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Jin, H. and Audus, K.L. (2005) Effect of bisphenol A on drug efflux in BeWo, a human trophoblast-like cell line. *Placenta, 26 Suppl.A*, S96-S103. PMID: 15837075

Keywords:
Bisphenol A (BPA), P-glycoprotein (P-gp), BeWo cells, Drug efflux and 17β-estradiol (E2)

Abstract:
Bisphenol A (BPA) is a monomer of polycarbonate plastics that has estrogenic activities and has been shown to be a substrate for multidrug resistant efflux mechanisms, specifically, P-glycoprotein. Since the natural hormone estrogen reverses multidrug resistance in some cell types, we hypothesized that BPA might have a similar activity in trophoblasts. We have used BeWo cells as an *in vitro* model for human trophoblasts and calcein AM as a substrate for drug efflux mechanism to characterize BPA interactions with placental P-glycoprotein. We found that chronic exposure of BeWo cells to BPA did not alter intracellular calcein accumulation in a fashion that would be reflective of changes in P-glycoprotein expression. However, BeWo cells acutely exposed to BPA pre-treatment were observed to have a significantly decreased calcein accumulation suggesting a direct stimulatory effect on P-gp. Addition of cyclosporin A, a P-glycoprotein inhibitor and substrate, completely reversed BPA's effects on calcein accumulation and resulted in a net increase, relative to controls, in calcein accumulation by the BeWo cells. BPA was found not stimulate P-gp ATPase. Therefore, our results suggested that BPA stimulated drug efflux by BeWo cells probably by direct effects on P-glycoprotein.

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Text of paper:

Effect of Bisphenol A on Drug Efflux in BeWo, a Human Trophoblast-like Cell Line

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Running Head: Bisphenol A Increases Drug Efflux

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1. Introduction

Bisphenol A (BPA) is a monomer of epoxy resins and polycarbonate plastics. It is widely used for the production of plastics, resins and coatings (1), including the inner coating of food cans and in dental sealant. BPA can enter the body by adsorption or ingestion since a significant amount of BPA has been detected in liquids from the plastic lining of food cans (2) and in the saliva of patients from dental composites or sealants (3). Additionally, many aquatic ecosystems appear to be polluted with BPA (1,4,5). BPA has been detected in early gestation of human pregnancy in serum at ng/mL levels and is significantly higher (up to 5-fold and greater) in amniotic fluid suggesting accumulation early in fetuses. Moreover, accumulation appears highest in the placenta and to favor male fetuses (6,7).

BPA is thought to mimic the action of estrogen *in vitro* and *in vivo* through binding to the estrogen receptor α (ERα) and β (ERβ) and regulating target gene expression. For example, BPA has been shown to activate ERα and ERβ (8,9), induce the expression of estrogen responsive genes and stimulate cell proliferation in MCF-7 breast cancer cells (10), and *in vivo*, disrupts growth and differentiation of the rodent reproductive tract and alters the release of prolactin from rat pituitary (11-14). Thus, BPA is generally considered to be an environmental estrogen and is an endocrine disrupter.

The human placenta serves as an interface for regulating xenobiotic distribution between fetal and maternal blood supplies. The rate limiting barrier in the placenta is a single layer of syncytial trophoblasts which appear to regulate drug and chemical exposure of the fetus through a group of transporters (15), a number of which are functionally expressed proteins and are localized to the maternal plasma membrane of the trophoblast (16-18). The prototype multidrug resistant mechanism in the placenta is the multidrug resistant gene product 1 (MDR1) also known as P-glycoprotein (P-gp). In addition, known to be present in the placenta are the multidrug resistance associated proteins (MRPs)

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and breast cancer resistance protein (BCRP) (19). Steroid hormones are both transported and modulators of these multidrug resistance mechanisms. For example, progesterone, one of the primary hormones of pregnancy, depending on concentration, has been reported to show inhibitory effects (above 10 μM) and stimulatory effects (below 1 μM) on P-gp activity. Progesterone, however, influences P-gp transport through an allosteric binding site (20,21). 17β-estradiol (E2), another primary hormone of pregnancy, is found to reverse BCRP mediated drug resistance (22) and the estrogen receptor antagonist, tamoxifen, reverses P-gp-mediated drug resistance *in vitro*. The nature of estrogen interations with P-gp are less clear than observed for progesterone (23,24). BPA also been shown to be substrate for P-gp. Accordingly, tissues expressing P-gp may therefore form a barrier to absorption of both natural and environmental estrogens and related structures (25).

