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# Low concentrations of Bisphenol A and *para*-Nonylphenol affect extravillous pathway of human trophoblast cells

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## ABSTRACT

Bisphenol A (BPA) and *para*-Nonylphenol (*p*-NP) are chemicals of industrial origin which may influence human reproductive health. The effects of these substances in the prenatal life is an important topic that is receiving greater attention in the developed countries. In this study, human trophoblast cells HTR-8/SVneo were exposed to BPA and *p*-NP ( $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M) and incubated for 24, 48 and/or 72 h then, examined for the main physiological processes which characterize the extravillous trophoblast. Cell proliferation showed no changes while the processes of cell migration and invasion were both reduced by BPA and *p*-NP. For each chemical, the activity was higher at lower concentrations with a maximum activity between  $1 \times 10^{-13}$  and  $1 \times 10^{-11}$  M ( $p < 0.05$  for  $1 \times 10^{-9}$  and  $p < 0.001$  for  $1 \times 10^{-11}$  M). Co-culture studies with human umbilical cord endothelial cells (HUEVC) revealed that trophoblast/endothelial interaction was significantly reduced by *p*-NP at  $1 \times 10^{-11}$  M. Moreover, both chemicals were inducing differentiation of HTR-8/SVneo toward polyploidy by the process of endoreduplication. The estrogen-receptor antagonist ICI significantly reduced *p*-NP action, while it had no effect on BPA treated cells. In conclusion, *p*-NP and BPA act on trophoblast cells altering key physiological processes in placenta development. The exact mechanism of action of the chemicals in human trophoblast still needs to be clarified.

## 1. Introduction

Placenta is the fetal organ interposed between the fetus and the mother which provides transfer of nutrients, gases as well as waste products, the secretion of hormones and acts as an protective immune barrier between the mother and the fetus (Gude et al., 2004). Because of its strategic location in the uterus, this organ is the link between environment and the fetus. Thus, any potentially harmful substance coming from the environment may have a negative effect impact on this organ. Maternal contamination with environmental substances may occur by cutaneous absorption, by ingestion of either contaminated food or water and by breathing contaminated air (Caserta et al., 2011). Among the environmental substances potentially contaminating pregnant mothers, Bisphenol

A (BPA) is receiving increased attention due to its negative effects on humans' health (Rochester, 2013). Despite that, BPA is still largely used in drinking glasses, food-storage containers, the lining of food beverage containers, dental sealants, medical equipment, tubing and consumer electronics (Vandenberg et al., 2007). Another potentially hazardous chemical is *p*-Nonylphenol (*p*-NP), a metabolite of alkylphenol ethoxylates used as surfactant in the manufacturing industry and present in detergents, paints, pesticides, personal care products, and plastics (de Weert et al., 2008). Many reports showed that BPA and *p*-NP affect different human reproductive processes including increased incidence of infertility, genital tract abnormalities and carcinogenesis in estrogen-sensitive tissues (Fowler et al., 2012; Sharpe and Skakkebaek, 1993; Skakkebaek et al., 1998; Sonnenschein and Soto, 1998). *In vivo* studies showed that exposure to BPA determined prostate and mammary cancers, earlier puberty, morphological changes in the ovary, uterus, and vagina, disruption of estrous cyclicity, body weight problems, alteration in the mammary gland (Howdeshell et al., 1999; Markey et al., 2003; Munoz-de-Toro et al., 2005; Nikaido et al., 2004). Studies in rats showed that exposure to *p*-NP increased calbindin-D9K mRNA expression in maternal and neonatal uteri and interfered with estrous cycle and pubertal onset (Hong et al., 2003, 2004; Kim et al., 2002a; Nikaido et al., 2004).

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Much work has been done in the last decade in our laboratory to investigate the effect of these two substances on human placenta. In particular, by using BeWo trophoblast cell line and a primary model of chorionic villous explants, we demonstrated that both these substances, BPA and p-NP, increased the secretion of the Chorionic Gonadotropin ( $\beta$ -hCG), the fundamental hormone in human pregnancy (Bechi et al., 2006, 2010). We also reported that p-NP at very low levels, ranging from  $1 \times 10^{-13}$  to  $1 \times 10^{-9}$  M, was able to alter placental secretion of cytokines mainly, GM-CSF and IL-10, two key mediators for normal placental development and fetal growth (Bechi et al., 2010). p-NP was also inducing trophoblast differentiation and cell apoptosis with a higher potency than 17 $\beta$ -estradiol (Bechi et al., 2006). In collaboration with a Danish group, using dual perfusion system, we also demonstrated that BPA is rapidly transferred across a placenta cotyledon (Morck et al., 2010).

Trophoblast cell migration and invasion in the maternal tissues are pre-requisites for normal embryo implantation. These processes give rise to several extravillous trophoblast (EVT) subtypes that move deeply into the decidua up to the spiral arteries. The invasion of trophoblast into the uterine stroma does not occur beyond the decidua and the first third of the underlying myometrium. The process stops when these cells differentiate into polyploid cells by endoreduplication thus forming the trophoblast giant cells (Bischof and Irrminger-Finger, 2005). Failure in EVT differentiation is associated with several complications to pregnancy including miscarriage (Burton et al., 2010; Hustin et al., 1990), placenta accreta (Chakraborty et al., 2002), pre term birth (Kim et al., 2002b), preeclampsia and fetal growth restriction (Burton and Jauniaux, 2004; Khong et al., 1986; Sibai et al., 2005).

Herein, using the HTR-8/SVneo cell line originally generated from villous explants at early pregnancy (Graham et al., 1993) and widely used to model the physiologically invasive EVT (Irving et al., 1995; Takao et al., 2011), we investigated whether exposure to BPA and p-NP affects the behavior of these cells analyzing the main physiological processes which characterize the extravillous trophoblast pathway e.g. trophoblast proliferation, invasion, migration as well as its interaction with endothelial cells and differentiation into polyploid giant cells.

