conducted a research and investigation process, specifically performing the experiments, write the initial draft, critical review, commentary or revision.

Francisco Filipak Neto: Participated with ideas and formulation of research goals. Worked in the formal analysis and application of statistical and worked in the original draft specifically with critical review, commentary or revision.

Micheli de Marchi and Erick Laurent Moggio: Participated with ideas and research goals, development or design of methodology for cell culture. Also worked in the original draft specifically with critical review, commentary or revision.

Izadora Volpato Rossi, Bruna Sabatke, Marcel Ivan Ramirez: Participated in the analysis, identification and characterization of microvesicles involving methodological replication or reproducibility of results and worked in the original draft specifically with critical review, commentary or revision.

Miguel Clodomiro dos Santos Lucena: Participated in the design and development of methodology in the immunoblotting and protein involved with Epithelial-Mesenchymal Transition, preparation and creation of results and worked in the original draft specifically with critical review, commentary or revision.

Adriane Regina Todeschini: Participated as co-director of the study with ideas and formulation of research goals. Include its participation in the preparation, creation and design and development of methodology. Also worked in the original draft specifically with critical review, commentary or revision.

Ciro Alberto de Oliveira Ribeiro: Participated as the director of the study, presenting ideas, formulation of research goals and creation of models. Also provided the study materials, reagents, materials, laboratory samples, animals, instrumentation, computing resources, or other analysis tools, and worked in the original draft specifically with critical review, commentary or revision, including management and coordination responsibility for the research activity planning and execution.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The study was supported by fellowship from the Brazilian agency CAPES (Coordination for the Improvement of Higher Education Personnel) and financial support from CNPq (National Council for Scientific and Technological Development – Finance Code 428830/2018–8). The authors thanks for the Center for Electron Microscopy at Federal University of Parana for ultrastructural analysis.

References

Abdallah, M.A., Harrad, S., 2014. Polybrominated diphenyl ethers in UK human milk: implications for infant exposure and relationship to external exposure. Environ. Int. 63, 130–136. https://doi.org/10.1016/j.envint.2013.11.009.

Alonso, M., Casado, S., Miranda, C., Tarazona, J.V., Navas, J.M., Herradón, B., 2008 Mar. Decabromobiphenyl (PBB-209) activates the aryl hydrocarbon receptor while decachlorobiphenyl (PCB-209) is inactive: experimental evidence and computational rationalization of the different behavior of some halogenated biphenyls. Chem. Res. Toxicol. 21 (3), 643–658. https://doi.org/10.1021/tx700362u. Epub 2008 Mar 1. PMID: 18311929.

Andaloussi, S.E.L., Mäger, I., Breakefield, X.A., Wood, M.J.A., 2013. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat. Rev. Drug Discov. 12 (5), 347–357. https://doi.org/10.1038/nrd3978. Epub 2013 Apr 15.

Babich, H., Borenfreund, E., 1990. Cells in Culture as Determined with the Neutral Red Assay. SAGE Journals n. 0, pp. 6–8. https://doi.org/10.1177/026119299001800116.1.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1006/abio.1976.9999.

Brito, P.M., Biscaia, S.M.P., Souza, T.L., Ramos, A.B., Leão-Buchir, J., Roque, A.A., Bellan, D.L., Trindade, E.S., Filipak Neto, F., Oliveira Ribeiro, C.A., 2020. Oral exposure to BDE-209 modulates metastatic spread of melanoma in C57BL/6 mice inoculated with B16-F10 cells. Chemosphere 260, 127556. https://doi.org/10.1016/j.chemosphere.2020.127556 0045-6535.

Chen, Y., Li, J., Liu, L., Zhao, N., 2012. Polybrominated diphenyl ethers fate in China: a review with an emphasis on environmental contamination levels, human exposure and regulation. J. Environ. Manag. 113, 22–30. https://doi.org/10.1016/j.jenvman.2012.08.003.

Collins, J.J., Budinsky, R.A., Burns, C.J., Lamparski, L.L., Carson, M.L., Martin, G.D., Wilken, M., 2006. Serum dioxin levels in former chlorophenol workers. J. Expo. Sci. Environ. Epidemiol. 16 (1), 76–84. https://doi.org/10.1038/sj.jea.7500439.

Craene, B., Berx, G., 2013. Regulatory networks defining EMT during cancer initiation and progression. Nat. Rev. Cancer 13 (2), 97–110. https://doi.org/10.1038/nrc3447.