Considering that the human placenta expresses P-gp (15-18), reports of BPA accumulation in the placenta and significant fetal exposure (6,7) are seemingly inconsistent with the presence of P-gp in the trophoblast. To consider this discrepancy, we have investigated how BPA might interact with P-gp in a human trophoblast model. Specifically, we are hypothesizing that BPA, similar to the natural steroid hormones, may alter P-gp efflux of drugs by the trophoblast. The availability of a human trophoblast-like cell line, BeWo, which forms an asymmetric monolayer to facilitate trans-cellular transport (26) provides the opportunity to examine the function and regulation of P-gp and other transporters associated with the trophoblast. Importantly, P-gp which is expressed by human trophoblasts has been shown to be expressed and functional in BeWo cell line (27). We therefore have utilized the BeWo cell line to determine whether BPA alters P-gp protein expression and the degree to which the agent may interfere with the efflux mechanism directly.

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2. Materials and Methods:

2.1 Materials

Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Peniciline/streptomycin as a mixture (10,000 U/mL), non-essential amino acids (MEM) and Novex® 8% Tris-Glycine gels were from Invitrogen Life Tech (Carlsbad, CA). Tissue culture flasks, 12-well and 24-well tissue culture plates were purchased from Corning Costar (Corning, NY). Dulbecco's modified Eagle's medium (DMEM), sodium bicarbonate, trypsin-EDTA (10x) solution, cyclosporin A, 4,4'-Isopropylidenediphenol (Bisphenol A), and Hanks' Balanced Salt Solution (HBSS) were from Sigma (St. Louis, MO). Calcein AM was obtained from Molecular Probes (Eugene, OR). Antihuman P-glycoprotein (C219) mouse monoclonal antibody was from Signet Laboratories (Dedham, MA). Peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA). ECL Plus Western Blotting Detection System was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Human P-glycoprotein membranes were purchased from Gentest (Woburn, MA). Microplate fluorescence reader was purchased from Bio-TEK Instruments, Inc (Winooski, VT). All other chemicals were also commercially available.

2.2 BeWo Cell Culture

The BeWo clone (b30) was obtained from Dr. Alan Schwartz (Washington University, St. Louis, MO, USA). The cells were cultured as previously described (28). Briefly, cells were grown in DMEM with 10% heat-inactivated FBS complemented with 0.37% sodium bicarbonate, 100 U/mL of penicillin/streptomycin, 2 mM glutamine and 0.1 mM MEM. The cells were maintained in 75-cm^2 surface area flasks at pH 7.4 under 5% CO_2 and 95% humidity at 37 °C. To pass the cells, the cells were exposed to a trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA in PBS). For uptake assays, cells were plated onto 12-well standard tissue culture plates with a density of 10,000 cells/cm^2. Cells were used upon forming a confluent monolayer (approximately 5-6 days in culture).

2.3 Chronic treatment of BeWo Cells with Bisphenol A

Selected concentrations of BPA were prepared as 1000 x stocks in ethanol. For 48 hr pre-treatment, cells at about 50% confluence were exposed to selected concentrations of BPA. The medium was changed once before use and BPA at the same concentration was included in the change medium. The control cells were cultured in the media containing vehicle (0.1% ethanol) under the same conditions.

2.4 Calcein Accumulation Studies

The accumulation studies were performed according to the methods described elsewhere with some modifications (29). Calcein AM is a substrate for P-gp and calcein, the fluorescent product, has been shown to be a substrate for both P-gp and MRP (30). The experimental procedures were conducted at 37 °C unless otherwise indicated. Briefly, the BeWo cells at confluence were washed twice with HBSS supplemented with 25 mM D-glucose (HBSS-Glc) followed by the pre-incubation with HBSS-Glc buffer with or without potential P-gp inhibitors for 30-45 min. The accumulation of calcein was performed by incubating cells with calcein AM (1 μg/mL as final) in the presence of selected concentrations of BPA or the vehicle (0.1% ethanol) for 45 to 60 min. The accumulation was stopped by

rapidly rinsing the cells three times with 1 mL/well of ice-cold HBSS-Glc. Following the rinse, each well received 1 mL of lysis buffer (2% Triton X-100). After incubation for 2 hr, the cell lysate from each well was mixed by repeated pipetting. The lysate was assayed to determine the intracellular level of calcein using a microplate fluorescence reader. Cellular protein in each lysate was also assayed with a BCA protein kit (Pierce). The accumulation of calcein was normalized as pmol calcein/mg cell protein.