## 2. Materials and methods

### 2.1. Chemicals

BPA and p-NP were purchased from Sigma Aldrich (Sigma Chemical Co, St. Louis, MO). Both compounds were dissolved in 100% ethanol and stored in glass containers at room temperature. Stock solutions were appropriately diluted with the culture medium just before their use for treatment on cells.

### 2.2. HTR-8/SVneo cell treatment

HTR-8/SVneo cells, kindly provided by Dr. Charles Graham (Queens University, Kingston, Canada), were cultured in RPMI 1640 medium without phenol red (Sigma Chemical Co) supplemented with 5% fetal bovine serum (FBS) (Biobrom, Berlin, Germany), 100 U/ml penicillin/streptomycin, and 2 mM glutamine (Sigma Chemical Co) (complete medium). Cells were cultured in 75 cm<sup>2</sup> flasks in a humidified 5% CO<sub>2</sub>-95% air atmosphere at 37 °C until 80% confluence.

For each experiment, HTR-8/SVneo cells were seeded in 25 cm<sup>2</sup> flasks or in 96-well plates depending on the type of analyses, in complete medium; after 4 h, medium was replaced with new medium containing 1% FBS and cultures were incubated overnight. The day after, cells were exposed to BPA or p-NP at the concentrations of  $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M in fresh 1% FBS,

RPMI 1640. Control cultures were exposed to vehicle alone (0.1% ethanol in 1% FBS, RPMI 1640).

### 2.3. HTR-8/SVneo cell viability

The viability of HTR-8/SVneo cells was assessed using the trypan blue exclusion test. Cells at 70% of confluence, in 25 cm<sup>2</sup> flasks, were cultured in 1% FBS, RPMI 1640, containing BPA or p-NP ( $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M) for 24, 48 and 72 h. Control cultures were exposed to vehicle. At each time, the cells were detached with trypsin and an aliquot of 20  $\mu$ l of cell suspension was mixed (1:1) with Trypan blue dye (Sigma Chemical Co). Cell viability was detected by counting unstained cells by two independent investigators, under an optical microscope using a Burkert chamber. The results of at least three experiments with 5 replicates are presented as the means of viable cells for a single chemical or control (0.1% ethanol), at each time of incubation.

### 2.4. HTR-8/SVneo cell proliferation

Cell proliferation was determined using the nonradioactive 5-bromo-2'-deoxyuridine-based (BrdU) cell proliferation assay (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. In brief, HTR-8/SVneo cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well and then exposed to BPA or p-NP ( $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M) for 24, 48 and 72 h in RPMI 1640 (1% FBS). Control cultures were exposed to vehicle. At each time, the cells were labeled with BrdU for 4 h and then incubated with anti-BrdU antibody. BrdU incorporation was detected using a colorimetric substrate solution and absorbance was measured at 450 and 690 nm. The experiments were carried out at least three times in 5 replicates and the results are presented as percentage of control values for a single chemical, at each time of incubation.

### 2.5. HTR-8/SVneo cell migration and invasion

HTR-8/SVneo cell migration and invasion were evaluated in 24-well modified Boyden chamber as previously described (Gentilini et al., 2007; Prast et al., 2008). The effects of BPA and p-NP, at the concentrations of  $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M, were assessed by pre-treating the cells with the chemicals and incubated 1% FBS, RPMI 1640, for 24, 48 and 72 h, before being seeded in the Boyden chamber. Transwells with polycarbonate filters (6.5 mm; 8  $\mu$ m pore size) (Becton Dickinson, San Diego, CA, USA) were coated with 10  $\mu$ g/ml type IV collagen (Sigma Chemical Co) or 15  $\mu$ l Growth Factor Restricted (GFR) Matrigel (Collaborative Biotech, Bedford, MA), for the migration and the invasion assay, respectively. The pre-treated cells were then seeded in the upper chamber of Transwells at a density of  $5 \times 10^4$  cells/well in 100  $\mu$ l of 1% FBS, RPMI 1640, containing fresh treatments (BPA and p-NP, at the concentrations of  $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M). Medium with 10% FBS was placed in the lower chamber as a chemoattractant factor. The plates were incubated for 4 h or 24 h at 37 °C for the migration and the invasion assay, respectively. Subsequently, the cells that remained on top of the filters as well as the collagen or Matrigel coating were scraped off. Filters were fixed with methanol and acetone (1:1), stained with hematoxylin, placed on glass slides and mounted with 20% glycerol in phosphate buffer saline (PBS). The number of cells that had reached the lower side of the filter was quantified by visual counting of five random areas under a light microscope, by two independent investigators. The migration and invasion capacity was calculated as the percentage of cells that passed through the membranes in the presence of treatments with respect to controls (0.1% ethanol) for



single chemicals, at each time of pre-exposure. The experiments were carried out at least three times in triplicates.

## 2.6. HTR-8/SVneo cell interaction with endothelial cells

Human umbilical cord endothelial cells (HUVEC) were obtained from Cambrex (East Rutherford, NJ, USA) and cultured in 10% FBS, EBM-2 medium (Cambrex) under standard conditions of 5% CO<sub>2</sub>-95% air, at 37 °C.

HTR-8/SVneo interaction with HUVEC cells was determined by a co-culture system. Glass cover slips (ø 10 mm) were first coated with 150 µl of GFR Matrigel (Collaborative Research) in 24 well plates. HUVEC cells were stained with an orange cell tracker, CMTMR (Invitrogen, Carlsbad, CA, USA) for 30 minutes, then collected by trypsinization and finally added to the GFR Matrigel-coated glass cover slips at a concentration of  $1 \times 10^5$  cells/well. The cells were monitored by fluorescent Leitz Diaplan microscopy (Leica, Wetzlar, Germany) for endothelial tube formation over the next 12 h in culture. HTR-8/SVneo were cultured in 25 cm<sup>2</sup> flasks and treated with  $1 \times 10^{-15}$  M BPA,  $1 \times 10^{-13}$  M p-NP or 0.1% ethanol (controls) for 48 h. Cells were then labeled with a green cell tracker CMFDA (Invitrogen) for 30 minutes, collected and seeded at  $1 \times 10^5$  cells/well on the endothelial tube structures. Over the next 24–48 h, trophoblast-endothelial cell interaction was monitored and recorded by fluorescence microscope (Leica) coupled with a camera. Four random images were taken from each treatment. The cell interaction was quantified by ImageJ measuring the two fluorescence (orange for HUVEC and green for HTR-8/SVneo cells) in four fields, selected by two independent investigators. The results from three experiments in triplicates were expressed as ratio between HUVEC and HTR-8/SVneo cell fluorescence.

