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Running title: Comparing BPA and BPS effects on placental cells

Summary sentence: Bisphenol A and its analog Bisphenol S, emerging contaminants founded in human serum and placental fluids, significantly affected the biological functions of HTR-8/SVneo cells, through estrogen receptors and MAPK mediated processes.

Keywords: BPA, BPS, HTR-8/SVneo cells, Endocrine disruptors, Human trophoblast, MAPK

Abstract

Bisphenol A (BPA) is one of the most investigated compound as a suspected endocrine disrupting chemical. It has been found at nM concentrations in the maternal serum, cord serum, and amniotic fluid and also permeates placental tissues. Attempts are being made to replace BPA with the analog Bisphenol S (BPS). Also BPS was found in maternal and umbilical cord serum, and urine samples from a large population of pregnant women. A few studies investigated BPA impact on the placentation process, and even less are available for BPS. This work aimed to elucidate and compare the effects of BPA and BPS on physiological functions of HTR-8/SVneo cells, derived from extravillous trophoblast of first-trimester pregnancy. Proliferation and migration ability of trophoblast cells were assessed in vitro after exposure to BPA or BPS (10^{-13}–10^{-3} M). Further, induction of the inflammatory response by the bisphenols was studied. To provide insight into the molecular pathways implicated in the responses, experiments were carried out in the presence or absence of tamoxifen as estrogen receptors (ERs) blocker, and U0126 as ERK1/2 phosphorylation inhibitor. Data indicate that BPA significantly affects both proliferation and migration of HTR8/SVneo cells, through ER and ERK1/2 mediated processes. Differently, BPS only acts on proliferation, again through ER and ERK1/2 mediated processes. BPS, but not BPA, induces secretion of interleukins 6 and 8. Such effect is inhibited by blocking ERK1/2 phosphorylation. To the best of our knowledge, these are the first data showing that BPS affects trophoblast functions through ER/MAPK modulation.

1. Introduction

The increasing abnormalities in the female reproductive functions over the years highlighted a strong link between the exposure to industrial chemicals and the effects on human health [1–3]. Within this relationship, the concept of endocrine disrupting chemicals (EDCs) has taken a central role in the field of reproduction. EDCs comprise many different substances from industrial, agricultural, and domestic sources. They have been related with adverse effects on female reproductive system and may cause the development of gynaecological pathologies [4]. Further, exposure to EDCs may interfere with normal growth and development of the fetus and render the placenta unable to support the requested physiological functions [5]. Bisphenol A (BPA) is one of the most investigated substances as a suspected EDC. BPA is a monomer that was first developed as a synthetic estrogen in the 1890s and reported to have the efficacy of estrogens in stimulating the female reproductive system in rats in the 1930s [6]. Later, BPA has widely been used for its cross-linking properties in the manufacture of polycarbonate plastics and epoxy resins [7]. As an additive of plastics [8], BPA is released in food and water from plastics devices and from food packaging materials, as well as from personal care products and thermal receipts/papers, which constitute the main source of oral and dermal intake of BPA in humans. Epidemiological studies have reported that up to 95% of adults have detectable levels of BPA in their serum, saliva, and urine [9], within the nM range. Recent in vivo studies showed that BPA exposure is a known risk factor for the development of type 2 diabetes [10], obesity [11], earlier puberty [12], cardiovascular diseases [13], breast and ovarian cancer [14], altered liver function, oxidative stress and inflammation [15]. As to reproductive tissue, BPA has been found in maternal serum (0–154 ng/ml), amniotic fluid (0–8.38 ng/ml), cord serum (0–62.8 ng/ml), and placental tissue (0-104.9 ng/g) [9, 16–18]. Due to its high lipophilic property, BPA may permeate the placenta [19]. Given

the potential risks posed by BPA on human health and reproduction, attempts are being made to replace it with the analog Bisphenol S (BPS). Structurally similar to BPA, BPS was first synthesized in 1869 as a dye, and is currently used in a variety of industrial applications and introduced into many commercial products available on the market as "BPA-free" [20, 21]. Human exposure to BPS occurs through ingestion, inhalation, and dermal contact [22]. BPS was found in human urine, generally at concentrations and frequencies comparable to BPA [23]. However, the information available on adverse effects by BPS, in particular as a potential EDC, is rather poor. As a consequence of its structural similarity to BPA, BPS could potentially lead to similar endocrine disrupting capacity and effects on the reproductive system [24]. BPS was found in maternal (0.03–0.07 ng/ml) and cord serum samples (0.03–0.12 ng/ml) [25, 26], suggesting its possible ability to cross the placental barrier [27]. BPS was also detected in urine samples from the at-risk population, such as pregnant women [28, 29]. Emerging evidence suggest that BPS is capable of imitating properties of hormones [30], interacting with various physiological receptors, including estrogen receptors (ERs), androgenic and aryl hydrocarbon receptors [31]. Some in vitro studies observed that BPS affects estrogenic and antiandrogenic activities on cells derived from human ovarian and breast cancer, in a similar manner and potency of BPA [32, 33]. Considering its complexity, knowledge on potential EDC effects on placental function is still limited, especially regarding BPA and even more BPS. The development of the human placenta depends entirely on normal differentiation, proliferation, and invasion of trophoblast cells [34]. Impaired function of trophoblasts can lead to severe pregnancy complications [35, 36]. The proliferation and invasion of trophoblast cells represent a complex process, strictly regulated by many factors, including hormones, prostaglandins, cytokines and hypoxia, which either promote or inhibit proliferation and/or invasion [37]. Numerous signalling cascades/proteins are involved in the regulation of