2.5 Protein Extraction and Western Blot Analysis

The method for membrane isolation was as described previously with some modification (31). Briefly, the cells were harvested in Tris-Mannitol buffer [(50 mM Tris-HCl, pH 7.0 containing 300 mM mannitol and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] followed by centrifugation at 200 x *g* for 6 min. The cell pellet was lysed by homogenization in TMEP buffer (50 mM Tris, pH 7.0 with HCl, containing 50 mM mannitol, 2 mM EGTA, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 0.5 mM PMSF, 2 µg/mL chymostatin and 2 mM β-mercaptoethanol). Undisrupted cells and nuclear debris were removed by centrifugation at 500 x *g* for 10 min. The supernatant fluid was centrifuged at 15,000 x *g* for 15 min at 4°C. The resultant pellet referred to as membrane fraction was then resuspended in TMEP buffer with 30% glycerol (w/v) and the resultant supernatant solution collected was referred to as the soluble fraction. Both fractions were used immediately or saved at -70°C. The proteins from the soluble and membrane fractions were separated on Novex® 8% Tris-Glycine gels followed by western blot assay using anti-human P-gp. The immunoreactive proteins were detected with horseradish peroxidase (HRP) conjugated secondary antibodies and visualized by using an ECL plus chemiluminescence kit. Protein stained by anti-P-gp antibodies were analyzed after scanning and densitometry analysis by OptiQuant image analysis software (Packard Instrument Company).

2.6 Cytotoxicity Assays

Cell death was assayed 20-24 hours after the treatments by measuring the percentage of lactate dehydrogenase (LDH) released from BeWo cells into the media as described elsewhere (32). Briefly, the culture medium from the cells, 1 ml in total, was collected. The cells were collected by scraping into a 0.5 ml aliquot of 100 mM potassium phosphate (KP) buffer, pH 7.4. The cell suspension was sonicated for 2 sec. Both the media and cell suspensions were used for LDH efflux assay. The LDH assay mixtures were composed of 0.5 ml of culture media or 0.25 ml of cell suspension, 500 µl sodium pyruvate, and 850 µl of 100 mM KP buffer for media or 1.1 of ml 100 mM KP buffer for cell suspensions. LDH activities were determined by following the changes of absorbance at 340 nm upon addition of β-NADH (1 mg/mL). Release of LDH from the cells was expressed as:

LDH release (%) = [LDH in media/ (LDH in media + LDH in cells)] x 100%.

2.7 P-gp-ATPase Activity Assay

The P-gp-ATPase activity assay was conducted in a 96-well plate using human P-gp membranes according to the manufacturer's instructions (Gentest, Woburn, MA) with some modification. In brief, all drugs and human P-gp membranes were prepared in Tris-Mes buffer [50 mM Tris-Mes, pH 6.8, 2 mM EGTA, 2 mM dithiothreitol (DTT), 50 mM KCl, and 5 mM sodium azide]. Twenty-microliter of drug solution was loaded onto the plate followed by the addition of an equal amount of P-gp membrane solution (0.25 mg/mL final) with or without sodium orthovanadate (100 µM). The mixture was pre-incubated at 37 ˚C for 5 min with agitation. The reaction was started by the addition of 5 mM MgATP. After incubation for 1 hr at 37 ˚C with agitation, the reaction was stopped by adding 30 µl of 10% SDS-antifoam A solution. The amount of inorganic phosphate released from the reaction was immediately determined by a colorimetric reaction. The detection reagent containing 2% ascorbic acid (pH 5, freshly prepared), 7 mM ammonium molybdate and 3 mM zinc acetate was added to each finished reaction solution followed by the incubation at 37 ˚C for 1 hr in dark. The absorbance was read at 780 nM. ATPase activity was determined by the difference in P_i levels between 0 min (reaction stopped immediately with SDS) and a 60 min incubation period. To get vanadate-sensitive ATPase activity, P_i

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released in 60 min in the samples with vanadate was subtracted from the samples without vanadate. ATPase activity was expressed as nmole/mg protein/min.