## 2.7. HTR-8/SVneo cell differentiation into polyploid giant cells

### 2.7.1. Cell DNA content

Total DNA content in HTR-8/SVneo cells was evaluated by CyQuant<sup>®</sup> NF assay Kit (Invitrogen). Cells were seeded in 96 well plates at  $1 \times 10^4$  cells/well in 100 µl of 1% FBS, RPMI 1640 medium containing treatments (BPA or p-NP at the concentrations of  $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M) or 0.1% ethanol (controls). At 24, 48 and 72 h, the absorbance/fluorescence was measured using a Cytofluor 2350 fluorescence scanner (Millipore Corp, Bedford, MA). HTR-8/SVneo cells assayed with CyQuant<sup>®</sup> NF were also photographed by inverted fluorescence Olympus IX81 microscope (Olympus, Hamburg, Germany) at 72 h. Cells in five random fields per well were counted. Results from three experiments in triplicates are presented as percentage of cells with an enlarged nucleus ( $> 10 \mu\text{m}$ ) with respect to total cells counted in each field. In a second set of experiments cells were seeded in a 96 well plates at  $1 \times 10^4$  cells/well and exposed for 48 h to BPA or p-NP at  $1 \times 10^{-15}$  M with or without 1 µM estrogen-receptor antagonist (ICI).

### 2.7.2. Cell morphology

HTR-8/SVneo cells were grown in 25 cm<sup>2</sup> flasks and treated with medium containing BPA and p-NP at concentration of  $1 \times 10^{-15}$  M or 0.1% ethanol (controls) for 5 days. Cells were then detached with trypsin and seeded at a density of  $2 \times 10^4$  cells on cover slips in a 24-well plate in medium containing the same treatment. After 24 h, cells were washed three times with cold PBS, fixed with 4% paraformaldehyde (PFA) for 20 min at 4 °C, and washed again in PBS. Non-specific binding sites were blocked using 3% goat serum for 30 min. Cells were then incubated for 1 h at room temperature with mouse anti-cytokeratin 7 antibody (Sigma Chemical Co) diluted 1:100 in 1% goat serum. After three washes in PBS the cells were incubated with goat anti-mouse secondary antibodies (IgG) conjugated to Alexa Fluor A488 dye streptavidin conjugate (Invitrogen) diluted 1:100

for 30 min at room temperature. Cells were then washed in PBS and nuclei were identified with Hoechst 3342 nuclear stain (1 µg/ml) (Sigma Chemical Co). Digital images of cellular morphology were captured with a camera connected to a fluorescence microscope (Leica). Morphometric analysis performed in three separate experiments was determined by measuring the total area of the nucleus and cytoplasm in five random fields per cover slip by ImageJ.

## 2.8. Western blot

HTR-8/SVneo cells were seeded in 25 cm<sup>2</sup> flasks and were treated with BPA and p-NP ( $1 \times 10^{-15}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M) or 0.1% ethanol (controls) for 72 h. After two washes in PBS the cells were exposed to lysing buffer (50 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 1 mM sodium orthovanadate, 25 mM sodium fluoride and a protease inhibitor cocktail containing 4-(2-aminoethyl benzenesulfonyl fluoride), pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma Chemical Co). Protein lysates were clarified by centrifuging at  $13,000 \times g$  for 15 min at 4 °C and total protein concentration was determined by the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Thirty micrograms of total proteins was separated on 10% polyacrylamide gel in the presence of SDS and β-mercaptoethanol, and the proteins were electrotransferred to PVDF membranes (Thermo Scientific). Blotted membranes were incubated in 4% fat dry milk in 20 mM Tris-buffered saline pH 7.2 (TBS) for 3 h at room temperature and incubated overnight with rabbit polyclonal anti-human p57<sup>KIP2</sup> (diluted 1:1000) (Thermo Scientific), or mouse monoclonal anti-human beta actin (1:1000) (Santa Cruz Biotechnology) in TBS plus 4% fat dry milk. Next, the membranes were exposed to the respective peroxidase-labeled secondary antibodies (1:3000) (Bio-Rad Laboratories) in TBS plus 4% fat dry milk for 2 h at room temperature. The reaction was revealed by chemiluminescence kit (Thermo Scientific) according to the manufacturer's instructions. Images were digitalized with CHEMI DOC Quantity One (Bio-Rad Microscience), and densitometry analysis was performed by Quantity One software (Bio-Rad Microscience).

## 2.9. Statistical analysis

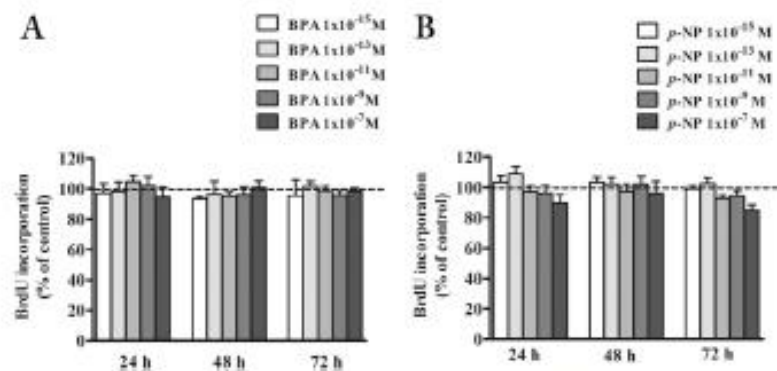
The data were analyzed with GraphPad Prism 4 using one-way analysis of variance (ANOVA), followed by Dunnett's test or Student's t-test when appropriate.  $p < 0.05$  was considered to be statistically significant.