Das, V., Bhattacharya, S., Chikkaputtaiah, C., Hazra, S., Pal, M., 2019. The basics of epithelial-mesenchymal transition (EMT): a study from a structure, dynamics, and functional perspective. J. Cell. Physiol. 234 (9), 14535–14555. https://doi.org/10.1002/jcp.28160.

Dong, Q., Yu, D., Yang, C.M., Jiang, B., Zhang, H., 2010. Expression of the reversion-inducing cysteine-rich protein with Kazal motifs and matrix metalloproteinase-14 in neuroblastoma and the role in tumour metastasis. Int. J. Exp. Pathol. 91 (4), 368–373. https://doi.org/10.1111/j.1365-2613.2010.00724.x.

Duan, Z., Li, Y., Li, L., 2018 Nov. Promoting epithelial-to-mesenchymal transition by D-kynurenine via activating aryl hydrocarbon receptor. Mol. Cell. Biochem. 448 (1–2), 165–173. https://doi.org/10.1007/s11010-018-3323-y. Epub 2018 Feb 13. PMID: 29442266.

European Union – POPs Poluentes Orgânicos Persistentes. Disponível em. https://ec.europa.eu/environment/archives/pops/index_en.htm, acesso em 05/01/2021.

Foster, Warren G., Maharaj-Briceño, Serena, Cyr, Daniel G., 2011. Dioxin-induced changes in epididymal sperm count and spermatogenesis. Ciência Saúde Coletiva 16 (6), 2893–2905. https://doi.org/10.1590/S1413-81232011000600027.

Frederiksen, M., Thomsen, C., Frøshaug, M., Vorkamp, K., Thomsen, M., Becher, G., Knudsen, L.E., 2010. Polybrominated diphenyl ethers in paired samples of maternal and umbilical cord blood plasma and associations with house dust in a Danish cohort. Int. J. Hyg Environ. Health 213 (4), 233–242. https://doi.org/10.1016/j.ijheh.2010.04.008.

Fujisawa, N., Yoshioka, W., Yanagisawa, H., Tohyama, C., 2018. Roles of cytosolic phospholipase $A_2\alpha$ in reproductive and systemic toxicities in 2,3,7,8-tetrachlorodibenzo-p-dioxin-exposed mice. Arch. Toxicol. 92 (2), 789–801. https://doi.org/10.1007/s00204-017-2081-z.

Gao, Z., Bu, Y., Liu, X., Wang, X., Zhang, G., Wang, E., Ding, S., Liu, Y., Shi, R., Li, Q., Fu, J., Yu, Z., 2016. TCDD promoted EMT of hFPECs via AhR, which involved the activation of EGFR/ERK signaling. Toxicol. Appl. Pharmacol. 298, 48–55. https://doi.org/10.1016/j.taap.2016.03.005.

Gillies, R.J., Didier, N., Denton, M., 1986. Determination of cell number in monolayer cultures. Anal. Biochem. 159 (1), 109–113. https://doi.org/10.1016/0003-2697(86)90314-3.

He, W., He, P., Wang, A., Xia, T., Xu, B., Chen, X., 2008. Effects of PBDE-47 on cytotoxicity and genotoxicity in human neuroblastoma cells in vitro. Mutat. Res. 649 (1–2), 62–70. https://doi.org/10.1016/j.mrgentox.2007.08.001.

Hillegass, J.M., Murphy, K.A., Villano, C.M., White, L.A., 2006 Sep. The impact of aryl hydrocarbon receptor signaling on matrix metabolism: implications for development and disease. Biol. Chem. 387 (9), 1159–1173. https://doi.org/10.1515/BC.2006.144.PMID:16972783.

Hofmann, U.B., Westphal, J.R., Zendman, A.J., Becker, J.C., Ruiter, D.J., van Muijen, G. N., 2000. Expression and activation of matrix metalloproteinase-2 (MMP-2) and its co-localization with membrane-type 1 matrix metalloproteinase (MT1-MMP) correlate with melanoma progression. J. Pathol. 191 (3), 245–256. https://doi.org/10.1002/1096-9896(2000)9999:9999<::AID-PATH632>3.0.CO;2-#.

Honn, K.V., Powers, W.E., Sloane, B.F., 1986. Mechanisms of cancer metastasis. In: Developments in Oncology, vol. 40. Springer, Boston, MA. https://doi.org/10.1007/978-1-4613-2635-9_18.