2.8 Cytoplasmic Esterase Activity Assay

BeWo cells grown in 150 cm^2 surface area flasks with about 95% confluence were washed 3 times with 37 ˚C PBS and harvested using a cell scraper. The cell suspension was centrifuged at 450 x *g* for 6 min and the cell pellet was resuspended in a small amount of PBS. The cell suspension was lysed with sonication followed by centrifugation at 15,000 x *g* for 20 min. The soluble fraction was collected and used for analyzing endogenous esterase activity. The esterase activity assay was performed in a 96-well black-sided plate. The drugs were loaded in to each well and the reaction was started by the addition of the soluble fraction isolated from cells as described above. The mixture was incubated at 37 ˚C for 45 to 60 min with agitation. The formation of calcein in each sample was determined using a microplate fluorescence reader.

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3. Results

3.1 Effect of BPA on Accumulation of Calcein by BeWo Cells

To investigate whether BPA affected drug efflux in BeWo cells, calcein AM was applied to cells with the presence of selected concentrations of BPA or vehicle alone (0.1% ethanol) as a control. Compared to the control group, the intracellular accumulation of calcein was largely inhibited by BPA in a dose-dependent manner as shown in Figure 1A. These results suggest an activation of the efflux of calcein from the BeWo cells. BeWo cells were then grown under conditions of chronic exposure to BPA for 48 hours and then assayed for calcein accumulation. Control experiments in parallel provided for acute and chronic exposure to either BPA or vehicle (0.1% ethanol alone). Figure 1B results indicated that the chronic exposure of BeWo cells to BPA did not significantly alter calcein accumulation. However, conditions where BeWo cells were acutely treated, a significant decrease in calcein accumulation was typical.

3.2 Influence of BPA on P-gp Protein Expression

Although chronic exposure of BeWo cells to BPA did not show significant changes in the functional assays of calcein efflux, we did use immunoblots to assess cytoplasmic and membrane-bound P-gp protein after 48 hr pretreatments. As shown qualitatively in the blot and quantitatively in the density summaries in Figure 1C, high concentrations of 17β-estradiol decreased membrane- and supernatant-associated P-gp in BeWo cells by a very small amount (i.e., approximately 25%). By contrast to the 17β-estradiol effects and probably more consistent with the functional data in Figure 1B, 48 hr pretreatments with high concentrations of BPA increased membrane- and supernatant-associated P-gp in BeWo cells by only a modest amount (i.e., 55%). These results were consistent through three replications of different BeWo cell preparations.

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3.3 Effect of Combinations of BPA, 17β-Estradiol and Cyclosporin A (CsA) on Calcein Accumulation by BeWo Cells

To investigate whether BPA and 17β-estradiol affect drug efflux in a competitive manner, 17β-estradiol was added to the uptake solution along with BPA. As shown in Figure 1D, BPA in the combination of 17β-estradiol appeared to have an additive effect on reducing calcein accumulation. Like BPA, 17β-estradiol produces a significant, dose-dependent (1 nM to 10 μM) decrease in calcein accumulation (data not shown). To investigate whether the BPA-induced decrease in calcein accumulation could be blocked by CsA, a known P-gp inhibitor (33), CsA was included in our experiments as a potential competitor. Results summarized in Figure 1E show that on addition of CsA alone, calcein accumulation by BeWo cells was increased as expected. In combination with BPA, CsA reverses BPA effects on calcein accumulation by BeWo cells suggesting competition for interactions with P-gP.

3.4 BPA and Cytotoxicity in BeWo Cells

Near confluent BeWo cell monolayers were exposed to BPA concentrations ranging from 0.1 μM to 200 μM for 24 hrs. At concentrations of 150 μM or greater, BPA caused marked cell detachment from the culture surface as well as LDH release from the cells that was >20% relative to vehicle (0.1% ethanol) treated cells which exhibited LDH release of <5% of an untreated control (data not shown).

3.5 Effect of BPA on P-gp ATPase Activity

Substrates for P-gp generally activate ATPases associated with the protein. P-gp ATPase activity was examined under the conditions indicated in Figure 2. Verapamil was used as a positive control.

