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Placental transfer of pesticides studied in human placental perfusion

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Abstract

To investigate the transplacental transport of pesticides, the pyrethroid cypermethrin and the fungicide azoles, propiconazole and bitertanol were tested in the placental perfusion model. Cypermethrin, propiconazole and bitertanol were also tested in the BeWo cell transfer model. The pesticides were chosen with the selection criteria: use in Denmark, significant treated areas and knowledge on hormone-disrupting effects. Propiconazole and bitertanol showed rapid transfer and adsorbance to the system in both placental perfusion and BeWo cell system, whereas cypermethrin had a slower transport across the placental cell layers in the two models. There was no difference between data of the single pesticides and their mixture in either placental perfusion or BeWo cell transfer model. Both the placental perfusion model and the BeWo cell model metabolized the pesticides and released metabolites into both foetal and maternal circulation. Using human exposure models, this study shows the potential exposure of the human foetus to pesticides cypermethrin, propiconazole and bitertanol and their metabolites.

KEYWORDS

BeWo cell model, bitertanol, cypermethrin, foetal exposure, pesticides, placental transport, propiconazole

1 | INTRODUCTION

The widespread use of pesticides makes it difficult to avoid human exposure. Some pesticides have been shown to be potentially neurotoxic or hormone-disrupting.[1] The endocrine-disrupting properties of pesticides are of public concern because the population may be exposed to a mixture of pesticides and other endocrine-disrupting compounds (EDCs) at work and from residues in the diet and environment, and the hormone-disrupting effects can be seen at very low levels of exposure. Most pesticides are metabolized to multiple metabolites further contributing to the mixed exposure. Chemicals that affect the same tissue, regardless of their specific mechanism of action, often display dose-additive effects when present in combination.[2] In an in vitro study, a mixture of azole fungicides including propiconazole inhibited the testosterone production in an approximately additive response, compared with the predicted effect based on each single fungicide.[3]

The foetus is particularly vulnerable to endocrine-disrupting effects as it is in rapid growth and development and highly sensitive to hormonal changes. EDCs including some pesticides can interfere with synthesis, secretion, transport, metabolism, binding action or elimination of endogenous hormones such as sex hormones and thyroid hormones, and thereby disrupt the normal hormone homeostasis of the body.[4,5] The hormonal interference of EDCs can result in permanent changes, which may reveal themselves in the adult life.[6] A correlation has been observed between adverse health outcomes in the offspring and the level of EDCs such as organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs) and perfluorinated substances (PFAS) in maternal blood during pregnancy and cord blood.[7-10] There are a number of reports on the endocrine-disrupting effects of pesticides mediated via direct interaction with nuclear hormone receptors such as the estrogen receptor,[11-13] androgen receptor,[11,14,15] and thyroid hormone receptor[16,17] as well as on the aryl hydrocarbon receptor.[17,18] Epidemiological studies showed that prenatal exposure to pesticides may relate to the risk of cryptorchidism.[19] The pesticides bitertanol, propiconazole, cypermethrin, malathion and terbuthylazine alone and as mixtures were shown to affect steroidogenesis causing increase in progesterone and decrease in testosterone in vitro and in vivo.[20]

Foetal exposure occurs through the placenta, which exchanges substances between maternal and foetal circulation with different transfer kinetics according to the substance properties. Factors influencing the placental transport include: adsorbance to placental tissue, molecule characteristics and placental metabolizing capacity. Molecules that are non-ionized and lipid-soluble with small molecular weight can easily cross membranes, but the rate of transport of a given substance across the placenta may depend on the potential metabolism in the placenta, and on transport and transfer proteins in the blood and placenta.

The transfer pathways across the placental syncytiotrophoblast include passive diffusion and facilitated diffusion driven by the concentration gradient through a transporter, and active transport, which is energy-dependent. Large molecules can be transported by endocytosis. The kinetics of passive diffusion across human placental membranes is modelled by the positive control substance antipyrine in the placental perfusion model. The syncytiotrophoblast is a multinucleated fused cell layer, and therefore, transcellular diffusion represents the passive diffusion in the full term placenta, whereas the BeWo trophoblast cell model represents paracellular diffusion as well as transcellular diffusion during the pregnancy.

Placental transfer studies of pesticides and other EDCs in a placental perfusion model have previously been performed in our group[21]: some showing restricted placental transfer of glyphosate and 2,3,7,8-tetrachlorodibenzo-dioxin (TCDD), suggesting accumulation in the placenta,[22-24] and others, such as benzoic acid, caffeine and glyphosate, and bisphenol A, were rapidly transported across the placenta and BeWo cell models by passive diffusion, suggesting unrestricted foetal exposure.[22,23,25]