trophoblast cell activity. Among them, mitogen-activated protein kinase (MAPK) pathways play a critical role in a wide range of biological processes [38, 39]. Further, cytokines and growth factors are secreted by trophoblast cells, and regulate the functional activity of the trophoblast via paracrine and autocrine mechanisms, control placenta development, and maintain immunological tolerance in the mother-fetus system [40, 41]. The present study aims to elucidate and compare the effects of BPA and BPS on the proliferation and migration ability of extravillous trophoblast HTR-8/SVneo cells, as well as on the secretion of inflammatory mediators. These cells preserve well-known factors controlling proliferation, migration and invasion, thus representing a good model for the in vitro study of molecular mechanisms at the basis of placentation, as well as of early events modulating placental development [42]. To provide insights into the molecular pathways implicated in cellular responses, involvement of the ERs and modulation of the ERK1/2 dependent pathway by BPA and BPS were investigated.

2. Materials and methods

2.1. Chemicals

Anti-phospho-ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-rabbit conjugated with horseradish peroxidase immunoglobulin G (IgG), and U0126 (MEK1,2 inhibitor) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Roswell Park Memorial Institute RPMI 1640 medium, Fetal Bovine Serum (FBS, EU origin), Charcoal Stripped Fetal Bovine Serum (CS-FBS), glutamine, penicillin/streptomycin, anti β-tubulin, anti-mouse IgG conjugated with horseradish peroxidase, Bisphenol A (BPA), Bisphenol S (BPS), Tamoxifen (TAM) and all other reagents were from Merk Life Science (Milan, Italy). Stock solutions were prepared in DMSO as follows: 0.1 M BPA, 0.1 M BPS, 0.01 M U0126 and 0.01 M TAM.

104 2.2. Cell culture and treatments

105 The HTR-8/SVneo human trophoblast cell line was kindly provided by Dr. Charles H. Graham
106 (Queen's University, Ontario, Canada [43]. HTR-8/SVneo cells originated from an explant culture
107 of the human first-trimester placenta; they exhibited the following intrinsic mechanisms: adhesion,
108 migration and invasion [44]. Cells were grown in RPMI 1640 medium supplemented with 10%
109 FBS, 2 mM L-glutamine, and 2% penicillin/streptomycin, and were maintained at 37◦C in a
110 standard atmosphere containing 5% CO2.For the experiments, cells were treated with trypsin and
111 removed from culture flasks, then seeded in 6-well, 12-well or 24-well culture plates at a density
112 of 1.4 × 104 cells/ml, and cultures were incubated overnight. After 24 h culture, cells were exposed
113 to bisphenols (BPA or BPS, dissolved in DMSO) at different concentrations for 3 days. When
114 indicated, cells were incubated with 10 μM U0126 (ERK1/2 inhibitor) or 0.1 μM TAM (ER
115 antagonist). Because of the hormonal activity of phenol red and FBS [45], experiments were
116 performed in phenol-red-free RPMI, supplemented with 5% CS-FBS. Dextran treated charcoal is
117 used to selectively remove hormones without nonspecific loss of other serum components. Final
118 DMSO concentration in the medium never exceeded 0.1%. In each experiment, control cells were
119 exposed to the vehicle alone to ascertain absence of effects.

120 2.3. E-SCREEN assay

121 The E-screen assay was originally developed to evaluate the proliferative effect of estrogen-like
122 compounds on MCF-7 cells, a human breast cancer cell line, that endogenously overexpresses ERs
123 [46]. Since HTR-8/SVneo cells do possess both nuclear- and membranelocalized- ERs [47], we
124 verified the possibility to use the E-screen assay in the present investigations. Preliminary trials
125 demonstrated that HTR-8/SVneo cells incubated with increasing amounts of 17β-Estradiol (E2)

126 are induced to proliferate, starting from the concentration 10^{-12} M, and reaching the maximum
127 response at 10^{-8} M (Supplemental Figure S1). The E-screen assay is then used for the first time on
128 HTR-8/SVneo, with a modification with respect to the original method, i.e. cells are exposed to
129 potential estrogenic compounds for three days instead of five days. In fact, previous observations
130 by our laboratory indicated that HTR-8/SVneo cells replicate at higher rate (~22 h) than MCF-7
131 cells (~32 h). Cell proliferation was then determined by a colorimetric assay based on the reduction
132 of a tetrazolium salt, MTT (3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by
133 metabolically active cells [48]. Optical density was assessed at 570 nm with background correction
134 at 650 nm, and results are expressed as Proliferative Effect (PE), the ratio of the cell number
135 achieved after BPA or BPS exposure, and the cell number in the control cells.