## 3. Results

In order to use BPA and p-NP at the concentrations selected ( $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M) in functional assays on HTR-8/SVneo cells, we first evaluated their possible impact on cell viability, by trypan blue exclusion (data not shown). In accordance with previous reports on trophoblast, no decrease in cell viability was observed at any of the concentrations tested of either BPA or p-NP (Bechi et al., 2013; Morck et al., 2010).

### 3.1. BPA and p-NP do not affect HTR-8/SVneo cell proliferation

HTR-8/SVneo cell proliferation was evaluated by BrdU incorporation. As shown in Fig. 1 no changes were observed with any treatment (BPA or p-NP), at any concentration ( $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M) and any time of exposure, with respect to control cultures (0.1% ethanol), at the same time of exposure.

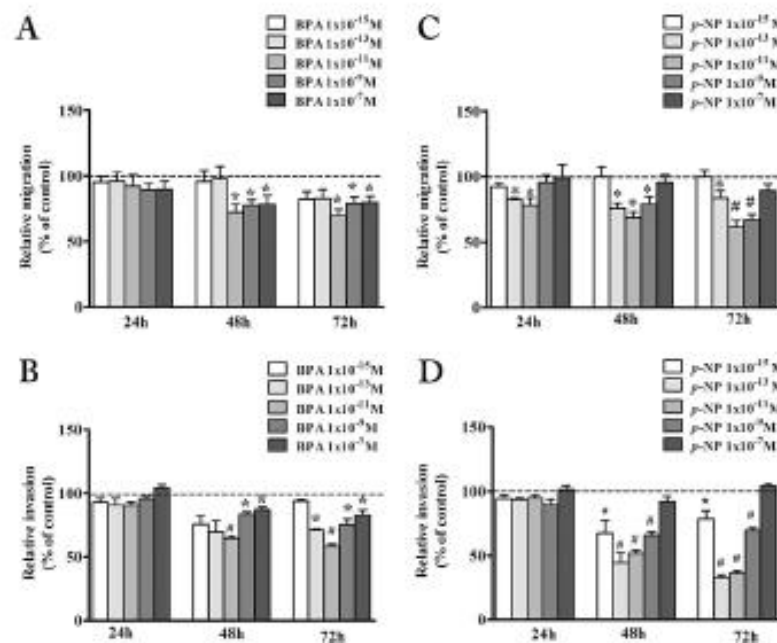


**Fig. 1.** Effect of BPA and p-NP on HTR-8/SVneo cell proliferation. HTR8/SV-neo cells were treated with BPA (A) and p-NP (B) at the concentrations of  $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M for 24, 48 and 72 h. Cell proliferation was determined by the BrdU incorporation assay. Data are expressed as percentage vs control vehicle-treated cultures (ethanol 0.1%; dotted line) and reported as means  $\pm$  SEM. The results represent at least  $n = 3$  experiments performed in five replicate for each data point.

### 3.2. BPA and p-NP impair HTR-8/SVneo cell migration and invasion

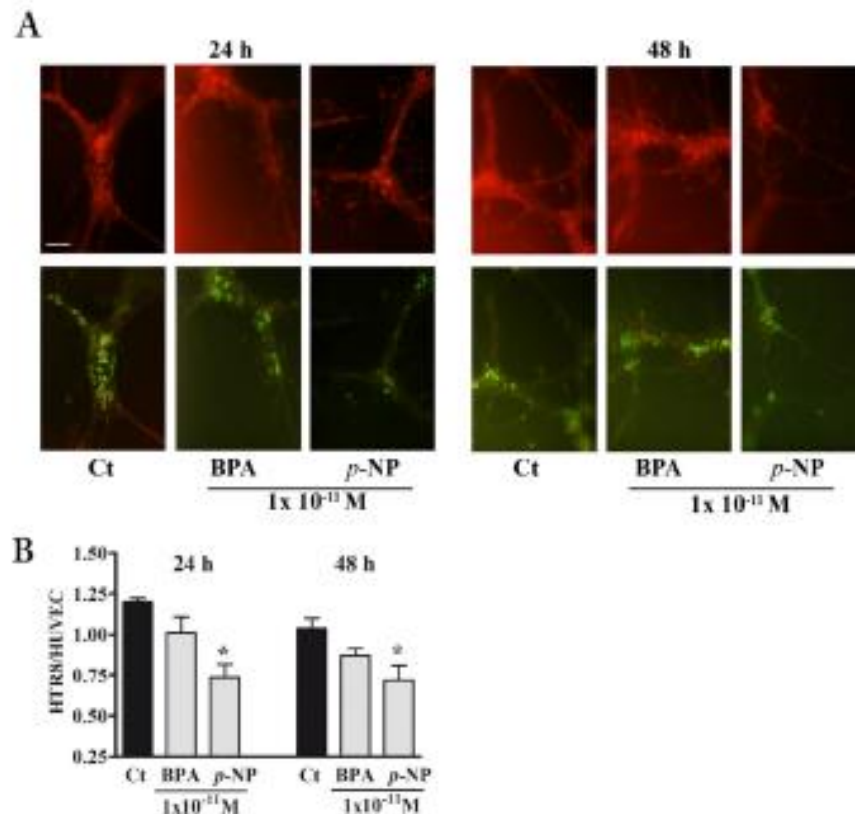
To assess whether BPA and p-NP altered the migration and invasion properties of HTR-8/SVneo, the cells were pre-treated with the compounds for 24, 48 and 72 h, then plated into polycarbonate membranes coated with a three-dimensional layer of collagen (cell migration) or GFR Matrigel (cell invasion) for 4 and 24 h for cell migration and invasion, respectively. For each chemical,

activity was higher at concentrations of  $1 \times 10^{-17}$  and  $1 \times 10^{-13}$  M and at longer exposure (48 and 72 h) (Fig. 2). The p-NP concentrations of  $1 \times 10^{-13}$  and  $1 \times 10^{-11}$  M were active in the first 24 h of incubation on the migration process (Fig. 2C) while the highest ( $1 \times 10^{-7}$  M) p-NP concentration had no effect on either cell migration or invasion at any time of incubation (Fig. 2C and D). A significant reduction in trophoblast invasion was observed when cultures were treated with p-NP at  $1 \times 10^{-15}$ . No significant effect was shown for BPA at 24 h either on cell migration or invasion (Fig. 2A and B).