In this study, we investigated the transplacental transport of the three pesticides: propiconazole, bitertanol and cypermethrin in the placental perfusion model and the BeWo cell line monolayer transport model. Propiconazole and bitertanol are fungicides (azoles), which have shown hormone disruptive effects in in vitro and animal studies.[14,26-28] Cypermethrin is a pyrethroid insecticide that has shown oestrogenic effects in in vitro and animal studies.[14,29-31] In addition, a mixture of the three pesticides, studied in the HOPE (hormone-disrupting effects of currently used pesticides) project, induced estrogen receptor transactivity and aromatase activity, and additively antagonized androgen receptor transactivity.[14] The present placental transport study was a part of the HOPE project.[14,20,32,33] The three pesticides (the fungicide azoles propiconazole and bitertanol, and the pyrethroid cypermethrin) were studied individually and in a mixture (1:1:1). The dose of each compound in the mixture was selected on the basis of previous toxicological data, and the highest dose was equivalent to the highest dose at which only subtle effects were expected on maternal body weights and litter size.[33] No information is available about actual in utero human exposures; however, the tested doses are assumed magnitudes higher. Transplacental studies of the three chosen pesticides have been performed in animal studies, and in human studies by studying the foetal tissue after delivery: propiconazole was tested for endocrine-disrupting effects along with four other azoles in an animal study, and the results showed de-masculinization of both adult males and offspring and foetotoxic effects in the form of post-implantation loss and late resorptions.[28] Transplacental toxicology evaluation of cypermethrin in an animal study did not reveal any teratogenic effects, but indicated that cypermethrin may be transplacentally genotoxic by a marginal increase in the percentage of DNA damage in the foetal blood and liver cells.[34] In a study of maternal and foetal exposure to selected pesticides, pyrethroids including cypermethrin were found in the meconium in 2.5% of cases.[35] In a study on concentration of pesticides in breast milk, cypermethrin was found in all samples, and a correlation with parity was found, suggesting bioaccumulation.[36] These studies suggest a potential foetal exposure. To investigate the transport kinetics of the three chosen pesticides, we used a human placental perfusion model, and the BeWo trophoblast monolayer transport model.

Thus, the focus was on pesticides that are relevant to human exposure, with the following selection criteria: currently used or risk of exposure in Denmark during the

study period, available analysis methods, and knowledge on hormone-disrupting effects from literature or ongoing projects.[16,20,33]

2 | MATERIALS AND METHODS

2.1 | Pesticides

For studies of the three pesticides in a mix: propiconazole PESTANAL®, analytical standard (CAS no. 60207-90-1), bitertanol PESTANAL®, analytical standard (CAS no. 55179-31-2) and cypermethrin, PESTANAL®, analytical standard (CAS no. 52315-07-8) were all purchased from Sigma-Aldrich (St. Louis, USA). Radioactively labelled substances: propiconazole [dioxolane-4-^{14}C] and cypermethrin [benzyl-7-^{14}C] (Izotop, Budapest, HU), were used for single-compound transport studies. The test compounds were dissolved in dimethyl sulfoxide (DMSO, CAS 67-68-5) for the in vitro and ex vivo studies, from Sigma-Aldrich (St. Louis, USA).

2.2 | Cell culture

The choriocarcinoma BeWo b30 cell line was obtained from Prof. Margaret Saunders (Bioengineering, Innovation, & Research Hub [BIRCH], St. Michael's Hospital, Bristol NHS Foundation Trust, Bristol, UK) with permission from Dr Alan Schwartz (Washington University, St. Louis, MO, USA). Cells were cultured in DMEM-F12 (Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12; Sigma-Aldrich, Schnelldorf, Germany) with phenol red and supplemented with the following: 10% foetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel), 4 mmol/L L-glutamine (Panum Institute, University of Copenhagen), and 1% penicillin/streptomycin (penicillin 20 000 IU/mL, streptomycin 5 mg/mL, Panum Institute, Copenhagen University). The cells were cultured, and experiments were performed under sterile conditions at 37°C with 5% CO_2 in a humidified atmosphere. At 75%-80% confluence, cells were subcultured using trypsin-EDTA solution (Sigma-Aldrich, Schnelldorf, Germany). Experiments were conducted in supplemented DMEM-F12 media without phenol red.

2.3 | Cytotoxicity assay

2.3.1 | Exposure

BeWo b30 cell were seeded at 10 000 cells/well in 96-well plates and grown for 24 hours under cell culture conditions. The semiconfluent cells were exposed to the pesticides, propiconazole, bitertanol, cypermethrin or a mixture of these pesticides (mix3) in concentrations 5×10^{-9} to 1×10^{-5} mol/L for 24 hours. Medium with 0.25% dimethylsulphoxide (DMSO; Sigma-Aldrich, Ayershire, UK) was used as negative control. Triton X-100 (Applichem, Darmstadt, Germany) 0.1% in media was used as positive control for the cytotoxicity assay.

2.3.2 | MTT assay

MTT assay was used to assess the cell viability after exposure to pesticides.[37] After 24-hour exposure, the medium containing pesticides was removed, and cells were washed three times in PBS and incubated with 100 μL/well MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) in a concentration of 0.5 mg/mL for 2 hours. The MTT was removed, and 100 μL/well DMSO was added to dissolve the crystals. The plate was shaken at 900 rpm for 1 minute, and absorbance was measured at 550 nm using a Multiscan FC plate reader (Thermo Fisher Scientific, Denmark).