Instituto Nacional de Câncer José Alencar Gomes da Silva (a), 2020. Estimativa 2020: incidência de câncer no Brasil [Internet]. INCA, Rio de Janeiro. Disponível em: https://www.inca.gov.br/sites/ufu.sti.inca.local/files/media/document/estimativa-2020- incidencia-de-cancer-no-brasil.pdf.

Kim, Y.H., Shim, Y.J., Shin, Y.J., Sul, D., Lee, E., Min, B.H., 2009. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces calcium influx through T-type calcium channel and enhances lysosomal exocytosis and insulin secretion in INS-1 cells. Int. J. Toxicol. 28 (3), 151–161. https://doi.org/10.1177/1091581809336885.

Leonel, J., Sericano, J.L., Secchi, E.R., Bertozzi, C., Fillmann, G., Montone, R.C., 2014. PBDE levels in franciscana dolphin (Pontoporia blainvillei): temporal trend and geographical comparison. Sci. Total Environ. 493, 405–410. https://doi.org/10.1016/j.scitotenv.2014.06.003.

Leonetti, C., Butt, C.M., Hoffman, K., Hammel, S.C., Miranda, M.L., Stapleton, H.M., 2016. Brominated flame retardants in placental tissues: associations with infant sex and thyroid hormone endpoints. Environ. Health: a global access science source 15 (1), 113. https://doi.org/10.1186/s12940-016-0199-8.

Liu, Q., Wang, K., Shao, J., Li, C., Li, Y., Li, S., Liu, X., Han, L., 2017. Role of taurine in BDE 209-induced oxidative stress in PC12 cells. Adv. Exp. Med. Biol. 975 Pt 2, 897–906. https://doi.org/10.1007/978-94-024-1079-2_71.

Marchi, M., Moggio, E.E., Filipak Neto, F., Brito, P.E.M., Silva Filho, B.F., Oliveira Ribeiro, C.A., 2021. BDE-209 and TCDD modulate the expression and activity of ATP-binding cassette (ABC) transporters in murine melanoma cells (B16-F1). Int J Cancer Sci Ther 3 (2), 2–7. https://doi.org/10.31487/j.IJCST.2021.02.01.

McGuinn, L.A., Ghazarian, A.A., Ellison, G.L., Harvey, C.E., Kaefer, C.M., Reid, B.C., 2012. Cancer and environment: definitions and misconceptions. Environ. Res. 112, 230–234. https://doi.org/10.1016/j.envres.2011.10.009.

Mesquita, L.G., Diniz, S.F., Queiroz, F. T. H. de, Souza, L. A. de M. e, Pavani, T.A. e S., Sant'anna, L.B., Pérez, M. de A., Pérez, L.K., Tavares, C.P., 2020. Câncer de Pele e Renda Familiar: um Estudo Ecológico. Revista Brasileira de Cancerologia 66 (4), 1–5. https://doi.org/10.32635/2176-9745.RBC.2020v66n4.949.

Mohankumar, K., Hariharan, V., Rao, N.P., 2016. Heavy metal contamination in groundwater around industrial estate vs residential areas in coimbatore, India. J. Clin. Diagn. Res.: J. Clin. Diagn. Res. 10 (4) https://doi.org/10.7860/JCDR/2016/15943.7527. BC05–BC7.

Moretti, S., Nucci, N., Menicali, E., Morelli, S., Bini, V., Colella, R., Mandarano, M., Sidoni, A., Puxeddu, E., 2020 Jan 7. The aryl hydrocarbon receptor is expressed in thyroid carcinoma and appears to mediate epithelial-mesenchymal-transition. Cancers 12 (1), 145. https://doi.org/10.3390/cancers12010145. PMID: 31936153; PMCID: PMC7016998.

Munshi, A., Hobbs, M., Meyn, R.E., 2005. Clonogenic cell survival assay. In: Blumenthal, R.D. (Ed.), Chemosensitivity. *Methods In Molecular Medicin*, vol. 110. Humana Press. https://doi.org/10.1385/1-59259-869-2:021.

Murray, I.A., Patterson, A.D., Perdew, G.H., 2014 Dec. Aryl hydrocarbon receptor ligands in cancer: friend and foe. Nat. Rev. Cancer 14 (12), 801–814. https://doi.org/10.1038/nrc3846. PMID: 25568920; PMCID: PMC4401080.