136 2.4. Western blotting analysis

137 Western blotting procedures were carried out as previously reported [49]. After the experimental
138 treatments, cells were lysed in ice-cold lysis buffer containing phosphatase inhibitors (1 mM
139 sodium ortovanadate and 50 mM sodium fluoride) and proteins were electrophoresed, transferred
140 to nitrocellulose membrane and probed with specific antibodies. MAPK activity was assessed by
141 using rabbit anti-phospho-ERK1/2 or anti-total-ERK1/2 as primary antibodies (1:1000) and
142 horseradish-peroxidase-conjugated goat anti-rabbit IgG (1:2000). Immunoblots were developed
143 by enhanced chemiluminescence reagent, and a densitometric analysis of the films was performed
144 by Image Master equipped with TotalLab software ver. 1.0 (Amersham-Pharmacia). Given the
145 inherent variations in film development, densitometry data were normalized to an external
146 reference sample (REF) (unexposed cells) loaded in each immunoblot. MAPK activity was
147 evaluated as phospho/total ERK1/2% of phosphorylation with respect to control. β-tubulin
148 immunodetection was used as the control to assess equal protein loading.

149 2.5. Transwell cell migration assay

150 Transwell inserts (PET membrane, 8-μm pore size, Millicell Hanging, Merck Life Science, Milan,
151 Italy) were used for the cell migration assay, according to manufacturer's instructions. Briefly,
152 after three days of treatment with BPA or BPS, HTR-8/SVneo cells were trypsinized, resuspended
153 in RPMI with 1% FBS and seeded in the upper chamber of each insert at a density of 3×10^4
154 cells/well; the lower chambers contained 750 μL RPMI supplemented with 10% FBS. After 24 h
155 of incubation at 37°C the non-migrated cells on the upper surface of the membrane were removed
156 by gentle swabbing. Cells that had reached the lower side of the filter were fixed with methanol
157 for 30 min, stained for 10 min with crystal violet 0.3%, and quantified by visual counting of ten
158 randomly selected fields from each filter under a light microscope. Data were normalized against
159 cell count. Migration capacity was calculated as the percentage of cells that passed through the
160 membranes in the treated- with respect to control- cells.

161 2.6. Luminex assay

162 Analysis of inflammatory cytokines and cytokine-induced vascular endothelial growth factor
163 (VEGF) was performed in the medium of trophoblast cell culture, where soluble factors can be
164 released after bisphenols exposure. After the experimental treatments, the medium was collected
165 in 2 mL tubes, centrifuged at 1000 × g for 10 min at 4°C, and supernatant was kept at −80°C until
166 analysis. Secretions of IL-6 (interleukin 6), IL-8 (interleukin 8), IL-1β (interleukin 1β), TNF-α
167 (tumor necrosis factor-α), chemokine CCL2 (CC Motif Chemokine Ligand 2), and VEGF were
168 analysed using the immunological kit for Human Magnetic Luminex Screening Assay (Bio-techne
169 s.r.l. Milan, Italy). The analysis was performed using the BioPlex200 equipment (BIO-RAD
170 Laboratories srl, Milan, Italy). Sample concentrations of the different compounds were estimated

through a standard curve using a 5-order polynomial curve and expressed pg/mL (Bio-Plex Manager software 5.0). Data were normalized against cell count.

2.7. Data analysis

Data groups were compared using one-way ANOVA with SigmaPlot software (ver 13, Systat Software Inc.), and followed by Dunnet post-hoc test; a statistical difference was accepted when $P < 0.05$. All experiments were independently repeated at least three times.

3. Results

3.1. BPA and BPS exposure increase HTR-8/SVneo cell proliferation

Cells were exposed to increasing concentrations of BPA or BPS (from 10^{-13} Mto10^{-3} M) for three days. As reported in Figure 1, both BPA and BPS affected the proliferation of HTR-8/SVneo cells. At concentrations ranging from 10^{-11} M to 10^{-7} M BPA or BPS, cell proliferation was significantly increased compared to the control cells. The most active concentration was 10^{-7} M for BPA and 10^{-9} M for BPS, reaching the maximum proliferative effect of 1.89 and 1.62, respectively. BPA at 10^{-5} M concentration significantly increased HTR-8/SVneo cell proliferation, whereas no effect was observed at an equimolar concentration of BPS. At the highest concentration tested (10^{-3} M) of BPA or BPS, the proliferative effect was significantly lower than 1. This result can be ascribed to toxic effects of the compounds on HTR-8/SVneo cells.

3.2. MAPK (ERK1/2) pathway is involved in the stimulatory effects induced by BPA and BPS

The ability of BPA and BPS to modulate the MAPK pathway, and its involvement in the proliferative effect, were investigated by assessing ERK1/2 phosphorylation through western blot analysis. As reported on Figure 2, BPA triggered ERK1/2 phosphorylation in the 10^{-9} –10^{-5} M

concentration range. A significant increase of ERK1/2 phosphorylation was observed at 10^{-9} M and 10^{-7} M BPS. In the presence of U0126, a selective MAPK inhibitor, BPA and BPS effects on ERK1/2 phosphorylation were significantly reversed.