2.4 | BeWo cell transport

The BeWo b30 cells were seeded in a density of 100 000 cells/cm^2 onto Transwell® inserts (n = 6) (polyester [PE] filters 3 μm pore size, 1.12 cm^2 growth area, 0.5 mL apical [maternal] chamber, 1.5 mL basolateral [foetal] chamber). Before seeding of cells, wells were coated with human placenta collagen (2.9 mg/mL diluted 1:3 with 70% EtOH) by adding 1 mL to the apical chamber and drying for 3 hours. The transport studies were conducted when a confluent monolayer was formed (5-6 days). The trans-epithelial electrical resistance (TEER) was measured using an EndOhm apparatus. TEER values above 30 Ω were used as a cut-off value for a confluent BeWo cell monolayer,[38] together with visual inspection of the cells by light microscopy. Transport study was conducted at 37°C, and the plates with inserts were only removed from the incubator when sampling. Blank wells (n = 3) (coated with human collagen, but without cells) were used as control and to calculate the trans-epithelial electrical resistance for each compound (measured TEER with cells minus measured TEER in blanks). At T0, the selected pesticide was added to the apical chamber in a concentration of 1 μmol/L in 0.5 mL transport medium. The basolateral chamber was filled with 1.5 mL fresh transport medium. At different time points (0, 1 minute, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 24 hours), 100 μL samples from the basolateral chamber and 10 μL samples from the apical chamber were collected and placed in a 6-mL scintillation tube. The basolateral chamber was refilled with 100 μL fresh medium after each sample. After the last samples had been

collected, the TEER value was measured again, and the cell layer on the filter was washed three times in ice-cold HBSS, and each filter was cut out and placed in a scintillation tube. Immediately after collection, all samples were mixed with 2 mL scintillation medium for radiolabelled substances, the non-radiolabelled were frozen on dry ice and stored at −20°C until analysis. The concentration in each sample was calculated using a standard curve, and the data were corrected for the previous samplings and refilling of the basolateral chamber when calculating the amount of pesticide transported across the BeWo cell monolayer.[23] To compare transport rate in the BeWo cell transport system, the basolateral/apical (BA) ratio was calculated by dividing basolateral concentrations with apical concentrations. This is parallel to the foeto/maternal (FM) ratio used in placental perfusion when comparing transport rates.

2.5 | Placental perfusion

Placentas were received after uncomplicated pregnancies and vaginal births or caesarean sections. Written maternal consent was obtained before birth, and the study was approved by the regional scientific ethics committee and the national data protection agency. The placentas were injected immediately with Krebs Ringer buffer containing heparin and transported to the perfusion laboratory for examination and perfusion as described elsewhere.[39] In brief, a single foetal artery/vein pair supplying an isolated part of the placenta was cannulated for perfusion. The maternal side of the placenta was diffusely perfused, and the perfusion buffer was re-circulated for the duration of the perfusion. Perfusion buffer consisted of supplemented DMEM-F12 without phenol red as in BeWo cell transport experiments; in placental perfusions, the media was supplied with heparin 25 IU/mL (Copenhagen University Hospital Pharmacy) and Human Serum Albumin (HSA) dialysed in Krebs Ringer buffer, 30 g/L in the maternal reservoir and 40 g/L in the foetal reservoir (20% solution, product # 109697, CSL Behring Gmbh). The foetal medium was gassed with 95% N_2 and 5% CO_2, and the maternal medium was gassed with 80% O_2 and 20% CO_2. The whole set-up was located in a 37°C heated flow bench. Samples from maternal and foetal reservoir were taken before addition of test substances (cypermethrin, propiconazole, bitertanol or a mixture of these three pesticides (mix3), final concentration 0.5 or 1 µmol/L) and control substance antipyrine (final concentration 100 µg/mL; Aldrich-Chemie, Steinheim, Germany) to the maternal circulation, and after 0, 5, 15, 30, 60, 120, 180, 240, 300, 330 and 360 minutes of perfusion. For radiolabelled compounds, the 0.5 mL samples were centrifuged for 5 minutes at 4000 g, and the supernatant was transferred to vials. Two millilitre of Ecoscint (XR LS-372) scintillation liquid (BN instruments A/S) was added to 200 µL of supernatant.

For non-radiolabelled samples in the perfusions studying a mix of the three pesticides, 1 mL supernatant was stored at −20°C until analysis by GC-MS and LC-MS-MS. Another 200 µL of supernatant was stored at −20°C until antipyrine detection by HPLC.

The placental perfusion demonstrates the transplacental transport kinetics of an added substance over time, the concentration changes in the maternal compartment and the appearance of the substance in the foetal medium. As an expression of transport, the FM ratio is used to compare substance transport kinetics across concentration differences and gives a single curve that can be analysed for structure and area.[40]

2.5.1 | System adherence test

In the BeWo transwell set-up, a system adherence test was performed using non-radioactively labelled cypermethrin. The substance was added to the system without addition of cells or collagen present. Studies using non-radioactive cypermethrin demonstrated greater adsorption to the polycarbonate transwell filters than to the polyester (PE) transwell filters. For this reason, PE transwell filters were used in the BeWo monolayer transport studies.