Neidhart, B., Kowalska, M., Valentin, J., Gall, F.M., Ren, Q., Riedl, R., Pot, S., Rottmar, M., 2021. Tissue inhibitor of metalloproteinase (TIMP) peptidomimetic as an adjunctive therapy for infectious keratitis. Biomacromolecules 22 (2), 629–639. https://doi.org/10.1021/acs.biomac.0c01473.

Nunes, Helga Caputo, 2018. Potential de diferenciação de células-tronco mesenquimais tecido adiposo humanas expostas ao 2,3,7,8-tetraclorodibenzo-p-dioxina (TCDD) e bisfenol A (BPA)/Helga Caputo Nunes. - Botucatu (São Paulo, Brazil).

Otarola, G., Castillo, H., Marcellini, S., 2018 Apr. Aryl hydrocarbon receptor-based bioassays for dioxin detection: thinking outside the box. J. Appl. Toxicol. 38 (4), 437–449. https://doi.org/10.1002/jat.3575. Epub 2017 Dec 26. PMID: 29277905.

Overwijk, W.W., Restifo, N.P., 2001. B16 as a Mouse Model for Human Melanoma. Current Protocols in Immunology. https://doi.org/10.1002/0471142735.im2001s39 (Chapter 20), Unit–20.1.

Pereira, L.C., Souza, A.O., Tasso, M.J., Oliveira, A.M.C., Duarte, F.V., Palmeira, C.M., Dorta, D.J., 2017. Exposure to decabromodiphenyl ether (BDE-209) produces mitochondrial dysfunction in rat liver and cell death. J. Toxicol. Environ. Health, Part A 80 (19–21), 1129–1144. https://doi.org/10.1080/15287394.2017.1357370.

Przybylo, J.A., Radisky, D.C., 2007. Matrix metalloproteinase-induced epithelial-mesenchymal transition: tumor progression at Snail's pace. Int. J. Biochem. Cell Biol. 39 (6), 1082–1088. https://doi.org/10.1016/j.biocel.2007.03.002.

Reilly P, T, Bellevue H, F, Woster M, P, vensson K, C, 1998. Comparison of the in vitro cytotoxicity of hydroxylamine metabolites of sulfamethoxazole and dapsone. Biochemical Pharmacology 55 (6), 803–810. https://doi.org/10.1016/s0006-2952(97)00547-9.

Robinson, B.B., Han, A., 2006. Role of immunohistochemistry in elucidating lung cancer metastatic to the ovary from primary ovarian carcinoma. In: Hayat, M.A. (Ed.), Handbook of Immunohistochemistry and in Situ Hybridization of Human Carcinomas, pp. 537–545. https://doi.org/10.1016/S1874-5784(05)80116-3, 4.

Roy, R., Yang, J., Moses, M.A., 2009. Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. J. Clin. Oncol.: official journal of the American Society of Clinical Oncology 27 (31), 5287–5297. https://doi.org/10.1200/JCO.2009.23.5556.

Salgado S, IC, Ferreira B, M, Luz Z, J, Filipak Neto, F, Oliveira Ribeiro A, C, 2018. Tribromophenol affects the metabolism, proliferation, migration and multidrug resistance transporters activity of murine melanoma cells B16F1. Toxicology in vitro 50, 40–46. https://doi.org/10.1016/j.tiv.2018.02.005.

Sanabria, M., 2014. Toxicologia reprodutiva de ratos adultos expostos ao 2,3,7,8-tetraclorodibenzo-p-dioxina (TCDD) in útero – Botucatu (São Paulo, Brazil).

Scheau, C., Badarau, I.A., Costache, R., Caruntu, C., Mihai, G.L., Didilescu, A.C., Constantin, C., Neagu, M., 2019. The role of matrix metalloproteinases in the epithelial-mesenchymal transition of hepatocellular carcinoma. Anal. Cell Pathol. 26, 9423907 https://doi.org/10.1155/2019/9423907.

Schecter, A., Pavuk, M., Päpke, O., Ryan, J.J., Birnbaum, L., Rosen, R., 2003. Polybrominated diphenyl ethers (PBDEs) in U.S. mothers' milk. Environ. Health Perspect. 111, 1723–1729. https://doi.org/10.1289/ehp.6466.