3.3. BPA but not BPS impairs HTR-8/SVneo cell migration

In vitro effects on the migration capacity of HTR-8/SVneo cells were examined after incubation at different concentrations of BPA or BPS (from 10^{-13} Mto10^{-5} M) for three days. A significant reduction of trophoblast cell migration was observed after treatment with BPA at 10^{-7} M and 10^{-5} M concentrations (−22% and − 53% versus control) (Figure 3). BPS exposure did not induce any statistically significant change on HTR-8/SVneo cell migration, at any concentration tested.

3.4. The effects of BPA and BPS on cell proliferation and migration are mediated by ER and MAPK signalling pathways

To reveal the involvement of ER and ERK1/2 signalling pathways in mediating the effects induced by BPA and BPS on HTR-8/SVneo cell proliferation and migration, further experiments were performed in the presence of TAM, a specific antagonist of ER, and U0126. The effects of TAM or U0126 were tested using one of the peak active concentrations of BPA or BPS. Blocking ER with TAM, the effect of both BPA and BPS on cell proliferation (Figure 4A), and the effect of BPA on HTR-8/SVneo cell migration (Figure 4B) were abolished. The proliferative effects induced by BPA and BPS were fully reversed by U0126, which decreased to levels lower than control (Figure 4A). Treatment with U0126 also recovered the migration ability of trophoblast cells, which was inhibited by BPA (Figure 4B).

3.5. BPS but not BPA affects inflammatory cytokines secretion in HTR-8/SVneo cells

Cells were incubated with different concentrations of BPA or BPS (from 10^{-11} Mto10^{-5} M) for three days, then secretion of different inflammatory proteins was measured: IL-6, IL-8, TNFα, IL-1β, CCL2, and VEGF. Interestingly, IL-6 and IL-8 secretion was affected by BPS exposure (Figure 5A and B), with significant increases at 10^{-11} M that were counteracted by treatment with the U0126 inhibitor. No effect of BPA was detected on interleukin secretion, with respect to the basal levels. Stimulation of HTR8/SVneo cells with BPA or BPS treatment did not affect CCL2 secretion (Figure 5C). In our experimental conditions, basal levels and secretion of IL-1β TNF-α and VEGF from HTR-8/SVneo cells were always below the sensitivity of the assay.

4. Discussion

BPA and its analog BPS are ubiquitous emerging contaminants of increasing concern. The real-world exposure to BPA and BPS occurs daily at low concentrations, but relevant for human health risk [50]. Emerging evidence suggest high potential of adverse biological effects on reproductive functions after exposure to these chemicals [51–53]. The aim of this work was to elucidate and compare the effects of BPA and BPS on biological activities of HTR-8/SVneo cells, derived from extravillous trophoblast of first-trimester pregnancy. A wide range of concentrations of BPA and BPS (from 10^{-13} Mto 10^{-3} M) were used to perform in vitro experiments, which comprises the nM range often documented in human fluids. To the best of our knowledge, these are the first data showing that BPS affects trophoblast functions through ER/MAPK mediated pathways.

Due to the observed effects on humans, particularly those related to hormonal regulation of reproductive processes [54], BPA has been qualified as a xenoestrogen [55]. Based on the chemical similarity to the natural hormone E2, BPA can bind with ERs (ERα and ERβ), although displaying 1000 to 2000-fold less affinity with respect to E2 [56]. BPS has been reported to have activity and potency similar to those of BPA [32]. Since the ERs signalling pathways are involved in the cell

proliferation equilibrium, we investigated the possible physiological consequence of BPA and BPS exposure in triggering estrogen-like proliferation on extravillous trophoblast cells. The findings demonstrated that both BPA and BPS increase HTR-8/SVneo cells proliferation, starting from the concentration 10^{-11} M (Supplemental Figure S2), one order of magnitude higher than natural estrogen E2, whose effect on HTR-8/SVneo cells is significant at 10^{-12} M (Supplemental Figure S1). Similar to other EDCs, BPA and BPS displayed non monotonic dose–response curves. More specifically, they showed an inverted U-shaped curve, inducing a significant biological effect at low concentrations, reaching the maximum at 10^{-9} M and 10^{-7} M, and causing cytotoxic responses at the highest doses. The proliferative effect of both BPA and BPS was abolished by the treatment with the ER-blocker TAM, which confirms that both bisphenols mimic the effect of E2. Similar findings, obtained after exposure of cells at different BPA concentrations, were observed on the human choriocarcinoma cell line BeWo [57, 58], JEG-3 cells [59], and human ovarian carcinoma cell line OVCAR3[60]. To the extent of our knowledge, no studies are currently available on BPS effects on reproductive tissues. MAPKs are responsible for converting many cellular and extracellular stimuli into specific responses controlling cell proliferation, differentiation, apoptosis, embryogenesis, and regulation of inflammatory and stress responses [61]. Transduction pathways mediated by phosphorylated ERK1/2 play an essential role in the placenta development [62] and is reported to facilitate trophoblast differentiation [63]. Several data support that BPA-dependent estrogenic activity flows through the ER-mediated extranuclear signals activation, that results in the ERK1/2 phosphorylation [64]. Previous studies have revealed that BPA activates ERK1/2 phosphorylation in human adrenal and breast [65], placenta [66], and also HTR-8/SVneo cells [67]. Present data demonstrate that phosphorylation of ERK1/2 in trophoblast cells is activated by treatment with both BPA and BPS. Since TAM prevented their effect, we concluded

