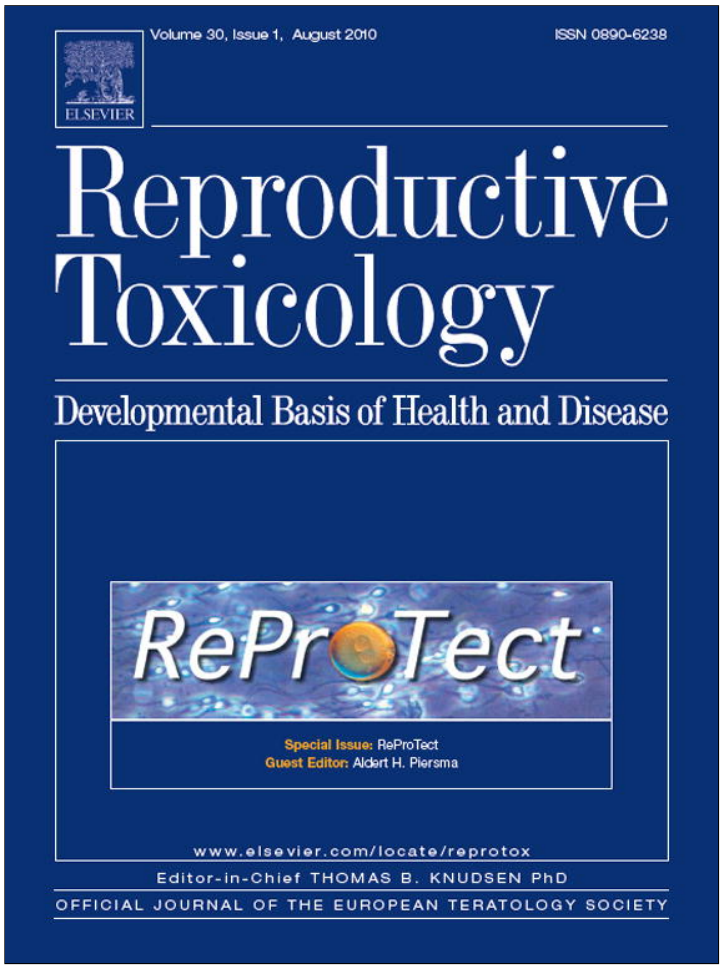

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Placental transport and *in vitro* effects of Bisphenol A

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ABSTRACT

Bisphenol A (BPA), an estrogen-like chemical, leaches from consumer products potentially causing human exposure. To examine the effects of BPA exposure during pregnancy, we performed studies using the BeWo trophoblast cell line, placental explant cultures, placental perfusions and skin diffusion models, all of human origin. Results showed BPA cytotoxicity in BeWo cells with an apparent EC50 at 100–125 μM. BPA exposure significantly increased β-hCG secretion and caspase-3 expression in placental explants at an environmentally relevant concentration of 1 nM. In the transport studies, a rapid transfer of BPA was observed across the term placentae and the BeWo cell monolayer. Further, transdermal transport of BPA was observed. These results indicate that fetal BPA exposure through placental exchange occurs with potential adverse implications for placental and fetal development. This battery of test systems within the realm of human implantation and fetal development represents important elements in risk assessment of reproductive toxicity.

1. Introduction

In 2004, the annual production of Bisphenol A (BPA) in the US used in polycarbonate, epoxy resins and other consumer products was estimated to be 2.3 billion pounds [1]. The majority of the BPA products on the market are in direct contact with food or liquid. When changes in heat or acidity occur, or if the product contains residual monomers, BPA can be released with risks of human exposure [2]. The major human route of exposure is assumed to be dietary, though dermal occupational exposure is also seen. BPA has also been found in ambient air and dust samples at up to 208 ng/m^3 and 17 μg/g, respectively [3]. This implies three routes of exposure: oral ingestion, inhalation, and dermal exposure.

Human exposure to BPA has been estimated by the European Commission to be 1.46 μg/kg bodyweight/d for an adult [2], and measurements have revealed levels in plasma ranging from 0.3 to 18.9 ng BPA/mL [4–7]. BPA has also been detected in umbilical cord blood (0.2–9.2 ng/mL), indicating transport of BPA across the placental barrier, in placental tissue (up to 104.9 ng/g) [6], and in amniotic fluid (0–8.38 ng/mL) [4,5].

BPA has the ability to interact with human estrogen receptors of both α, β, and γ subtypes [8,9], and *in vitro* experiments have revealed significant endocrine activity of BPA through estrogen and androgen receptors [10,11]. In women with polycystic ovary syndrome, higher levels of serum BPA concentrations were found compared to controls [12], and high levels of total BPA in the urine correlated with diagnosed cardiovascular diseases and diabetes [13]. In small rodents, *in utero* exposure to 2.4 μg BPA/kg bodyweight/d induces premature puberty, measured as vaginal opening [14]. Interference with normal development of reproductive organs and mammary glands has also been reported after BPA exposure pre- and postnatally in female and male mice to 250 ng/kg-d and 50 μg/kg-d, respectively [15,16]. Recently, adverse effects of BPA on behavioural and mental development and function, as well as impairment of sexual differentiation in exploratory behaviour, has been reported after *in utero* treatment to 10 μg BPA/kg-d in mice [17]. However, there are also studies showing no effects of BPA treatment [18–20].