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Relative to the positive control and addition of calcein AM as a substrate, BPA did not show any effect on P-gp-ATPase activity. P-gp ATPase activity was greatly inhibited by 200 µM BPA, however that was a concentration found to be cytotoxic by morphological examination and the LDH efflux assay as summarized immediately above.

3.6 Effect of BPA on Cytoplasmic Esterase Activity

To determine that BPA does not influence calcein accumulation in BeWo cells by altering release of the fluorescent product, calcein, from calcein AM, cytoplasmic esterase activity was assayed in the soluble portion of BeWo cell lysates. BeWo cell esterase activity in the presence of BPA and representative P-gp and esterase inhibitors is summarized in Figure 3. Neither BPA nor CsA altered esterase activity associated with calcein generation in BeWo cells. However, 10 mM of paraoxon, an esterase inhibitor, was used as a positive control and was a significant inhibitor of calcein generation by BeWo cells.

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4. Discussion

A collection of reports suggest very strongly that estrogens and progesterones have significant influence on both the function (20-24) and the expression (34) of multidrug resistance mechanisms. In this study we have examined whether BPA, an environmental estrogen, interacts with P-gp through either altering the efflux mechanism itself or through changes in protein expression. Previously investigators have demonstrated that BPA is at least a substrate for P-gp in the intestine (25), although no evidence previously existed to indicate that BPA may influence expression of the P-gp protein.

We were able to establish in this study that BPA indeed does have an influence on P-gp as evidenced by a significant decrease in calcein accumulation in BeWo cells treated acutely with the agent. Moreover, we showed that pretreatment of BeWo cells for 48 hours had little effect on calcein accumulation. These results suggest a direct stimulatory action of BPA on the efflux mechanism. This was further supported in part by immunoblots which showed very small increases in membrane- and supernatant-associated P-gp. Similar findings were recently reported where an increase occurred in ERα positive cells treated with 17β-estradiol (35). It is noteworthy that in this BeWo cell clone, 17β-estradiol had no such effect on cytosolic P-gp expression. Preliminary immunoblot work in our laboratory shows that this BeWo clone highly expresses ERβ and is apparently negative for ERα and may provide an explanation for the lack of the 17β-estradiol effect in this cell line. The fact that BPA has modest effects on P-gp in contrast to 17β-estradiol would suggest different mediating mechanisms are involved in the BeWo cells. Overall BPA has a low affinity for estrogen receptors relative to 17β-estradiol and therefore also points to the likelihood of other mechanisms. In the literature, BPA was shown to activate at least nine nuclear receptors in murine placenta, including ERβ in the placenta of only male fetuses (36). BPA at 10 μM can significantly increase drug metabolism via stimulating the human orphan nuclear receptor-mediated transcription in HepG2 cells. Specifically, cytochrome P450

type 3A4 through PXR/SXR which also is known to mediate P-gp expression (37). Consequently, extensive confirmatory studies will be necessary to examine how BPA and 17β-estradiol might influence P-gp expression through nuclear receptor mechanisms.

The acute presence of BPA was able to greatly stimulate drug efflux activity suggesting that there might be a direct interaction between BPA and drug efflux transporter. In this study, we looked at whether BPA competes for 17β-estradiol efflux. We found instead that BPA in the combination with 17β-estradiol inhibited, apparently in an additive manner, the accumulation of calcein. This may suggest interaction of the two agents in concert at the same site on P-gp, although interaction with more than one site is also possible (21). The fact that BPA's effects were strongly reversed by CsA, a strong P-gp inhibitor and substrate, suggests that BPA is altering the efflux of calcein AM and calcein by the BeWo cells. Shapiro et al. (21) has shown that studies can be developed to elucidate binding site interactions on P-gp and that is an objective of future studies with BPA and the natural steroid hormones in the trophoblast.

We ruled out BPA induced changes in toxicity to explain our observations for calcein accumulation in BeWo cells. BPA for instance was found to be no more cytotoxic to BeWo cells than other cell types. Cell damage was measured using LDH efflux assays and observed only at concentrations (>150 μM) well above those used in these studies for examining P-gp activity. Similar results were obtained in TM4 cells when after a 16 hr exposure to 200 μM of BPA, cell viability was dramatically decreased (38).