that the ERK1/2 stimulation was induced through the interaction of bisphenols with ERs. Further, ERK1/2 activation is involved in the effect of BPA and BPS on trophoblast proliferation, as the effect was abolished in the presence of the U0126. Overall, BPA and BPS, similarly to E2, activate the ER-dependent signals that culminate with the activation of rapid extra-nuclear pathways in HTR-8/SVneo cells. We conclude that bisphenols effects on trophoblast cells proliferation and migration require the activation of two consequential signals, ERs and ERK1/2 pathways; by blocking one of them the downstream effects are abolished. The present study shows for the first time an ER and MAPK mediated effect of both BPA and BPS on trophoblast cell proliferation. Previous studies reported that BPA did not affect cell proliferation [67–69]. In a further study [70] a concentration-dependent decrease in the proliferation of HTR8/SVneo cells exposed to BPA or BPS was observed. Therefore, additional work is advisable for a better understanding of these modulations. The trophoblast is an embryonic tissue that exerts a crucial role during implantation and placentation, and its migration and invasion capacity represents a pre-requisite for normal embryo implantation.

Numerous studies indicated that exposure to BPA might be associated with severe pregnancy-related complications affecting both mother and fetus [71]. BPA can affect placentation, a significant factor determining pregnancy outcome. It has been associated with implantation failure, miscarriage, premature delivery, and it may also contribute to infertility and subfertility [72, 73]. Few studies about the effect of BPA on the trophoblast migration were performed [67–69, 74], while no information is available yet on the effect of BPS on this process. Present results indicate that only BPA affected cell migration ability. The inhibitory effect of BPA was abolished by TAM, suggesting an estrogen-like effect mediated through ERs. Besides, the migratory ability of cells was recovered by treatment with the inhibitor U0126, demonstrating that ERK1/2 pathways are

relevant for BPA reduction of cell migration. In this study, BPS exposure, unlike BPA, never affected migration of HTR-8/SVneo cells. Similar to cell proliferation, also data on BPA effect on HTR8/SVneo cell migration are conflicting. Some studies [67, 68, 74] observed that migration and invasion were reduced following BPA exposure, while others [69] assessed that BPA enhances cell migration through ERK1/2 stimulation. The present work reports a reduction of migration at micromolar BPA concentrations. The discrepancy among the above studies may be related to the complexity of the migration process modulation. In fact, the control of trophoblast functions is strictly dependent on the balance of various factors [75], which can be influenced by BPA or BPS exposure thus interfering with the intricate role of the trophoblast in human placentation. Among other examples, a very recent investigation reports the important modulation by nutrient transfers, which is affected by BPA [76]. Therefore, further work is advisable to clarify BPA and BPS effects on physiological response of trophoblast cells, possibly performed on ex-vivo preparations thus avoiding possible influence by culture conditions. Previous studies indicated that cytokines may have a critical role in the functions of trophoblast cells; this is particularly true for interleukins [77–79] as key molecules involved in the biological processes related to migration and invasion of trophoblast cells [80]. IL-6 is widely expressed in the endometrium at implantation, and increased levels of placental IL-6 have been associated with preeclampsia, recurrent miscarriage, and infertility [81]. Previous studies provided some evidence that IL-6 enhances migration of HTR8/SVneo cells [82]. In addition, the HTR-8/SVneo cell line secretes IL-8, which is reported to increase cell migration and invasion [83]. However, the critical role of the cytokines on the embryo implantation is not yet understood. Herein, we investigated the effect of BPA and BPS on cytokines secretion by HTR-8/SVneo cells. BPS, only at 10^{-11} M, upregulated interleukins IL-6 and IL-8 secretion. We also assessed that IL-1β, CCL2, TNF-α and VEGF levels were not affected.

The effect of BPS on IL-6 and IL-8 protein levels was reversed by treatment with U0126. Thus, BPS induction of both IL-6 and IL-8 is mediated by the ERK1/2 signalling. BPA did not cause any effect on cytokines, although a slight but not significant stimulation was observed only at 10^{-11} M BPA on IL-8 secretion. These data are not sufficient to correlate cytokine secretion by HTR-8/SVneo with cell proliferation/migration modulated by BPA or BPS.