System adherence tests were performed in the placental perfusion system with the radiolabelled substances ^{14}C-cypermethrin and ^{14}C-propiconazol. This was done by recirculating the perfusion-medium with substances in the perfusion equipment without a placental lobule present. Samples were taken out and analysed according to protocol.

2.6 | Sample analysis

2.6.1 | Radiolabelled pesticides

Radioactively labelled substances cypermethrin and propiconazole used for single-compound transport studies were quantified using a liquid scintillator (Liquid Scintillation Analyzer, TRI-CARB 2300TR, Packard), and a calibration curve was included in each test round. The scintillation counter was programmed to count each sample twice for a maximum of 10 minutes. The second counting was used for analysis.

2.6.2 | Pesticides and metabolites quantified by GC-MS and LC-MS-MS

Mix3 (cypermethrin 1 µmol/L: propiconazole 1 µmol/L: bitertanol 1 µmol/L) was analysed using a common analytical method for determination of the polar pesticides and their

metabolites as described in Bossi et al.[33] The analytical limit of detection (LOD) for cypermethrin, bitertanol and propiconazole was 0.09, 0.72 and 0.62 ng/mL, respectively.

For the BeWo cell transfer experiments with non-radiolabelled substances, only samples from 0, 2, 4 and 24 hours were analysed. Four wells in a single study of mix3 experiments were analysed for propiconazol and bitertanol, and samples from the four wells were pooled before analysis for cypermethrin. For solo bitertanol experiments, the samples from six wells were pooled with two separate experiments.

For the placental perfusions, only samples from 0, 30, 60, 120, 180, 360 minutes were analysed from three repeated mix3 experiments for propiconazole and bitertanol.

2.6.3 | Control substance antipyrine quantified by HPLC

Antipyrine was detected on a LaChrom HPLC system equipped with a C-18 column and a SecurityGuard precolumn as described elsewhere.[22,41]

2.7 | Recovery

Recovery was calculated as previously described.[42] In the recovery equation below (1), C_M = counts in the maternal compartment at the end of perfusion, V_M = volume of the maternal compartment at the end of perfusion, V_A = volume analysed in the scintillation counter, C_F = counts in the foetal compartment at the end of perfusion, V_F = volume of the foetal compartment at the end of perfusion, C_j = counts in each sample removed during the perfusion from both maternal and foetal compartments for samples 1 through n, V_j = volume of each sample removed during the perfusion, C_C = counts from the cotyledon sample, M_{CT} = total mass of the perfused cotyledon, M_{CA} = mass of the cotyledon sample analysed in the scintillation counter, C_T = counts in the surrounding tissue sample, M_{TT} = total mass of the surrounding tissue, M_{TA} = mass of the sample of the surrounding tissue analysed in the scintillation counter, C_I = initial counts in the maternal compartment after adding the test substance, and V_I = initial volume of the maternal compartment.

$$\text{Recovery} = \frac{\left(\frac{C_M \cdot V_M}{V_A} + \frac{C_F \cdot V_F}{V_A} + \sum_{j=1}^{n} \frac{C_j \cdot V_j}{V_A} + \frac{C_C \cdot M_{CT}}{M_{CA}} + \frac{C_T \cdot M_{TT}}{M_{TA}} \right)}{\frac{C_I \cdot V_I}{V_A}} \cdot 100\%. \quad (1)$$

2.8 | Statistics

To test the statistical difference between the pesticides solo and the pesticides in mix, we used the non-parametric Kruskal-Wallis test for independent variables. The analyses were performed for placental perfusion studies and BeWo monolayer transfer studies. Analyses from placenta perfusion studies and BeWo cell layer studies were performed in SAS Statistical Software version 9.2.

Data from the cell viability study were analysed with GraphPad Prism, GraphPad Software Inc, La Jolla, CA, USA. Data from each individual compound or the mix were analysed by a one-way ANOVA followed by Dunnett's test. Comparison between different compounds and the mix3 were analysed by a two-way ANOVA followed by Bonferroni's test.

The differences were deemed statistically significant when $P < 0.05$.

3 | RESULTS

3.1 | Toxicity assay

The MTT assay showed limited toxic effects of the three pesticides propiconazole, bitertanol, cypermethrin or their mixture (mix3) in concentrations up to 1×10^{-5} mol/L. Viability was normalized in respect to negative control and expressed in percentage to enable comparison between the single compounds and their mix. At some concentrations, there was significant difference between propiconazole and cypermethrin compared to the mixture (mix3), but the direction of the difference was not constant. Bitertanol did not show significant difference at any of the concentrations compared to the mixture. Transport study using BeWo cells was done with 1 μmol/L, which did not show significant difference between the three compounds and the mix3 (Figure 1).