Calcein AM was used in this study because it is cell membrane permeable, non-fluorescent, and a substrate for P-gp. Once calcein AM enters the cell, it is cleaved by cytoplasmic esterases to form the non-membrane permeant, and fluorescent calcein which is used to estimate drug accumulation in cells (39). In order to exclude the possibility that the decrease in calcein accumulation by BeWo cells might

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be due to the inhibition of cytoplasmic esterase activity, esterase assays were performed as part of our investigation. As indicated by our results, BPA did not significantly alter the metabolism of calcein AM to calcein.

Many substrates and modulators of P-glycoprotein have been shown to stimulate or inhibit vanadate-sensitive associated ATPases (31,40,41). As a consequence we examined that characteristic for BPA over a wide concentration range (100 nM to 10 μM). BPA was found not to alter P-gp ATPase activity in this study. This observation was unusual but not unlike observations made by others. For example, in the membrane vesicles from Sf9 cells infected with MDR-baculovirus, estradiol-17β (β-D-glucuronide), coincidentally a metabolite of estradiol, was identified as an endogenous substrate for P-gp. However, estradiol-17β (β-D-glucuronide) neither stimulated P-gp ATPase activity nor inhibited verapamil-stimulated ATPpase activity when applied at the concentration as high as 100 μM (42). Sharom (43) has also questioned whether drug-stimulated ATPase activity is representative of the transport function of P-gp.

The fact that our results thus far suggest that BPA can stimulate the export of drugs from cells without activating P-gp ATPase activity raises a number of possibilities in terms of mechanistic explanation. First, the possibility of involvement of MRP might be considered since calcein AM is known to be transported by P-gp and calcein can be transported by both P-gp and MRP1 (30). However, we have been unable to demonstrate functional MRP1 in this BeWo clone (27) and as shown here, and CsA strongly reversed BPA's effects on calcein accumulation. A second possibility is that BPA activation of P-gp could be due to a conformational change which allows calcein AM to be more easily accessed by the transporter. On P-gp, at least three drug binding sites are identified, namely as H site selective for Hoechst 33342 and cochicine, R site selective for rhodamine 123 and anthracyclines and P site for progesterone (21,44). Progesterone was found not to be transported by P-gp (34,45). It has been

postulated that the binding of progesterone allosterically induces a conformational change of P-gp, resulting in an enhancement of drug transport by the H and R sites. We presume that the stimulation of calcein AM transport could be due to the occupation of the progesterone site or another allosteric site by BPA. Since it is possible that steroids such as progesterones and estrogens have some counterbalancing effects on P-gp, carrying these studies out with a mixture of the steroids might also yield important considerations as suggested earlier. Finally, although BPA did increase P-gp efflux, we did not demonstrate efflux of BPA itself. BPA could interact with P-gp like progesterone, an observation that would be consistent with literature reports of BPA accumulation in the placenta and fetus (6,7). Future studies will require direct assay of BPA to further explore the mechanisms by which this agent interferes with P-gp and transverses the placenta.

In summary, in our present study, BPA appeared to directly stimulate P-gp. BPA only modestly stimulated P-gp protein expression and the altered expression did not contribute significantly to the transport of calcein. We submit that this study forms the basis for further exploration of BPA interactions with trophoblast transport mechanisms and for developing a better understanding of how the P-gp is regulated by endogenous and exogenous steroids.

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Figure Legends

Figure 1. Bisphenol A (BPA) effects on calcein accumulation and P-glycoprotein expression in BeWo cells. Calcein uptake was performed with BeWo cells grown in 12-well culture plates. Bars represent means ± standard deviation for at least triplicate experiments. *: $p<0.001$ versus control group by Student *t*-test; **a:** $p<0.05$ versus control group; **b:** $p<0.05$ versus 5 μM CsA.

A) Concentration-dependent effects of BPA on calcein accumulation by BeWo cells.

B) Acute and chronic effects of BPA (10 μM) on calcein accumulation by BeWo cells. Bars summarize: 1 – Control vehicle treatment for 48 hours; 2 – Effect of a 48 hour BPA pretreatment and BPA washed away before assessing calcein accumulation; 3 – Effect of a 48 pretreament with BPA and BPA added during assessment of calcein accumulation; 4 – Effect of vehicle (0.1% ethanol) pretreatment and BPA added during assessment of calcein accumulation.