5. Conclusion

Our results indicate that BPA, already at sub-nanomolar concentrations, affects both proliferation and migration of HTR-8/SVneo cells through ER/MAPK mediated processes. BPS, at the same concentration range, enhances proliferation, also acting through ER/MAPK pathways. BPS has no effect on HTR-8/SVneo cell migration. Thus, BPA and BPS share estrogen-like activity and modulation of ERK1/2 signaling, while targeting different biological processes in trophoblast cells. Furthermore, BPS while not BPA, increases interleukins secretion through ERK1/2 phosphorylation. The physiological significances of this effect, observed only at a subnanomolar concentration, needs to be further elucidated. In the current state of knowledge, the replacement of BPA with BPS does not seem to ensure the safety of human health.

6. Data availability

The data underlying this article are available in the article and in its online supplementary material. Further information underlying this article will be shared on reasonable request to the corresponding author.

7. Author contributions

MP: running of experiments, graphical representation and writing of first draft. ES:

conceptualization and running of cytokines release measurements; data analysis. EF: conceptualization, supervision, funds, and writing of final draft. PV: theoretical organization of experiments, data analysis and writing of final draft.

8. Conflict of interests

The Authors declare that there are no conflict of interests regarding the publication of this work.

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Figures

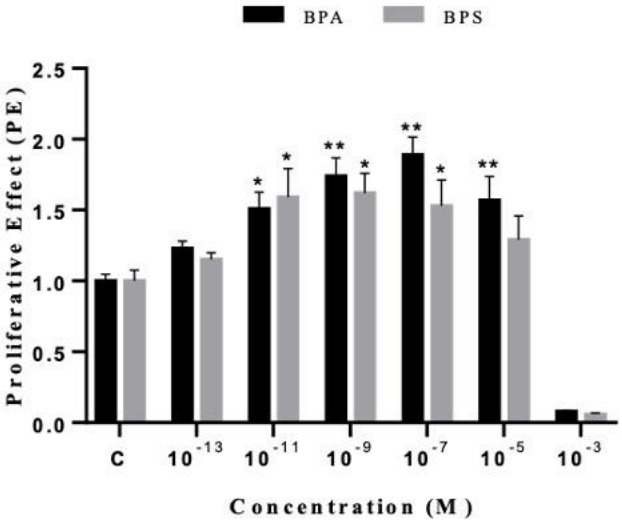


Figure 1: Effect of BPA or BPS on HTR-8/SVneo cell proliferation. Data are expressed as PE and reported as means ± SE of 5 different experiments, each performed in quadruplicate. *p < 0.05, **p < 0.01 related to control (C, control cells exposed to vehicle alone, PE=1).

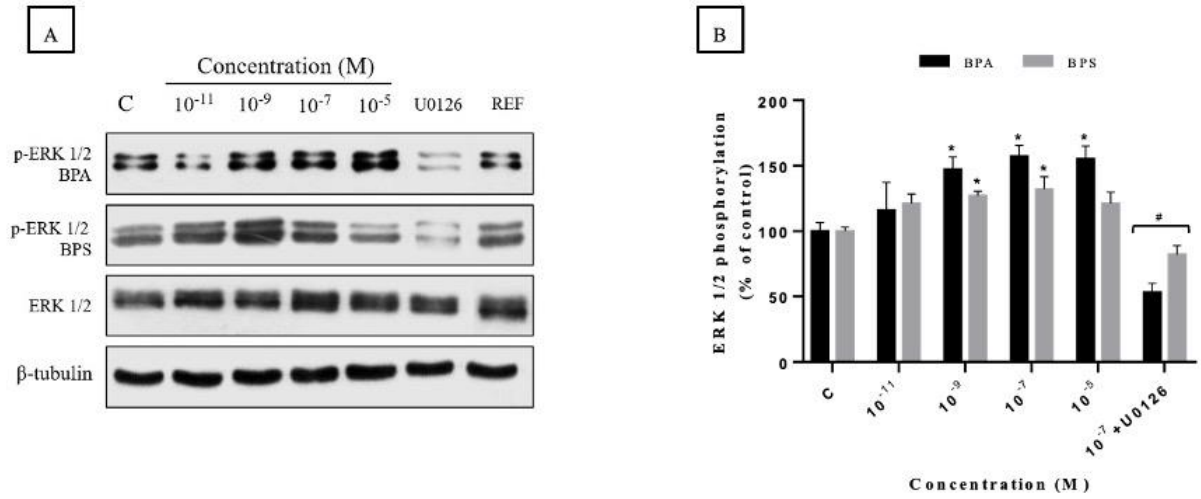


Figure 2: Effect of BPA or BPS on ERK1/2 phosphorylation in HTR-8/SVneo cells. [A] Representative immunoblots relative to phosphorylated and total ERK1/2. U0126= BPA or BPS 10^{-7}M treatment with the ERK1/2 inhibitor U0126. REF= external reference sample, loaded in each immunoblot. β-tubulin levels were also measured to assess equal loading of proteins. [B] Densitometric analysis of ERK1/2 phosphorylation, evaluated as the ratio between the phosphorylated and total forms, expressed as % of control (C) and reported as means ± SE of 4 different experiments. *p < 0.05 related to C (control cells exposed to vehicle alone), # p < 0.05 related to 10^{-7} M BPA or BPS.