**Fig. 2.** Effect of BPA and p-NP on HTR-8/SVneo cell migration and invasion. The HTR-8/SVneo cells were treated with BPA and p-NP at the concentrations of  $1 \times 10^{-15}$ ,  $1 \times 10^{-13}$ ,  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M for 24, 48 and 72 h. Cell migration (A and C) was evaluated as the number of cells reaching the lower side of collagen coated Transwell filters after 4 h; cell invasion (B and D) was evaluated as the number of cells reaching the lower side of Matrigel coated Transwell filters after 24 h. Data are expressed as percentage vs control vehicle-treated cultures (ethanol 0.1%; dotted line) and reported as means  $\pm$  SEM. The results represent at least  $n = 3$  experiments performed in triplicate for each data point. \* $p < 0.05$ ; # $p < 0.01$ .





**Fig. 3.** Effect of BPA and p-NP on trophoblast/endothelial cell interaction. (A) HUVEC cells were labeled with a red tracker and maintained in Matrigel coated wells. HTR-8/SVneo cells were pre-treated with BPA or p-NP, labeled with a green tracker and co-cultured for 24 and 48 h with HUVEC cells. (B) Trophoblasts-endothelial cell interaction was quantified measuring the red and the green fluorescence by ImageJ. The results are expressed as the ratio of HUVEC and HTR-8/SVneo cell fluorescence of  $n = 3$  experiments in triplicates. Ct: control vehicle-treated cultures (ethanol 0.1%). \* $p < 0.05$ . Bar = 75  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.3. p-NP but not BPA impairs HTR-8/SVneo interaction with HUVEC cells

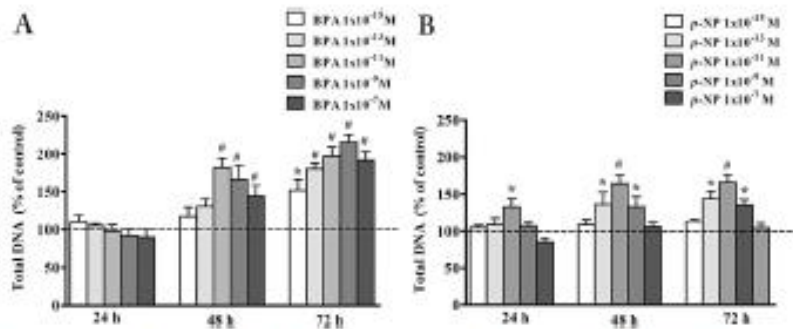
Interaction between HTR-8/SVneo and HUVEC cells was studied with an *in vitro* model of three-dimensional dual cell co-culture. For this analysis, HUVEC cells, labeled with a red tracker, were first cultured in wells coated with Matrigel in which they form the classical tube-like structure. HTR-8/SVneo cells were pre-treated with BPA or p-NP at  $1 \times 10^{-11}$  M for 24 and 48 h then labeled with a green tracker, and immediately added to the endothelial cell culture. Since remodeling of spiral arteries is associated with trophoblast invasion (Bischof and Irminger-Finger, 2005) we selected the concentration of  $1 \times 10^{-11}$  M, as the most active concentration in affecting trophoblast invasiveness. Trophoblast cells spontaneously interacted with the endothelial cells and fingerprinted the tube-like structure both in the control (0.1% ethanol) and chemical-treated cultures (Fig. 3A). Treatment with p-NP significantly reduced the interaction between the trophoblast and the endothelial cells as revealed by a lower ratio between the red and the green fluorescence, measured by a specific ImageJ software (Fig. 3B). The effect of p-NP was statistically significant at 24 h as well as 48 h of exposure while no effect was observed with BPA at equimolar concentration ( $1 \times 10^{-11}$  M) (Fig. 3B).

### 3.4. BPA and p-NP induce acquisition of polyploidy and differentiation of HTR-8/SVneo into placental bed giant cells

To test the possibility that treatment with BPA or p-NP might alter the process of trophoblast differentiation into polyploid bed giant cells, we performed genotype (cell DNA content) and phenotype (cell morphometry) analyses. In addition, polyploidy was investigated by assessing the expression of p57<sup>KIP2</sup>, a molecule associated to cell endoreduplication.

#### 3.4.1. Cell DNA content

The total DNA content of HTR-8/SVneo cells was measured by CyQuant<sup>®</sup> NF test (Fig. 4). Treatment with BPA determined an increase of total DNA content statistically significant at 48 h, with  $1 \times 10^{-11}$ ,  $1 \times 10^{-6}$  and  $1 \times 10^{-7}$  M and at 72 h, with all concentrations ( $1 \times 10^{-15}$  to  $1 \times 10^{-7}$  M) tested (Fig. 4A). Similarly to BPA, treatment with p-NP increased cell DNA content but unlike BPA and, similarly to that observed on cell migration and invasion, the effect of p-NP was clearly more potent with higher activity at lower concentrations ( $1 \times 10^{-15}$  and  $1 \times 10^{-11}$  M). As reported for cell migration and invasion, the effect of p-NP was already active at 24 h of exposure ( $1 \times 10^{-11}$  M) while BPA had no effect at this time (Fig. 4B).