In the present study, the potential adverse effects of BPA exposure during pregnancy were investigated by examining the effect on the trophoblast-derived BeWo choriocarcinoma cell line and explants from first trimester human placentae. Moreover, the fetal bioavailability of BPA was investigated with transport experiments

Abbreviations: BPA, Bisphenol A; TEER, transepithelial electrical resistance; IEMA, immunoenzymometric assay; P-gp, P-glycoprotein; P, permeability; P_e, apparent permeability.

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of full-term placentae, BeWo cell monolayers, and experiments on percutaneous penetration of BPA.

2. Materials and methods

2.1. Ethics

Human tissue collection strictly adhered to the guidelines outlined in the Declaration of Helsinki and the study was approved by the relevant ethics committees. Informed consent was obtained from all placenta donors.

2.2. BeWo cell cultures

The human choriocarcinoma cell line BeWo (Istituto Zooprofilattico Sperimentale, Brescia, Italy) was used to study *in vitro* effects of BPA on cell viability and β-hCG secretion. Cells were cultured in Ham's F-10 without phenol red (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 100 U/mL penicillin/streptomycin and 2 mM glutamine (Sigma Chemical Co.) in a humidified atmosphere with 20% oxygen and 5% carbon dioxide (CO_2) at 37 °C. Cells were grown in 96-well plates until 70–80% confluence. After overnight storage in 2% FBS, BeWo cells were exposed to BPA (10 pM to 1 mM) or medium plus vehicle (0.1% ethanol) controls. After 24 h of incubation, culture medium was stored at −80 °C for β-hCG analysis and cells were assessed for cell viability. Experiments were carried out in quadruplicate and repeated three times.

The b30 BeWo cell clone (kindly provided by Dr. Margaret Saunders, Bristol, UK, with permission from Dr. Alan Schwartz) was used for the transport assays. Cells of the b30 clone form a confluent monolayer when grown on a membrane, a feature which the original BeWo clone lacks [21] and therefore serve as a relevant model for placental transport studies [22,23]. Cells were cultured in Dulbecco's modified essential medium (DMEM) (D5671, Sigma Chemical Co.) with 10% FBS, 100 μg/mL penicillin/streptomycin and 2 mM L-glutamine. For experimental setup, the cells were grown on special polyester Transwells (Pore size 0.4 μm, 1.12-cm^2 growth area, apical volume 0.5 mL, basolateral volume 1.5 mL, Corning Costar, NY, USA) with an apical and basolateral chamber resembling the maternal and fetal circulation, respectively. Cells were seeded in the apical chamber (224,000 cells/mL), which was coated with human placental collagen (type IV, nr. C7521, Sigma Chemical Co.) (1 mg/345 μL 0.1% acetic acid, then diluted 1:4 in 70% ethanol). The resistance across the monolayer was measured by an EndOhm apparatus (World Precision Instr., Sarasota, FL). The experiments were conducted when the cells were confluent with a transepithelial electric resistance (TEER) above 30 Ω cm^2 (preferably 40–60 Ω cm^2).

2.3. Human chorionic first trimester villous explant cultures

First trimester human placentae (8–9 weeks of gestation) were obtained at the Hospital Campostaggia after elective terminations of pregnancy. Tissues were rinsed in cold phosphate buffered saline (PBS) and dissected as described by [24] within 2 h of obtaining the placental tissue. Briefly, small fragments of villous tips (15–20 mg wet weight) were placed on Millicell CM culture dish inserts (Millipore Corp, Bedford, MA), previously coated with 180 μL undiluted matrigel (Collaborative Research, Inc., Bedford, MA) and inserted in 24-well plates. Explants were cultured in serum-free DMEM/F12 without phenol red (Gibco, Grand Island, NY) supplemented with 100 μg/mL penicillin/streptomycin and 2 mM L-glutamine (Sigma Chemical Co.). Explant cultures were incubated overnight at 37 °C in 5% CO_2 for attachment to the matrigel, whereupon the culture medium was replaced with medium containing 1 nM BPA, or medium plus ethanol (controls). After 48 h, explants were removed from matrigel, washed in PBS, frozen and stored at −80 °C until processing for Western blot analysis. Culture medium was stored at −80 °C until assayed for β-hCG. BPA exposure and control cultures were carried out in triplicate; tissue samples and supernatants from separate explant cultures were pooled at the end of incubation.

2.4. Cell viability

After 24 h of incubation, 10 μL of the fluorescent dye, alamarBlue TM (aB or resazurin), was added to 100 μL culture medium of BPA-treated or untreated BeWo cells. After 4 h, the fluorescence was quantified as described by [25]. Blank values were obtained in wells with culture medium and aB in the absence of cultured cells. Data were expressed as percentage of control.

2.5. β-hCG secretion

The concentration of β-hCG in BeWo cells and explant medium was assessed by a commercial immunoenzymometric assay (IEMA) (Radim SpA, Pomezia, Italy). Results were expressed as mIU/mL for BeWo cells and in relation to protein concentration for explant cultures, determined from explant lysates by the Quick Start Bradford Protein Assay (Bio-Rad Laboratories).