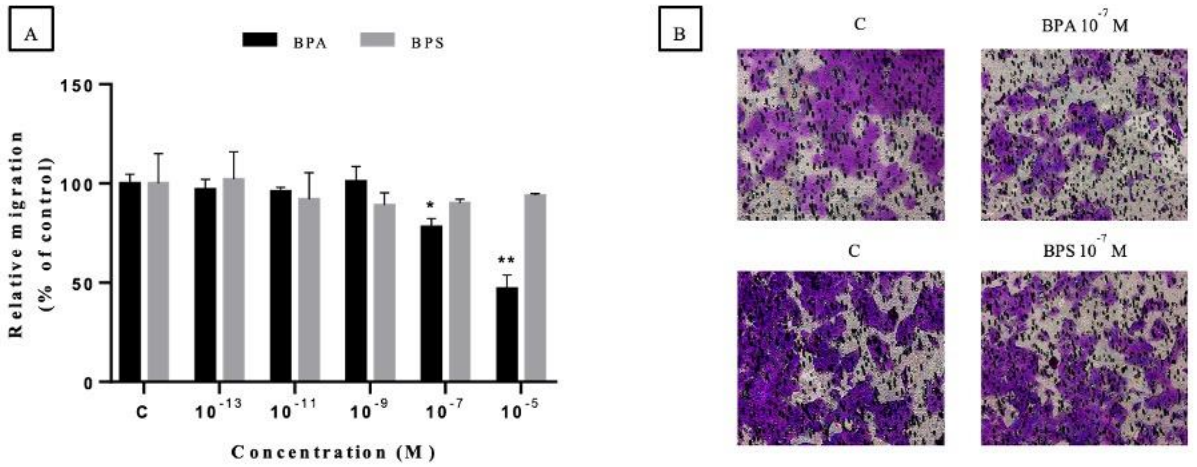
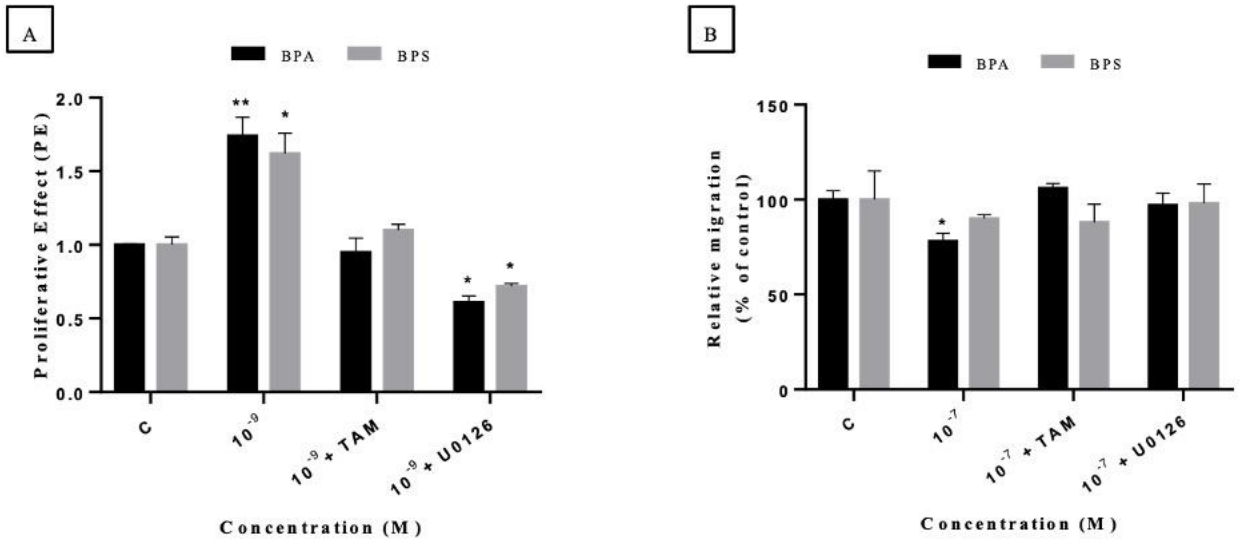


Figure 3: Effect of BPA or BPS on HTR-8/SVneo cell migration. [A] Densitometric data relative to cell migration, evaluated as the number of cells reaching the lower side of the membrane in Transwell inserts. [B] Representative light microscope images of Transwell migration assay (10 ×). Data are expressed as a percentage of the control (C) and reported as means ± SE of 4 different experiments. *p < 0.05, **p < 0.01 related to C (control cells exposed to vehicle alone).