**Fig. 4.** Effect of BPA and p-NP on HTR-8/SVneo cell DNA content. HTR-8/SVneo cells were treated with BPA (A) and p-NP (B) at the concentrations of  $1 \times 10^{-11}$ ,  $1 \times 10^{-10}$ ,  $1 \times 10^{-9}$ ,  $1 \times 10^{-8}$ ,  $1 \times 10^{-7}$  M for 24, 48 and 72 h. Measure of cellular DNA content was determined using CyQuant<sup>®</sup> NF. Data are expressed as percentage vs control vehicle-treated cultures (ethanol 0.1%; dotted line) and reported as means  $\pm$  SEM. The results represent at least  $n = 3$  experiments performed in triplicate for each data point. \* $p < 0.05$ ; \*\* $p < 0.01$ .

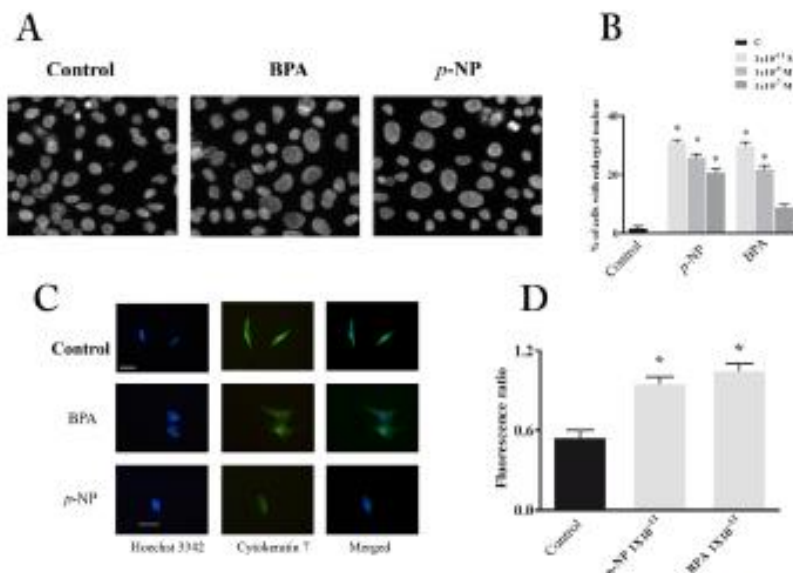
### 3.4.2. Cell morphology

The nucleus morphology of HTR-8/SVneo cells was examined using a specific nuclear dye CyQuant, after exposure to BPA and p-NP ( $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$ ,  $1 \times 10^{-7}$  M), for 72 h (Fig. 5A). The results showed a higher presence of cells with an enlarged nucleus both in BPA and p-NP treatment (Fig. 5A). Cell counting showed that the effect of both chemicals was dose-dependent with higher activity at lower concentrations ( $1 \times 10^{-11}$  and  $1 \times 10^{-9}$  M). The highest p-NP concentration ( $1 \times 10^{-7}$  M) had no significant effect (Fig. 5B). Nucleus and cytoplasm fluorescence using specific labels for the nuclear (Hoechst 33342) and the cytoplasmic (cytokeratin 7) compartments (Fig. 5C) showed enlarged nuclei, characteristic of polyploid cells, in the cultures treated with BPA ( $1 \times 10^{-11}$  M) (middle) and p-NP ( $1 \times 10^{-11}$  M) (bottom) whereas a tight epithelial morphology was maintained in

control cultures (0.1% ethanol) (top) (Fig. 5C). As shown in Fig. 5D, the ratio between the nucleus and the cytoplasm area measured by an ImageJ application was increased after exposure to BPA and p-NP with respect to control cultures.

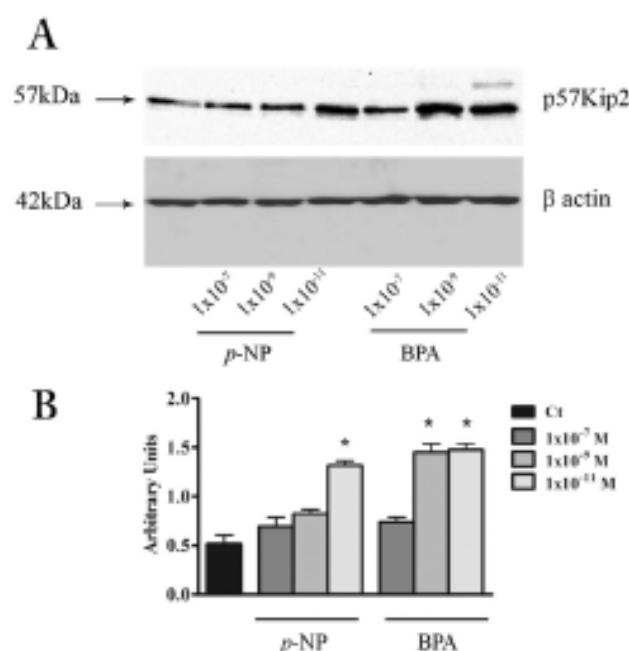
### 3.4.3. Expression of p57<sup>Kip2</sup>

To define the molecular pathway involved in the formation of enlarged nuclei observed in HTR-8/SVneo cells after exposure to BPA and p-NP, we checked the expression of protein p57<sup>Kip2</sup>, a known molecule preventing cell entrance into mitosis (Ullah et al., 2009). HTR-8/SVneo were treated with BPA and p-NP ( $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M) for 72 h, cell lysates were subjected to western blotting analysis using antibodies against human p57<sup>Kip2</sup>. A band at 57 kDa corresponding to the molecular mass of p57<sup>Kip2</sup> protein was