2.6. Western blot for caspase-3 expression

Villous explants were homogenized in ice-cold lysing buffer (Tris–HCl 50 mM, NaCl 50 mM, Triton X 100 1%, Na deoxycholate 1%, SDS 0.1%) containing 100 mM sodium orthovanadate 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 × *g* for 15 min at 4 °C. Protein concentration was determined by the Quick Start Bradford Protein Assay (Bio-Rad Laboratories). 150 μg of total proteins were separated on a 12% polyacrylamide gel in the presence of SDS and β-mercaptoethanol transferred to nitrocellulose filters (Hybond-C, Amersham International, Little Chalfont, UK). Membranes were incubated in blocking solution (5% [wt/v] powdered milk in 10 mM PBS, 0.1% Tween 20) for 1 h and exposed to 1.0 μg/mL of goat anti-human caspase-3 (R&D Systems, Abingdon, UK) overnight at 4 °C. Nitrocellulose filters were washed three times with PBST (0.1% Tween 20 in PBS 10 mM) and exposed for 1 h to the rabbit anti-goat antibody, labeled with peroxidase (Bio-Rad Laboratories) at room temperature. Bands were visualized with a chemiluminescence kit (Immun-Star Chemiluminescent Kit, Bio-Rad Laboratories) according to the manufacturer's instructions. The pro and cleaved caspase-3 enzyme bands were detected at 20 s and 15 min. Equal loading of the proteins was confirmed by staining the blots with a 10% (v/v) Ponceau S solution (2% Ponceau S in 30% trichloroacetic acid/30% sulfosalicylic acid, Sigma Chemical Co.). Densitometric analysis was performed by Quantity One Software (Bio-Rad, Milan, Italy), giving the ratio of the densitometric values obtained in caspase 3-specific chemiluminescence reaction and the major Ponceau S-stained membrane bands [26].

2.7. BeWo transport assay

The transport assay method was described and optimized recently [27]. In short, when a confluent monolayer was observed (day 6 or 7), the cells were washed three times and pre-incubated for 30–60 min in 37 °C Hank's balanced salt solution (HBSS) (H8264, Sigma Chemical Co.). HBSS containing 0.5 μM radiolabelled BPA (Ring-14C (U) 0.1 mCi/mL, ARC-1671, American Radiolabeled Chemicals Inc., St. Louis, USA) was added to the donor chamber and regular HBSS to the receiving chamber. The concentration of BPA was chosen to resemble levels detected in human blood samples [5,6]. Samples of 100 μL were collected at relevant time-points from the recipient chamber and the volume was replaced with HBSS. The experiments were conducted in both transport directions with and without the addition of 100 μM verapamil (Sigma Chemical Co.), which was previously reported to inhibit the P-glycoprotein (P-gp) transporter in BeWo cells [28]. After the experiment, a sample (100 μL) was collected from the donor chamber. All samples were mixed with 2 mL of scintillation buffer (XR LS-372, BN instruments A/S) and counted by liquid scintillation (TRI-CARB 2300TR, Packard). Standard samples of BPA and a calibration curve were included in each test round. To correct for the influence of the coated membrane on BPA permeability, blank tests without cells were performed in both transport directions with and without verapamil.

The permeability of BPA across the monolayer was calculated from equation A, where P = permeability, ΔQ = number of moles transported at Δt (= time point), A = the transport area and C_0 = the initial concentration of the substance on the donor side. The apparent permeability (P_e) was calculated by equation B, which is derived from the relationship presented in Utoguchi et al. where P_t = the total permeability across the cells and the membrane, P_e = the permeability across the cells alone and P_m = the permeability across the membrane alone [29].

$$\text{A}: \quad P = \frac{\Delta Q/\Delta t}{AC_0}\ \text{cm/s} \qquad \text{B}: \quad P_e = \frac{1}{(1/P_t) - (1/P_m)}\ \text{cm/s}$$

The permeability ratio of P_e in the basolateral to apical direction divided by P_e in the apical to basolateral direction was calculated. A ratio of 1.5 or higher designates active efflux [30].

2.8. Placental perfusions

Term placentae were collected after elective caesarean sections or vaginal deliveries from the University Hospital of Copenhagen. The *ex vivo* perfusion method was described in detail previously [31]. A dually perfused, closed system was used. Briefly, an appropriate vascular unit was cannulated and perfused with Krebs Ringer Buffer containing heparin (5000 IU/mL) and glucose (9 mM). Two cannules were gently placed in the maternal side of the cotyledon and the circulations were controlled by pumps to 9 mL/min (maternal) and 3.5 mL/min (fetal). Physiological levels of Human Serum Albumin (HSA, 20% solution, CSL Behring Gmbh, Marburg, Germany) were added to the maternal (30 or 50 g/L) and fetal (40 or 60 g/L) Krebs Ringer solutions. BPA binds to albumin [32] and HSA is therefore added to reflect physiological conditions. A limited amount of BPA (FM ratio of 0.29) is transported in perfusions without the addition of HSA [33]. The system was maintained at 37 °C and the maternal and fetal solutions were gassed with 95% O_2–5% CO_2 and 95% N_2–5% CO_2, respectively. The cotyledon was preperfused for 30 min to establish stable venous outflow and oxygen transfer. Radiolabelled BPA and non-labelled BPA (99%, Sigma Chemical Co.) and the positive reference substance antipyrine (Aldrich-Chemie, Steinheim, Germany) were added to the maternal compartment in final concentrations of 0.5 μM BPA (10% radiolabelled BPA) and 100 μg/mL antipyrine. As an expression of the placental transfer, the fetal–maternal concentration ratio (FM) was calculated. The perfusions were considered successful with fetal compartment leakage of ≤3 mL/h and a FM ratio for antipyrine above 0.75 by the end of perfusion. The recovery of the BPA initially added was calculated as described previously [34].