C) Immunoblot of P-gp protein in BeWo cells with BPA and 17β-estradiol. Cells were pretreated for 48 hr with: 1 – vehicle alone (0.1% ethanol); 2 – 17β-estradiol (10 μM); 3 – BPA (10 μM). Cells were harvested and the proteins from soluble (60 μg/lane) and membrane fractions (40 μg/lane) were separated by SDS-PAGE. The proteins were stained with monoclonal anti-human P-gp antibodies. The arrow head indicates the position of P-gp. Bar graphs show corresponding density measures for membrane- and supernatant-association blots after scanning and densitometry analysis by OptiQuant image analysis software.

D) Effect of 17β-estradiol (1 μM) and BPA (10 μM) on calcein accumulation by BeWo cells.

E) Effect of Cyclosporin A (CSA; 5 μM) and BPA (10 μM) on calcein accumulation by BeWo cells.

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Figure 2. Effect of bisphenol A (BPA) on P-glycoprotein ATPase activity. ATPase activity was measured under the following conditions: **1 –** buffer alone; **2 –** with 20 µM verapamil; **3 –** with 2.5 µg/mL Calcein AM; **4 –** with 100 nM BPA; **5 –** with 100 nM BPA plus 2.5 µg/mL Calcein AM; **6 –** with 1 µM BPA; **7 –** with 1 µM BPA plus 2.5 µg/mL Calcein AM; **8 –** with 10 µM BPA; **9 –** with 10 µM BPA plus 2.5 µg/mL Calcein AM; **10 –** with 200 µM BPA and **11 –** with 200 µM BPA plus 2.5 µg/mL Calcein AM. ATPase activity was determined by the amount of inorganic phosphate released from the reaction. The bars indicated the standard deviation with an N=3. **a:** $p < 0.05$ versus control and **b:** $p<0.05$ versus 2.5 µg/mL calcein AM

Figure 3. Effect of Bisphenol A (BPA) on BeWo esterase activity. The soluble portion of BeWo cell lysate was incubated with calcein AM under the conditions as follows: **1 –** vehicle alone; **2 –** 5 µM cyclosporine A; **3 –** 10 µM BPA alone; **4 –** 10 µM BPA plus 5 µM cyclosporin A; **5 –** 10 mM paraoxon alone; **6 –** 10 mM paraoxon plus 5 µM cyclosporin A. Bars represent means ± standard deviation for at least triplicate experiments. *: $p<0.01$ compared to control group by Student *t*-test.

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Figure 1

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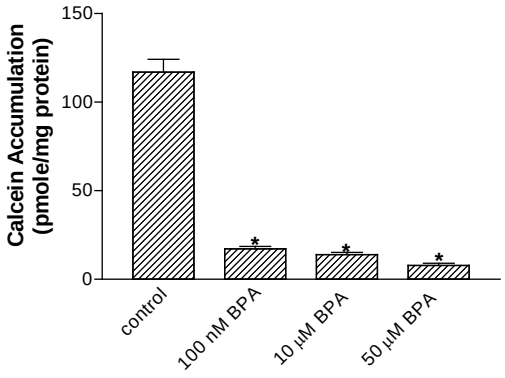
A.



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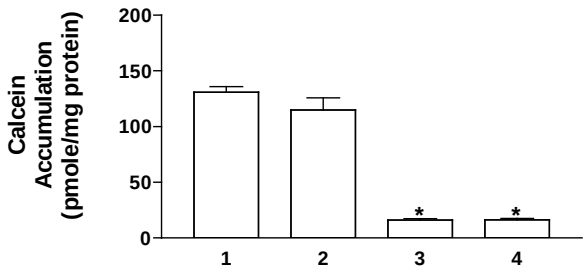