**Fig. 5.** Effect of BPA and p-NP on differentiation of HTR-8/SVneo into placenta giant cells. The HTR-8/SVneo cells were treated with BPA and p-NP at the concentrations of  $1 \times 10^{-11}$ ,  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M for 72 h. (A) Representative photographs with BPA  $1 \times 10^{-11}$  M and p-NP  $1 \times 10^{-11}$  M. C: ethanol 0.1%. Bar = 10  $\mu$ m. (B) Percentage of cells with enlarged nucleus with respect to total cells counted in five random fields and reported as means  $\pm$  SEM. \* $p < 0.05$ . The results represent  $n = 3$  experiments in triplicate. (C) Representative photographs of HTR-8/SVneo cells after 5 d of exposure to only vehicle (C: ethanol 0.1%), BPA  $1 \times 10^{-11}$  M and p-NP  $1 \times 10^{-11}$  M. Nuclei (blue) were stained with Hoechst 33342, cytoplasm compartment (green) were obtained using anti-cytokeratin 7. Bar = 25  $\mu$ m. (D) The ratio of nucleus/cytoplasm fluorescence in five random fields per cover slip from three separate experiments. Data are expressed as arbitrary units and reported as means  $\pm$  SEM. \* $p < 0.05$ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)





**Fig. 6.** Effect of BPA and p-NP on p57<sup>Kip2</sup> expression. (A) Representative western blot for p57<sup>Kip2</sup> protein in total cellular lysates after 72 h of exposure to BPA and p-NP at  $1 \times 10^{-7}$  M,  $1 \times 10^{-9}$  M and  $1 \times 10^{-11}$  M. (B) Densitometric analysis on  $n=3$  experiments performed in triplicate. Ci: control vehicle-treated cultures (ethanol 0.1%). \* $p < 0.05$ .

detected in all samples analyzed (Fig. 6A). Densitometry analysis showed a significant increase in p57<sup>Kip2</sup> expression in p-NP at  $1 \times 10^{-11}$  M treated cultures, while no changes were observed for p-NP at  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M. BPA at  $1 \times 10^{-11}$  and  $1 \times 10^{-9}$  M resulted in a significant increase in p57<sup>Kip2</sup> expression while no effect was observed at the highest concentration tested (Fig. 6B).

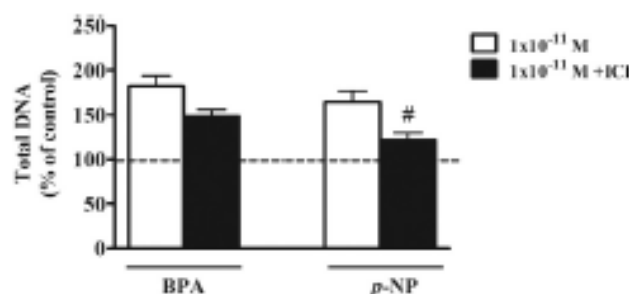
### 3.5. Estrogen-receptor antagonist (ICI) reverses the increased efficacy of p-NP on HTR-8/SVneo cell DNA content

In order to explore whether the mode of action of BPA and p-NP on trophoblast cells was via an estrogen-receptor dependent pathway, we performed experiments in the presence or in the absence of ICI ( $1 \mu\text{M}$ ) and tested the total DNA content using BPA and p-NP at  $1 \times 10^{-11}$  M, the most active concentration. As shown in Fig. 7 the treatment with ICI significantly reduced the p-NP efficacy while no effect was observed on BPA.

## 4. Discussion

The extravillous trophoblast differentiation program is a crucial physiological process in early human pregnancy contributing to placenta establishment and its development in the maternal uterus. Cytotrophoblast cells proliferate in multilayer cell columns anchoring the placenta to the maternal decidua. From the distal part of these columns, isolated extravillous trophoblast cells (EVT) migrate outwards onto the surface of the decidua, then acquire an invasive phenotype and move deeply into the decidualized endometrium up to the spiral arteries determining their remodeling. The EVT invasive phenotype ends as these cells finally differentiate into polyploid placenta giant cells (Bischof and Irminger-Finger, 2005).

Herein, we have shown that trophoblast exposure to low, non toxic, concentrations of BPA and p-NP, two widely diffused



**Fig. 7.** Effect of estrogen-receptor antagonist (ICI) in BPA and p-NP treated cultures on HTR-8/SVneo cell DNA content. HTR-8/SVneo cells were treated with BPA and p-NP ( $1 \times 10^{-11}$  M) in the presence or not of ICI for 48 h. Cellular DNA content was determined using CyQuant<sup>®</sup> assay. Data are expressed as percentage vs control cultures and reported as means  $\pm$  SEM. The results represent  $n=3$  experiments performed in triplicate. # $p < 0.01$ .

environmental chemicals, alters different biological processes occurring in the extravillous pathway.

Specifically using HTR-8/SVneo cells, a good cell model for studying the EVT pathway (Graham et al., 1993; Takao et al., 2011), we demonstrated that BPA and p-NP caused a decrease in cell migration and invasion, the effect being mostly characterized by both a hormetic and a biphasic behavior. In particular, activity of both chemicals was most effective at concentrations of  $1 \times 10^{-11}$  and  $1 \times 10^{-13}$  M and less effective at higher,  $10^{-7}$  M, and lower,  $10^{-15}$  M, concentrations. Moreover, activity of both chemicals was higher at longer (48 and 72 h) exposure. Decrease of cell migration and invasion was not attributable to a negative effect of the chemicals on cell viability or cell proliferation because none of these indices were significantly affected by BPA and p-NP exposure. Although no impairment of cell proliferation was shown, we found that total DNA content in HTR-8/SVneo after exposure to BPA and p-NP was increased. We highlighted that the increase in cell DNA content was due to an increasing differentiation into trophoblast giant cells. By morphological and morphometric analysis we found indeed that about 30% of HTR-8/SVneo cells treated with BPA or p-NP showed a nucleus size at least 2 times larger than that of cells exposed only to vehicle and a significant reduction of cytoplasm in favor of the nucleus. Once again, the highest effect was observed with the concentration of  $1 \times 10^{-11}$  M. The polyploidy is the result of multiple S-phases in the absence of cytokinesis leading to giant cell appearance with a single enlarged nucleus (Ullah et al., 2009). The DNA content of these giant cells generally ranges from 8N to 64N, although levels as high as 1000N have been reported (Ullah et al., 2008; Zybinska and Zybinska, 2005).