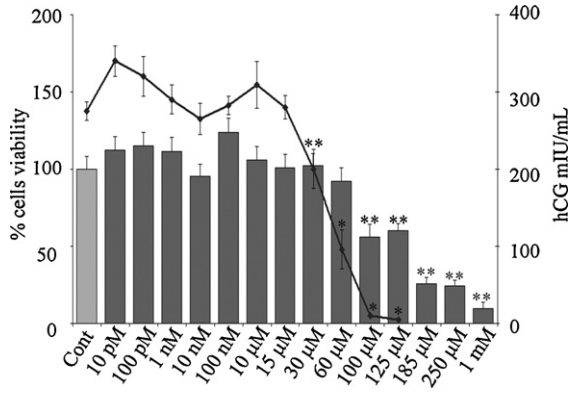


Fig. 1. Cell viability (histograms) in BeWo cells exposed for 24 h to BPA concentrations from 10 pM to 1 mM. Results are expressed as % of control cell viability. β-hCG concentration (lines) in culture medium of BeWo cells exposed for 24 h to BPA (10 pM to 1 mM). Results are expressed as mIU/mL of culture medium. C = control cultures exposed to 0.1% ethanol (vehicle). Data is the mean of 3 separate experiments. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

2.8.1. Perfusion sample analysis

Samples were collected before and after addition (null sample) of BPA, and at different time-points up to 150 min. Samples were centrifuged for 5 min at 4000 × *g* and supernatant and pellets were collected. Samples of the perfused cotyledon and the surrounding tissue were collected and BPA was extracted in 3 mL acetonitrile (J.T. Baker) for 3 days. All samples were analyzed for BPA content by liquid scintillation counting as described above.

For antipyrine analysis, the protein was precipitated by 200 μL ice-cold acetonitrile containing 10 μL/mL of internal standard phenacetin (4-acetophenetidide 97%, Acros Organics, Geel, Belgium) to each 200 μL sample. The samples were centrifuged for 10 min at 2400 rpm, and 200 μL of the supernatant were analyzed by HPLC as previously described [31].

2.8.2. Ethanol

BPA was dissolved in 96% ethanol (Kemetyl A/S, Koge, Denmark) resulting in a final ethanol concentration of 2‰ in the placental perfusions and 1‰ in BeWo experiments. Ethanol concentration in the samples was analyzed by headspace gas chromatography using a Restek BAC-1 or BAC-2 column, *tert*-butanol or 2-butanol as internal standard and a FID-detector.

2.9. Dermal absorption

Dermal absorption of BPA was investigated by the *in vitro* diffusion model (static diffusion cells, Franz type) using full thickness human skin (0.8–1.0 mm) and following OECD guideline 428 as previously described [35,36]. In short, the human skin was placed in the diffusion cells separating the donor and the receiving chambers. The diffusion cells were placed in water at 35 °C to ensure a skin temperature of approximately 32 °C. The integrity of the skin was evaluated by capacitance measurements. The skin was exposed to 17.5 mM BPA for 48 h in the donor chamber and samples were collected from the receiving chamber at regular time intervals. After the experiment, BPA remaining in the donor chamber and in the skin was determined. Samples as well as acetonitrile extracts of skin samples were analyzed by liquid scintillation counting.

2.10. Statistical analysis

Data from explants studies were compared by ANOVA followed by Dunnett's test. Data from BeWo transport experiments were compared by a two-tailed Student's *t*-test. *p*-Values ≤ 0.05 were considered statistically significant.

3. Results

3.1. BeWo cell viability and β-hCG secretion

BeWo cells were exposed to BPA in a wide range of concentrations (10 pM to 1 mM) or medium plus vehicle (0.1% ethanol) controls. After 24 h of exposure, cultures were examined for cell viability and β-hCG secretion. As shown in Fig. 1, cell viability was significantly reduced in BPA exposed cells at concentrations ranging from 100 μM to 1 mM, with an apparent EC_{50} at 100–125 μM. A significant decline in β-hCG secretion was seen at 30 μM BPA exposure ($p < 0.05$) and secretion dropped dramatically from 60 to 125 μM BPA ($p < 0.01$) (Fig. 1).