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Figure 1

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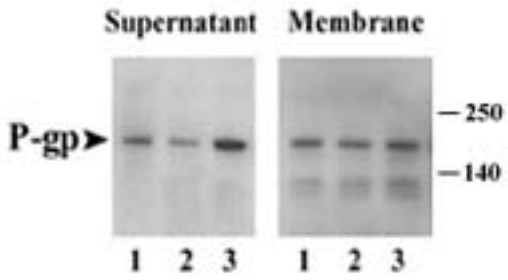
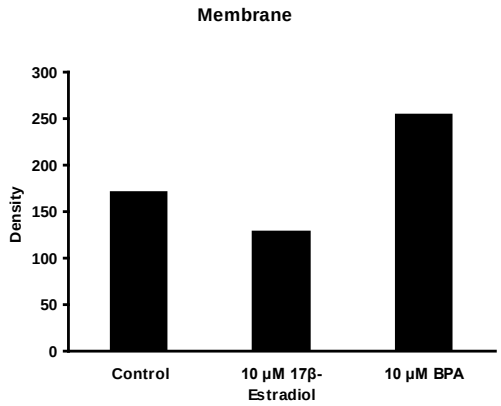
C.





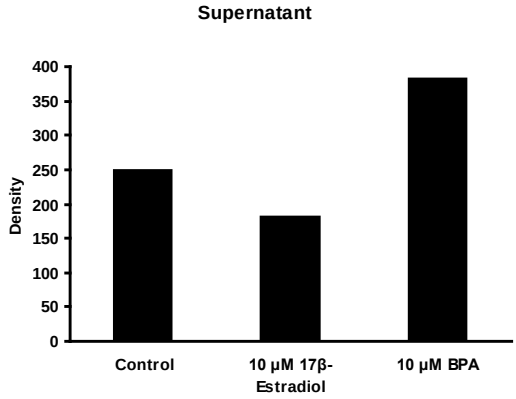


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Figure 1

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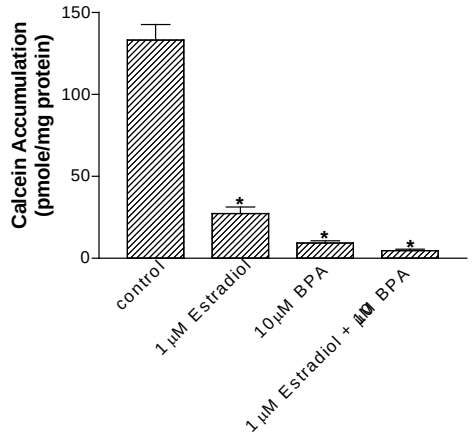
D.



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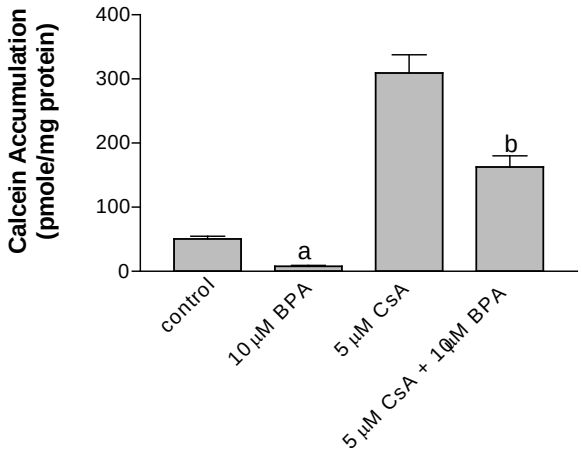

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Figure 2.

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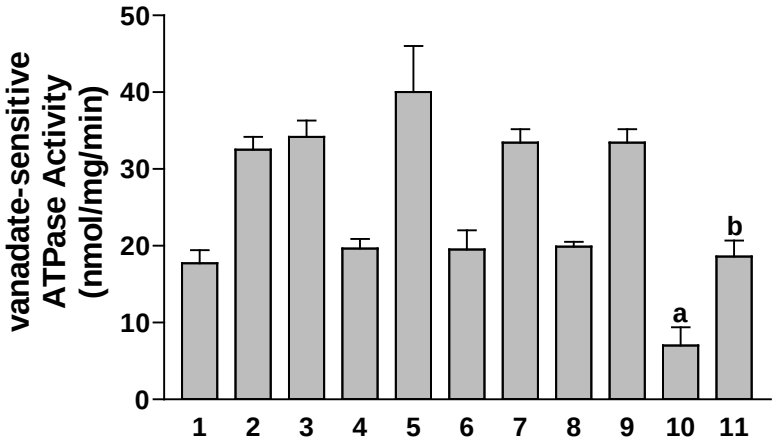

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Figure 3.
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