3.2 | Transport BeWo

In the mix3 study, samples from three wells were studied, with propiconazole, bitertanol and cypermethrin in a concentration of 1 μmol/L for each substance. For the analysis of cypermethrin, the samples were pooled.

One micromolar ^{14}C-propiconazole was used in two transfer studies of propiconazole solo 2× (cells n = 6 wells, blank n = 3 wells). In the BeWo transfer model, a rapid transfer kinetic was observed similar to passive diffusion across the cell monolayer. System recovery was 87%. No difference was found between the transfer of propiconazole across BeWo cell monolayer between the pesticide as solo and mixture experiments (n = 4 wells) (Basolateral/Apical-ratio, $P = 0.4$) (Figure 2A).

One micromolar bitertanol was studied in two experiments in the BeWo cell monolayer 2× (cells n = 6, blanks n = 3) and showed similar transport across BeWo cell monolayer to propiconazole, suggesting transport via passive diffusion. No
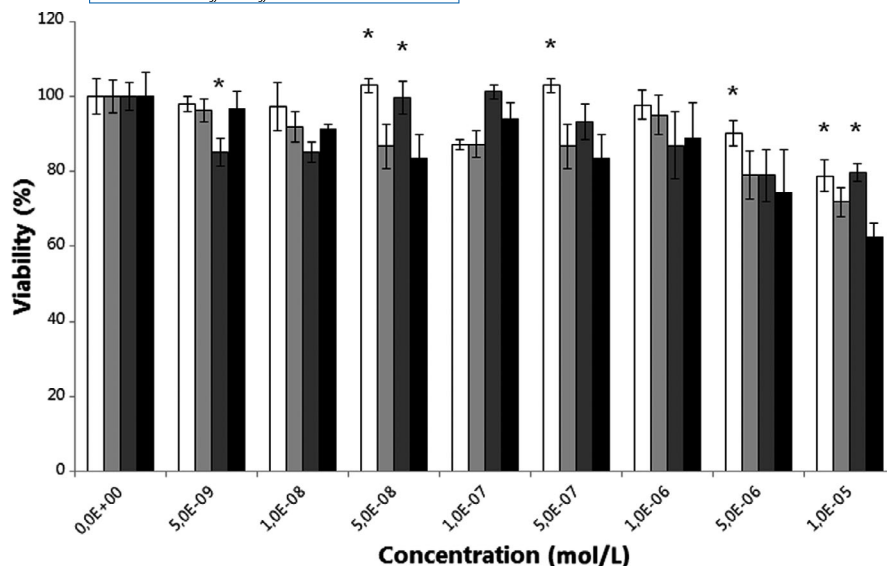
FIGURE 1 MTT viability assay of propiconazole (white bar), bitertanol (light grey bar), cypermethrin (dark grey bar), or their mixture (black bar), in concentrations up to 1×10^{-5} mol/L. Viability was normalized in respect to negative control and expressed in percentage to enable comparison between compounds and mix. At some concentrations, there was significant difference between cypermethrin and propiconazole compared to the mix (*$P < 0.05$), values are presented as mean ± SD.
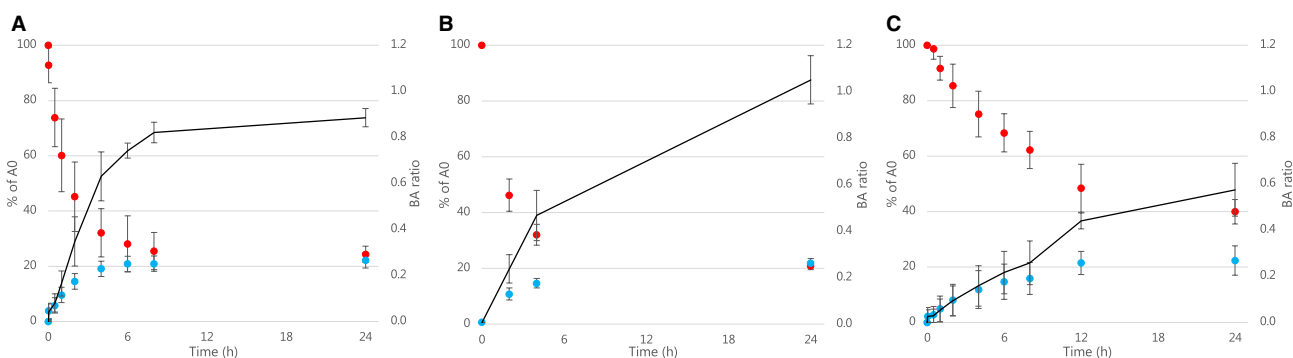

FIGURE 2 Transport of propiconazole (A), bitertanol (B) and cypermethrin (C) over BeWo cell monolayer during 24 h. The figures show the apical and basal concentration (left y-axis) and the BA ratio (right y-axis) of the studied pesticides. Apical (red circle) corresponds to the maternal side and basolateral (blue circle) corresponds to foetal side. Data presented are in % of substance added to apical side (1 μmol/L). Solo experiments were performed: propiconazole (2 studies × 6 wells, n = 12), bitertanol (2 studies × 6 wells n = 12), cypermethrin (3 studies × 6 wells n = 18), and one experiment with four wells was performed with the three substances together, (propiconazole, bitertanol and cypermethrin 1:1:1, mix3 n = 4, for cypermethrin samples were pooled before analysis, mix3 cypermethrin: n = 1). As the data showed no difference from solo experiments, the data from solo and mix3 experiments are presented together: propiconazole n = 16, bitertanol n = 16 and cypermethrin n = 19. Black line: BA ratio solo + mix3 transport studies. The data are presented as mean ± SD.

difference was found in the transport of bitertanol as solo and in mixture (BA ratio, $P = 0.3$) (Figure 2B).