3.2. Chorionic villous explant cultures

Explant cultures ($n = 6$) were exposed to 1 nM BPA or medium plus vehicle (0.1% ethanol) controls. The BPA concentration of 1 nM is environmentally relevant [3,8] and is non-toxic to trophoblast, as revealed from experiments in BeWo cells. After 48 h of BPA exposure, cultures were examined for β-hCG secretion and caspase-3 cleavage, which are specific markers for differentiation of trophoblasts and apoptosis. β-hCG secretion was significantly increased compared to controls (Fig. 2A). Western blot analysis with anti-caspase-3 antibody revealed two bands corresponding to the pro and the cleaved caspase-3 enzyme at 32 and 20 kDa, respectively (Fig. 2B). Densitometric analysis showed a significant decrease of pro-caspase 3 and a significant increase in the cleaved form following exposure to 1 nM BPA (Fig. 2C) indicating cell apoptosis.

3.3. Transcellular BeWo transport

The BeWo cell monolayer was confluent on day 6 after seeding. The TEER was $60 \pm 16\ \Omega\,cm^2$ before experiments and $34 \pm 6\ \Omega\,cm^2$ after completing the experiments. BPA crossed the BeWo cell monolayer and was detected in the receiving compartment after 5 min in both transport directions. The permeability (P_e) of BPA across the BeWo b30 monolayer was higher in the experiments performed in the fetal to maternal direction compared to the opposite direction. The P_e in the fetal to maternal direction without verapamil was significantly higher than the P_e in the same direction with 100 μM verapamil (*p*-value < 0.05, Fig. 3A), indicating that inhibition of the P-gp efflux pump reduces the fetal to maternal BPA transport. The permeability ratio of BPA was 2.4 after 15 min and 1.65 after 30 min, indicating active efflux of BPA by the BeWo cells. With verapamil present, the permeability ratio was 0.85 and 0.92 after 15 and 30 min, respectively, revealing an inhibitory effect of verapamil on BPA efflux (Fig. 3B).

3.4. Transplacental transport

A total of 6 perfusions were considered successful (for individual data see Table 1). No difference was observed in the transport of BPA between the two concentrations of HSA (see Section 2.8) or the means of delivery. All mothers were non-smokers and 4 had taken prescribed penicillin. The placentae were obtained after caesarean sections in 3 cases and after vaginal delivery in the remaining three.

The passage of BPA, ethanol and the reference substance antipyrine occurred in a similar manner during the 2.5-h perfusions (Fig. 4). All substances reached a mean FM ratio of approximately 1 after 90 min of perfusion. 19.4% of the added BPA was transferred across the barrier. The mean recovery of BPA was 71.8 ± 12.4% and the mean accumulation was 14.3 ± 5.5% in the perfused cotyledon and 12.7 ± 8.5% in the surrounding placental tissue (Table 1).

3.5. Dermal absorption

Within 48 h, 13% of the added BPA was recovered in the receiving chamber, which represents the percutaneous absorption. In the epidermis and dermis, 7.4% and 17% were recovered, respectively (Table 2).

4. Discussion

The BeWo cells have preserved many cytotrophoblastic properties and are considered an appropriate *in vitro* system to investigate

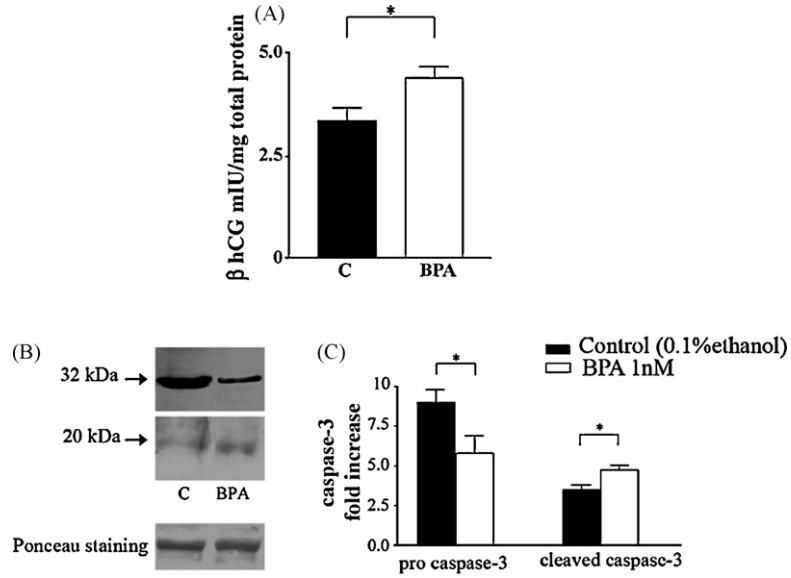
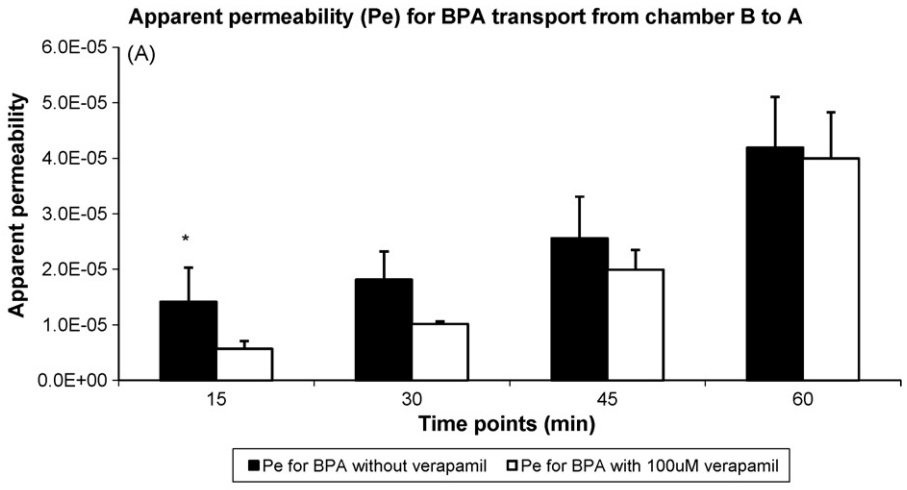