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566 **Figure 4: Effect of Tamoxifen and U0126 on HTR-8/SVneo cells exposed to BPA or BPS.** [A] Effect of 10^{-9} M
567 BPA or BPS with or without TAM or U0126 on cell proliferation. [B] Effect of 10^{-7} M BPA or BPS with or without
568 TAM or U0126 on cell migration. Data are reported as means ± SE of 4 different experiments. *$p < 0.05$, **$p < 0.01$
569 related to C (control cells exposed to vehicle alone).

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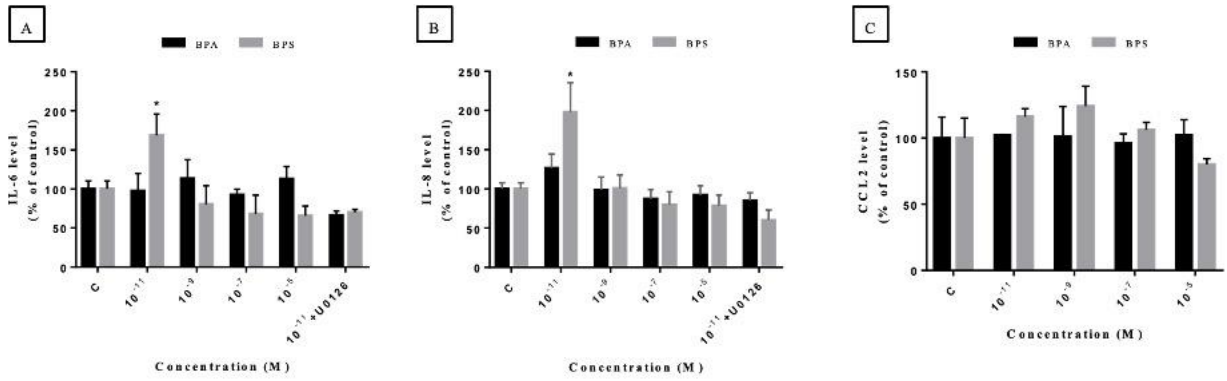

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572 **Figure 5: Effect of BPA or BPS on IL-6, IL-8, and CCL2 secretion by HTR-8/SVneo cells.** [A] IL-6 secretion
573 (C=23.86 pg/ml); [B] IL-8 secretion (C=27.70 pg/ml); [C] CCL2 secretion (C=11.72 pg/ml). Data are expressed as %
574 of control (C) and reported as means ± SE of 3 different experiments. *$p < 0.05$ related to C (control cells exposed to
575 vehicle alone).

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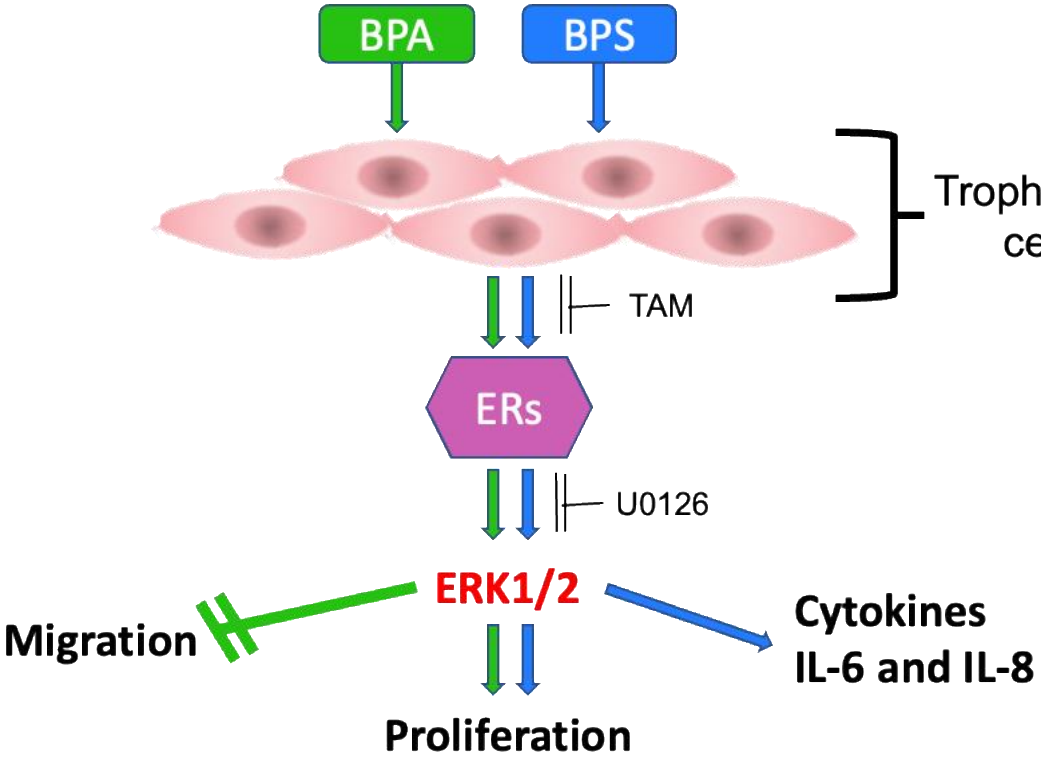
577 **Graphical abstract**



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