Three BeWo transport studies 3× (cells n = 6, blank n = 3) were performed adding 1 μmol/L 14C-cypermethrin. These showed transport of 14C-cypermethrin across the cell monolayer. At 24 hours, there was no difference in BA ratio of cypermethrin across BeWo cell monolayer between the solo pesticide and the mixture transfer experiments (n = 4 wells) ($P = 0.7$) (Figure 2C).

3.3 | Transport placental perfusion

Data from placental perfusions (n = 4) performed using ¹⁴C-propiconazole and propiconazole in mix3 (n = 3) are

illustrated in Figure 3A. No difference was found in the FM ratio between solo and mix perfusions ($P = 0.07$), and the data are therefore combined in the figure. Only data from the first 180 minutes are shown in the figure due to missing data in some perfusions and too few repetitions to calculate mean and standard deviation after this time point. The FM ratio of the perfusions showed rapid transport corresponding to the passive diffusion of the control antipyrine across placenta, with a FM ratio above 0.75 after 90 minutes of perfusion. The maternal and foetal data show that propiconazole rapidly disappears from the maternal reservoir and appears in foetal circulation, with an equilibrium at around 15% of added compound in both circulations after 30 minutes, indicating tissue adherence.

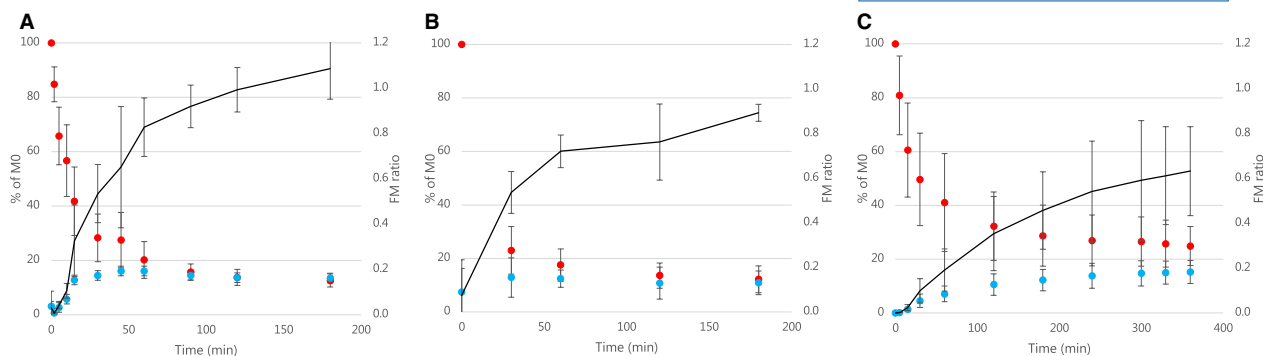Bitertanol in perfusion of mix3 (n = 3) showed a placental transport similar to propiconazole, with rapid decline in

FIGURE 3 Placental transport of the three HOPE pesticides. The figures show the maternal and foetal concentration (left *y*-axis) and the FM ratio (right *y*-axis) of the studied pesticides: (A) propiconazole solo and mix3 combined: 1 μmol/L n = 7, (B) bitertanol mix3: 1 μmol/L n = 3, (C) cypermethrin 0.5 and 1 μmol/L combined n = 7. Red circle: maternal concentration, blue circle: foetal concentration, black line: FM ratio. Propiconazole and bitertanol showed a rapid transport similar to passive diffusion during the 3-h perfusion. Cypermethrin was observed in the foetal circulation after 30-min perfusion, but the transport rate of cypermethrin was slower than passive diffusion and did not reach equilibrium in the 6-h perfusion. The data are presented as mean ± SD of % of measured M0.

maternal concentration indicating tissue adherence or metabolism (Figure 3B). Data from bitertanol solo-perfusions were not available due to analytical limitations.

Four placental perfusions with a maternal start concentration of 0.5 μmol/L ^{14}C-cypermethrin and three perfusion with a maternal start concentration of 1 μmol/L ^{14}C-cypermethrin were performed (Figure 3C). These showed a concentration-independent transport of ^{14}C-cypermethrin during the 6-hour placental study. Cypermethrin was observed in the foetal circulation after 30 minutes of perfusion, and after 6 hours of perfusion, the FM ratio was 0.5. This is a slower transport rate than passive diffusion. Data from cypermethrin mixture-perfusions were not available due to analytical limitations.