Fig. 2. (A) β-hCG secretion by explant cultures from first trimester of human pregnancy stimulated with 1 nM BPA or vehicle alone (0.1% ethanol, C) for 48 h. Data are the mean of 6 separate experiments. $^{*}p \leq 0.05$. (B) Representative Western blot performed on total explants culture lysates for caspase 3 expression. C: control cultures; BPA: 1 nM BPA treatment. Ponceau S staining shows equal protein loading. A band of 32 kDa, corresponding to pro-caspase-3 was revealed by chemiluminescence after 20 s of exposure in BPA-treated and control untreated explant cultures. Longer (15 min) blot exposure revealed a band of 20 kDa corresponding to the caspase-3 cleaved form. (C) Densitometric analysis performed on $n = 3$ separate experiments showed statistical differences ($^{*}p \leq 0.05$) between responses of explants treated with 1 nM BPA and their respective controls both for the pro and the cleaved form of caspase-3 enzyme.



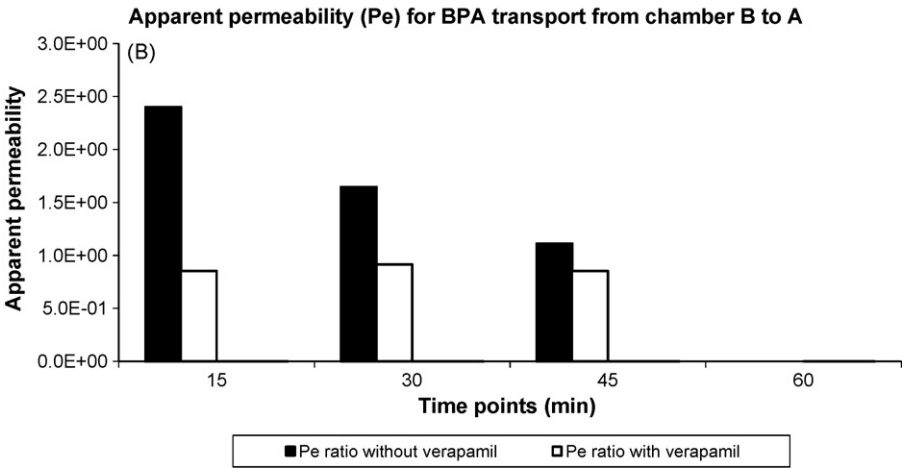


Fig. 3. Apparent permeability for BPA transport across the BeWo cell monolayer. (A) Comparison of the P_e for transport from B to A with and without verapamil. (B) Comparison of the permeability ratio with and without verapamil. Statistical differences between P_e were found between experiments with and without verapamil, determined by two-tailed Student's *t*-test ($^{*}p < 0.05$).

Table 1
Data from the individual placentae used for BPA perfusions, and the respective experiment control and recovery data. Recovery is expressed as % of added BPA. The individual placentae are displayed as numbers 1–6. BPA, Bisphenol A; SD, standard deviation; M, Maternal; F, Fetal; Y, Yes; N, No.

Parameter	Bisphenol A						Mean ± SD
	1	2	3	4	5	6	
Volume loss (M, mL)	4.5	14.4	−7.7	3.9	6.8	25.0	7.8 ± 11.0
Volume loss (F, mL)	10.4	4.7	10.8	−2.1	8.2	6.1	6.4 ± 4.8
Flow (fetal, mL/2 min)	6.9	7.3	7.3	6.5	7.2	6.5	7.0 ± 0.4
Time from birth to lab (min)	28	–	–	25	–	25	26 ± 1.7
Preperfusion (min)	41	71	43	60	32	42	48.2 ± 14.7
Maternal age (year)	35	39	29	38	27	19	31.2 ± 7.7
Placental weight (g)	718	662	889	667	829	799	761 ± 93
Cotyledon weight (g)	23	15	46	45	27	19	29 ± 13.2
Total perfused tissue (g)	73	65	100	108	83	67	82.6 ± 17.8
Cesarean section (Y/N)	Y	Y	N	Y	N	N	–
Gestation age (days)	–	271	290	267	285	–	278 ± 11
Smoker (Y/N)	N	N	N	N	N	N	–
Medicine (Y/N)	Y	Y	N	Y	Y	N	–
% BPA recovered in maternal perfusate	20	21	23	18	18	17	19.5 ± 2.3
% BPA recovered in fetal perfusate	22	16	22	22	15	18	19.1 ± 3.1
% BPA recovered in cotyledon	14	8	17	23	14	10	14.3 ± 5.5
% BPA recovered in surrounding tissue	14	4	19	6	7	26	12.7 ± 8.5
% BPA recovered in samples	5	6	5	4	4	7	5.2 ± 1.3
% BPA recovered red blood cells	<1	<1	<1	<1	<1	<1	–
Total BPA recovery	76	55	87	74	59	80	71.8 ± 12.4