The effect of BPA and p-NP to cause nuclear polyploidization was demonstrated also on hamster ovary cells (CHO-K1) (Tayama et al., 2008). Studies on mice trophoblast stem cells showed that treatment with a synthetic estrogen, diethylstilbestrol (DES), led to their differentiation toward the polyploid giant cell lineage. In particular, DES-treated pregnant mice exhibited abnormal early placenta development associated with an overabundance of trophoblast giant cells and an absence of diploid trophoblast (Tremblay et al., 2001). Moreover treatment with low doses of DES in pregnant mice was associated with fetal death whereas 100% fetal mortality was associated with the higher dose (Scott and Adejokun, 1980). During placental formation, differentiation of trophoblast stem cells in giant cells is regulated by cyclin-dependent protein kinases (CDK) activity (Ullah et al., 2008). Recent analysis has revealed that suppression of CDK1 activity by the CDK specific inhibitor p57<sup>Kip2</sup> triggered genome endoreduplication. This inhibition prevents entrance into mitosis and cells begin a new round of genome duplication leading to nuclear polyploidization (Ullah et al., 2009). Since p57<sup>Kip2</sup> is



involved in the differentiation process, we investigated its expression by western blot analyses. We showed that the levels of this protein increased after treatment with  $1 \times 10^{-11}$  M of BPA and p-NP confirming that both chemicals induced HTR-8/SVneo cells to differentiation into giant cells via endoreduplication process.

Finally, we investigated the effect of BPA and p-NP on remodeling of spiral arteries by HTR-8/SVneo extravillous trophoblast cells. By a dual co-cultures *in vitro* model, which is a suitable system to highlight trophoblast/endothelial interaction (Hunkapiller and Fisher, 2008), we demonstrated that treatment of trophoblast cells with p-NP ( $1 \times 10^{-11}$  M) reduced their spontaneous interaction with endothelial cells. The chemical concentrations shown to be active are even lower than those found in human fluids and tissues. As for human pregnancy, BPA and p-NP have been found in amniotic fluid, fetal cord serum, placenta and maternal blood with levels in these tissues varying from 2 to 36 nM for BPA and from 0.1 to 1 nM for p-NP (Huang et al., 2014; Schonfelder et al., 2002; Tan and Ali Mohd, 2003).

Despite intense research and data accumulated in the last years on the hazards of these compounds for reproductive health, insufficient evidence exists regarding their effects on the placenta. Previous studies by us and others have shown that low doses of BPA and p-NP (pM–nM) act on human trophoblast by inducing apoptosis as well as cytokines and hormone secretion (Bechi et al., 2006, 2010; Benachour and Aris, 2009). In accordance with studies in other cells (Vandenberg et al., 2012) we recently found that trophoblast exposure to several environmental chemicals at low (pM–nM) concentrations altered hCG secretion, in a hormetic manner (Bechi et al., 2013). Hormetic responses at low-doses are remarkably common in *in vivo* and *in vitro* studies of environmental chemicals in humans and in animals (Vandenberg et al., 2012).

A very recent study aimed to investigate the potential association between maternal BPA exposure and preeclampsia, a harmful complication in pregnancy (Lecerc et al., 2014). The authors showed a significant accumulation of BPA in the placenta of women with preeclampsia in comparison to normotensive women (Lecerc et al., 2014). As preeclampsia is associated with an inadequate trophoblast invasion and remodeling of maternal spiral arteries (Lyall et al., 2001; O'Tierney-Ginn and Lash, 2014), two features resulting in the present study after exposure to BPA and p-NP, it can be assumed that maternal exposure to these chemicals, during pregnancy, may cause or contribute to the pathogenesis of the disease.

Even though the chemicals tested are well recognized for their estrogenic activity, the mechanism of their action on trophoblast cells still needs to be elucidated.

Both estrogen receptor alpha (ER- $\alpha$ ) and estrogen receptor beta (ER- $\beta$ ) are expressed by human placenta (Bechi et al., 2006) while in HTR-8/SVneo cells, the only ER- $\beta$  isoform has been detected at both mRNA and protein level (Cervellati et al., 2013). Interestingly Bukovsky et al. have shown that of ER- $\beta$  hormone binding domain plays a key role in the regulation of trophoblast differentiation (Bukovsky et al., 2003). BPA and p-NP are known to bind to ER- $\alpha$  and ER- $\beta$ , however the not detectable expression of ER- $\alpha$  in HTR-8/SVneo cells suggests a possible involvement of the beta isoform. Other mechanisms of action and receptors have been identified as possible targets for BPA and p-NP that may also be relevant in placenta. In particular low concentrations of BPA have been reported to trigger effects via G protein-coupled receptor (GPCR-30) and estrogen-related receptor gamma (Morice et al., 2011; Okada et al., 2008; Pupo et al., 2012; Tohime et al., 2014). p-NP as well as BPA can act also via a signaling pathway involving the glucocorticoid receptor and retinoid X receptors (Vandenberg et al., 2012). To explore the possibility of an estrogen-receptor mediated pathway in the BPA and p-NP action on HTR-8/SVneo trophoblast cells, we performed experiments using an estrogen-receptor antagonist (ICI). Our study, showing that addition of ICI was able to reduce p-NP effect

of about 30% while it did not show any effect on BPA treatment, suggests that other mediators for the full action of BPA and partially for p-NP can exist.

## 5. Conclusions

All together, our data are a clear evidence of the potential impact of environmental substances on human placentation. Although it is difficult to translate the data obtained *in vitro* with pathophysiological conditions, on the basis of extensive literature, we can speculate that a defective placentation caused by maternal exposure to environmentally relevant concentrations of BPA and p-NP might contribute to the complications for pregnancy and fetal health.

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