3.3.1 | Recovery

For the two radiolabelled substances ^{14}C-cypermethrin and ^{14}C-propiconazole, the recovery in placental perfusion

experiments was 59 ± 15% and 55 ± 17%, respectively. This relatively low recovery can be explained by the system adherence test which showed a remaining concentration of 62% and 48% of added ^{14}C-cypermethrin and ^{14}C-propiconazole at the end of perfusion. This means that about half of the added pesticides are lost from the perfusion by adsorbing to the chamber and tubing. This is important to realize but it does not affect the transport ratio of the substances. In the BeWo transfer system, recovery was 87% for ^{14}C-propiconazole, which indicates a lower system adherence in the BeWo cell model.

3.4 | Metabolites

Both in the BeWo cell model and in the placenta perfusion, a considerable metabolism was shown. In the placental perfusion model, the metabolites 1,2,4-triazole (metabolite of propiconazole and bitertanol) and 3-phenoxybenzoic acid (3-PBA) (a metabolite of cypermethrin) were found at increasing
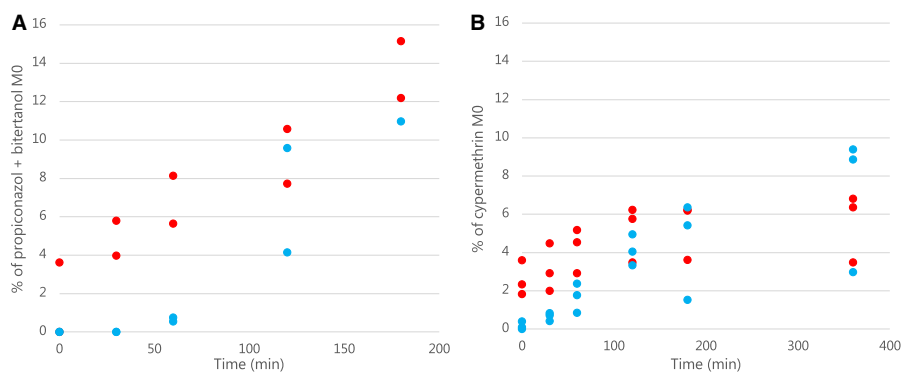

FIGURE 4 Concentration of 1,2,4-triazole (A) and 3-PBA (B) in samples from mix3 (n = 3 perfusions), (C) 3-PBA in samples from 24 h transport study of mix3 (n = 4 wells). Red circle maternal/apical concentration, blue circle: foetal/basolateral concentration. The concentration of 3-PBA on both apical and basolateral side increased throughout the study. The data are presented as mean ± SD of % of measured M0 for propiconazole and bitertanol for triazole, and cypermethrin for 3-PBA, respectively. For triazole, only samples from two perfusions were available so the individual data are presented.

concentrations over time in both foetal and maternal circulation (Figure 4A,B). In the BeWo cell model, 3-PBA was shown in increasing concentrations in both basolateral and apical chambers (Figure 4C). This shows the ability of the placenta to metabolize the investigated pesticides creating substances with other chemical properties than the added substance. Both mother and foetus are exposed to the metabolites.

4 | DISCUSSION

4.1 | Data analysis

The data from this study show the human placental transport, metabolism and system adherence of three pesticides. Radiolabelled substances were used for single-compound transport studies, available with the ^{14}C label in the main part of the molecule. Non-labelled substances were used for mixture studies to distinguish the substances and identify selected metabolites. The data presented using non-radiolabelled substances were based on pooled samples in the analyses as our access to analyses were limited. This decreases the power of the study. In one pooled sample from the BeWo mixture study, the concentration of cypermethrin seemed to reach 120% in the maternal circulation. This is presumed to be an artefact of sampling or analysis as the cells are not able to produce cypermethrin, but because we pooled the samples there is only the one data outcome at this time point, and we chose to include the datapoint as measured in the presented mean. The analysis method used for labelled substances does not distinguish between the labelled parent substances and labelled metabolites on both maternal and foetal side. This is a drawback of using radiolabelled substances.

The system adherence test showed some accumulation to the system in both the placental perfusion and the BeWo model. This may explain the relatively low recovery of the pesticides in the two models, but does not add anything to the discussion of the physiological transport of the pesticides. The recovery should not be affected by metabolism as it was calculated using data from the labelled substances.

4.2 | Transport properties of pesticides

There is a growing concern that exposure to even low concentrations of pesticides in the foetal period, when development of the reproductive and nervous system occurs, can cause irreparable damage. Due to the inter-species differences in placental structure and transport, the transplacental transport of pesticides is investigated in human placental tissue.