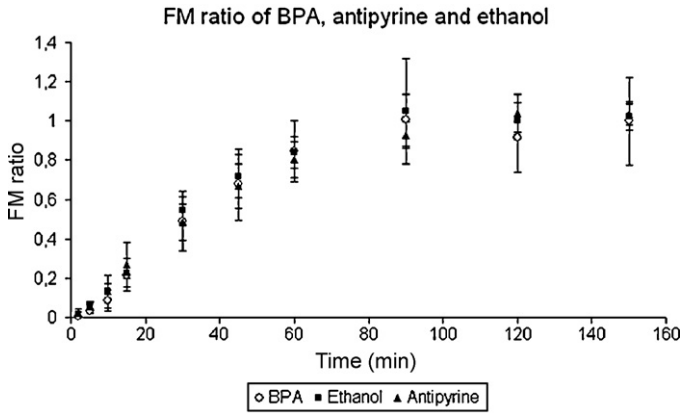


Fig. 4. Fetal–maternal (FM) ratio of BPA, antipyrine, and ethanol. The transport of BPA, antipyrine, and ethanol occurred in a similar manner during the perfusions. An FM ratio of approximately 1 was reached after 90 min, indicating concentration equilibrium in the fetal and maternal compartments.

placental transport [22,37]. To identify toxic concentrations of BPA in human placenta, we analyzed cell viability and β-hCG secretion in a test range of 10 pM to 1 mM using *BeWo cells*. We found that BPA significantly reduced cell viability and β-hCG secretion at 100 μM BPA (22.8 μg BPA/mL) and 30 μM BPA (6.85 μg BPA/mL), respectively. Based on these findings we selected a lower, non-toxic BPA concentration in the functional study on explant villous cultures, where exposure to BPA concentrations (1 nM or 0.2 ng/mL) significantly increased β-hCG secretion and caspase 3 cleavage. It should be noted that the expression of P-glycoprotein in these BeWo cells is lower than the expression of P-gp in the b30 BeWo clone and in human placenta [38–40]. The toxicity studies carried out with the cells from the original BeWo clone were undertaken to provide initial estimates of toxicity in order to select the concentrations that would be employed in the subsequent experiments of this study, but since the expression of P-gp is reduced in these cells, it is expected that other model systems utilized in this study which have a more complete efflux system (i.e., the b30 clone and human placenta), would tolerate higher amounts of BPA before reaching toxic limits due to having comparatively reduced intracellular levels of BPA.

β-hCG is a marker of continuous endocrine activity of trophoblast cells and more specifically of the syncytiotrophoblast, the epithelial covering of chorionic villi [41–43]. While the decreased β-hCG release at high BPA concentrations indicates loss of trophoblast function, the significantly increased β-hCG release at low, non-toxic BPA concentrations may reflect cell differentiation to syncytiotrophoblast. Cell apoptosis by caspase-3 fragmentation at low BPA concentrations also suggests that BPA affects the differentiation of trophoblast, with an increase in epithelial shedding as a consequence.

BPA was transported across the BeWo monolayer in both transport directions. In the experiments without verapamil the apparent permeability (P_e) from the fetal to the maternal side was higher than in the maternal to fetal direction, with a ratio of 2.4 and 1.65 after 15 and 30 min, respectively. A ratio above 1.5 indicates active efflux of the substance. With P-gp inhibited by 100 μM verapamil, the permeability ratio between the two transport directions only reached 0.85 and 0.92 at 15 and 30 min, respectively. Thus, the permeability from the fetal model compartment to the maternal model compartment is significantly higher in the experiments without verapamil, compared to the experiments where the P-gp is inhibited. P-gp expression has been detected in BeWo cells of the b30 clone [28] and BPA has been shown to be a substrate for the P-gp transporter [44,45]. These results therefore indicate that part of the BPA is actively effluxed by the P-gp protein in an attempt to protect the fetus. The majority of the added BPA, however, remains in the maternal compartment. Efflux of other P-gp substrates by BeWo cells such as vinblastine, vincristine and digoxin has also been reported [28].

Transfer across the human placenta depends on several factors, including the size and physiochemical properties of the substances. A report on parameters important for placental and dermal trans-

Table 2
Percutaneous penetration of BPA, given as mean values ± SD from 11 independent Franz cells. The overall recovery was 82.1% of the applied dose.