Shaverdashvili, K., Wong, P., Ma, J., Zhang, K., Osman, I., Bedogni, B., 2014. MT1-MMP modulates melanoma cell dissemination and metastasis through activation of MMP2 and RAC1. Pigment cell & melanoma research 27 (2), 287–296. https://doi.org/10.1111/pcmr.12201.

Sjödin, A., Päpke, O., McGahee, E., Focant, J.-F., Jones, R.S., Pless-Mulloli, T., Toms, L.-M.L., Herrmann, T., Müller, J., Needham, L.L., Patterson, D.G., 2008. Concentration of polybrominated diphenyl ethers (PBDEs) in household dust from various countries. Chemosphere 73, S131–S136. https://doi.org/10.1016/j.chemosphere.2007.08.075.

Steil J, G, Buzzo A, JL, Oliveira Ribeiro A, C, Filipak Neto, F, 2022. Polybrominated diphenyl ethers BDE-47 and BDE-99 modulate murine melanoma cell phenotype in vitro. Environmetal Science and Pollution Research 29, 11291–11303. https://doi.org/10.1007/s11356-021-16455-0.

Théry, C., Clayton, A., Amigorena, S., Raposo, G., 2006. Isolation and Characterization of exosomes from cell culture supernatants and biological fluids. Current Protocols in Cell Biology 3, 22.1-22.3.22.29.

Tkach, M., Théry, C., 2016. Communication by extracellular vesicles: where we are and where we need to go. Cell 164 (6), 1226–1232. https://doi.org/10.1016/j.cell.2016.01.043.

van Roy, F., Berx, G., 2008. The cell-cell adhesion molecule E-cadherin. Cell. Mol. Life Sci. : CM 65 (23), 3756–3788. https://doi.org/10.1007/s00018-008-8281-1.

Veldhoen, M., Hirota, K., Westendorf, A.M., Buer, J., Dumoutier, L., Renauld, J.C., Stockinger, B., 2008 May 1. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature 453 (7191), 106–109. https://doi.org/10.1038/nature06881. Epub 2008 Mar 23. PMID: 18362914.

WHO (World Health Organization), 2020. http://www.who.int/mediacentre/factsheets/fs225/en/. (Accessed 4 November 2020) accessed on.

Wu, N., Herrmann, T., Paepke, O., Tickner, J., Hale, R., Harvey, L.E., La Guardia, M., McClean, M.D., Webster, T.F., 2007. Human exposure to PBDEs: associations of PBDE body burdens with food consumption and house dust concentrations. Environ. Sci. Technol. 41 (5), 1584–1589. https://doi.org/10.1021/es0620282.

Xu, T., Xie, H.Q., Li, Y., Xia, Y., Sha, R., Wang, L., Chen, Y., Xu, L., Zhao, B., 2018. Dioxin induces expression of hsa-miR-146b-5p in human neuroblastoma cells. J. Environ. Sci. 63, 260–267. https://doi.org/10.1016/j.jes.2017.06.038.

Yu, L., Han, Z., Liu, C., 2015. A review on the effects of PBDEs on thyroid and reproduction systems in fish. Gen. Comp. Endocrinol. 219, 64–73. https://doi.org/10.1016/j.ygcen.2014.12.010.

Zhi, H., Wu, J.P., Lu, L.M., Li, Y., Chen, X.Y., Tao, J., Mai, B.X., 2018. Decabromodiphenyl ether (BDE-209) enhances foam cell formation in human macrophages via augmenting Toll-like receptor 4-dependent lipid uptake. Food Chem. Toxicol. 121, 367–373. https://doi.org/10.1016/j.fct.2018.09.024.

Zhou, B., Wang, X., Li, F., Wang, Y., Yang, L., Zhen, X., Tan, W., 2017. Mitochondrial activity and oxidative stress functions are influenced by the activation of AhR-induced CYP1A1 overexpression in cardiomyocytes. Mol. Med. Rep. 16 (1), 174–180. https://doi.org/10.3892/mmr.2017.6580.

Zhu, P., Yu, H., Zhou, K., Bai, Y., Qi, R., Zhang, S., 2020 Jun 16. 3,3'-Diindolylmethane modulates aryl hydrocarbon receptor of esophageal squamous cell carcinoma to reverse epithelial-mesenchymal transition through repressing RhoA/ROCK1-mediated $COX2/PGE_2$ pathway. J. Exp. Clin. Cancer Res. 39 (1), 113. https://doi.org/10.1186/s13046-020-01618-7. PMID: 32546278; PMCID: PMC7298755.