Placental transfer studies of pesticides and other EDCs in our laboratory have previously shown limited placental transfer of the pesticides: glyphosate and TCCD, and accumulation of the pesticides in the placental tissue.[22-24] In this study, we investigated the placental transfer of three pesticides (propiconazole, bitertanol and cypermethrin) when added as single substances and their mixture to a placental perfusion model, and in a BeWo cell monolayer transfer model. Propiconazole and bitertanol were transferred by a rate that suggests transfer by passive diffusion, whereas cypermethrin had a slower transfer rate in both models. The relatively rapid transfer of the studied pesticides is critical in foetal exposure estimation, where the foetus is exposed in the same magnitude as the concentration in maternal blood. Transplacental transfer of cypermethrin has previously been investigated in an animal model, where it was shown to be genotoxic, by a rise in DNA damage in foetal blood and liver cells[34]; however, no other studies have confirmed this observation. In an epidemiological study of maternal and foetal exposure to pyrethroids and cypermethrin, these two pesticides were found in the meconium in 2.5% of the cases.[35] The same study showed neuronal developmental damage in 2 year olds that indicates prenatal exposure effects on the nervous system, which is at its most vulnerable in the foetus.

4.3 | Metabolites

The metabolites of bitertanol include p-hydroxy-bitertanol, 1,2,4-triazole, triazole alanine and triazolylacetic acid (TAA).[43] The metabolites of propiconazole are 1,2,4-triazole, triazole alanine and triazolylacetic acid (TAA).[44] 3-phenoxybenzoic acid (3-PBA), 3-(4-hydroxyphenoxy)benzoic acid O-sulphate ester and 3-(4-hydroxyphenoxy)benzoic acid are cypermethrin metabolites.[45]

In this study, we also demonstrated metabolism of the three pesticides by the placenta. The common metabolite, 1,2,4-triazole, of propiconazole and bitertanol,[46] and the cypermethrin metabolite, 3-PBA, were all detected, and their concentrations increased over time. 1,2,4-triazole is classified as an EDC[46,47] and suspected to be toxic to reproduction. The common pyrethroid metabolite, 3-PBA, exhibits anti-oestrogenic activity in vitro,[48] and anti-androgen activity.[49] Urinary concentrations of 3-PBA were found to be negatively associated with serum thyroid hormone levels in Korean adults.[50]

The production of metabolites by the placenta is demonstrated in this study to be independent of other maternal and foetal metabolizing organs, and the metabolites are found in both maternal and foetal circulation, increasing over time. This can add to the adverse effects of the pesticides, especially in cases where the metabolite is more toxic than the studied compound, as is the case of benzo[a]pyrene and the epoxide metabolite BPDE.[42,51] Studies on radioactively labelled substances do not demonstrate the metabolism of the substances. However, no difference was seen between transport of labelled and unlabelled substances; thus either

the metabolism must have had an equal effect on the maternal and foetal sides, or the metabolism did not represent an amount significant enough to affect the transport rate in the time frame studied.

4.4 | Mixture effects of pesticides

Weak endocrine disruptors like azole fungicides give rise to combination effects when they occur in mixtures. It is a new focus in regulatory risk assessment to not underestimate the risk of adverse effects associated with exposure to endocrine-disrupting chemicals. Assessment of toxic effects of mixed exposure are complicated and cannot be predicted from the single substance effects alone. Secondary effects, uptake kinetics, transport, metabolism and excretion must also be considered.[52,53] Foetal exposure occurs through the placental cell layers, transferring different substances by different transport methods resulting in different transport kinetics according to the physical-chemical properties of the substance, which could be affected by the mixture effect. Predicting additivity is closely linked to the number of chemicals, their potency and their concentration levels in the selected models. In vitro, we can predict additivity on one specific mechanism independently of the compound's ability to interact with this particular molecular target.[3] The fungicide prochloraz interacted antagonistically with the insecticide dimethoate in a study of the effect of a pesticide mixture on metabolism. A decreased metabolism of organophosphates caused by prochloraz would delay the formation of the more toxic oxon form and in that way decrease the joint toxic effect.[52] Antagonism could also occur as a result of chemical interactions in the growth media making pesticides less available to the test organisms.

No effects of mixed exposure were seen in this study, which could be attributed to the fact that two of the substances were transferred by passive diffusion, thereby not interacting with transport receptors and carrier substances.

4.5 | Regulatory remarks

Currently, propiconazole and cypermethrin are approved active substances in the EU for biocides and pesticides, although no products with propiconazole are authorized in Denmark. Bitertanol was approved in DK during the period of 1989-2011.[54] Propiconazol has been classified by harmonized classification as toxic for reproduction, category 1B, H360D (may damage the unborn child) and is under evaluation for endocrine-disrupting properties.[55] According to the Biocidal Product Regulation[56] and the regulation for Plant Protection Products,[57] active substances for these products may not be approved if they are documented endocrine-disrupting chemicals, unless exposure can be proven to be negligible. All active substances are therefore currently being evaluated for endocrine-disrupting properties according to the new criteria set out by the commission.[58,59]

5 | CONCLUSION

This study demonstrates the potential of pesticides to be transferred across placenta into the foetal circulation, and the ability of the placenta to produce metabolites, exposing the mother and foetus to both parent compounds and their metabolites. Moreover, our study demonstrates comparable results between the placental perfusion model, and the BeWo cell line transport model.

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