	Bisphenol A (μg)	Bisphenol A (% of applied dose)
Donor recovery	188 ± 38.2	44.5
Epidermis deposition	31.2 ± 6.9	7.4
Dermis deposition	73.1 ± 27.4	17.2
Skin deposition	104 ± 24.6	24.6
Receptor recovery	55.3 ± 23.1	13.0

port observed that small size and moderate lipophilicity ($\log K_{ow}$) increase transport rate [33]. With a molecular weight of 228.2 g/mol and a $\log K_{ow}$ of 3.4, BPA is a relatively small chemical with moderately hydrophobic properties. Ionized charged substances usually do not pass the lipid membranes of the placenta, however, BPA is not ionized at physiological pH-levels (pK_a between 9.59 and 11.3) [46]. The physiochemical properties of BPA therefore point towards a possibility of placental transfer of BPA by passive diffusion.

The metabolism of BPA was not investigated in these experiments and therefore some of the radioactivity detected in placental tissue or in the perfusate may represent metabolites of BPA including glucuronide conjugates. Placental perfusions revealed a rapid placental passage of BPA reaching equilibrium between maternal and fetal BPA concentrations within the perfusion period. The efflux of BPA by P-gp in the placental tissue was not investigated in the present study; however, further perfusions with the addition of verapamil would clarify the effect in the cotyledon. Since P-gp is expressed in the placenta [47], BPA efflux presumably occurs. However, since the concentration used in the transport studies is representative of reported levels in human plasma, and since a fetal–maternal equilibrium is reached, *in vivo* efflux is likely to reduce the transplacental transfer of BPA to the fetal circulation, but it does not prevent it. Together, the findings of BPA transport across the human term placentae and BeWo cell monolayer strongly implicate the potential for fetal exposure to BPA. Both the perfusion studies and the cell culture transport studies demonstrate transport of BPA to the fetal compartment. The data are similar, but not identical, due to several differences between these two models, such as differences in the tissue layers separating the compartments, flow patterns, and equilibration times [27].

The effect of BPA exposure on the human fetus is not known; however, animal studies have found several hormonal and developmental adverse effects associated with fetal exposure [14–17]. These findings raise concern about maternal exposure to BPA, since active concentrations are 30 times lower than the reported concentrations of 5.9 ng/mL in the serum of pregnant women in the U.S. [7]. BPAs effects on differentiation and apoptosis in first trimester trophoblast may alter early placental development, which may lead to pregnancy complications including preeclampsia and fetal growth restriction [48–50].

The present experiments on dermal exposure indicate that for an exposure period of 48 h, more than 1/3 of the administered BPA will potentially be available for systemic exposure. The only previous study on skin penetration of BPA used pig skin with only 10 h of exposure, which is too short for a chemical with a lag-time as long as BPA, and no penetration was reported [51]. However, they observed an increasing deposition in epidermis and dermis over time, which is an indication that dermal penetration may occur after lag periods. Thus, their observations regarding deposition in the skin are in agreement with our observations, and supports our observation of a potentially significant dermal penetration and thereby exposure of the fetus following transplacental transport. Furthermore, since BPA has been detected in amniotic fluid [4,5], percutaneous fetal absorption and/or excretion of BPA from and/or into the amniotic fluid may occur.

The main barrier to percutaneous penetration is the stratum corneum. *In utero* the fetus has no proper skin barrier until the stratum corneum starts to develop around 24 weeks' gestation. After 24 weeks, there is a steady increase in the number of epidermal cell layers and in epidermal thickness, although it is not until around 34 weeks' gestation that a well-defined stratum corneum has completely developed [52]. Based on transepidermal water loss and percutaneous absorption studies, term infants seem to possess stratum corneum with adult barrier properties [53]. Therefore, the use of adult skin for transdermal absorption studies is in full agreement with the use of term placentae for the perfusion studies. Moreover, concomitant with formation of the stratum corneum *in utero*, vernix caseosa forms a natural multifunctional cream separating the skin surface from the amniotic fluid, which may further improve barrier properties [54]. As skin with fully developed stratum corneum is used in the present experiments, the already significant absorption observed will be expected to be an underestimate of the potential dermal absorption and thereby also systemic exposure in younger fetuses.

The *in vitro* and *ex vivo* methods used in the present study not only provide relevant information regarding placental and fetal exposure to BPA, but also represent important alternatives to animal testing [55]. In addition to *ex vivo* placental perfusion studies, *in vitro* testing on BeWo cells and placental explants can provide additional knowledge regarding possible effects of fetal exposure to xenobiotics. The *ex vivo* and *in vitro* transport systems employed in this investigation serve as qualitative methods for studying transplacental and dermal transfer. The transport of different substances may be compared and ranked according to their degree of transport; however, an exact quantitative answer of transport is not given from these methods. The transport across term placentae and BeWo cell monolayers gave similar results and the BeWo cell model may be used to predict placental transfer [27] to be followed by *ex vivo* perfusion studies for verification.

5. Conclusions

In conclusion, the *in vitro* results indicate that low, environmentally relevant concentrations of BPA can affect the hCG secretion of the early trimester trophoblast and increase apoptotic activity with potential adverse effects on placental development. Furthermore, BPA is transported across the term human placentae, the trophoblast cell monolayer, and the human skin, which strongly implicate the potential for fetal exposure to BPA, with the risk of hormonal and developmental disruption. These test systems within the realm of human implantation and fetal development, are important elements of reproductive toxicology risk assessment.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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