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Transfer and Metabolism of the Xenoestrogen Zearalenone in Human Perfused Placenta

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Background: Pregnancy is a sensitive condition during which adverse environmental exposures should be monitored thoroughly and minimized whenever possible. In particular, the hormone balance during gestation is delicate, and disturbance may cause acute or chronic long-term health effects. A potential endocrine disruption may be provoked by *in utero* exposure to xenoestrogens mimicking endogenous estrogens. The mycoestrogen zearalenone (ZEN), a toxic fungal secondary metabolite and mycotoxin found frequently in food and feed, constitutes a prominent example.

Objectives: We performed a comprehensive assessment of the transfer as well as phase I and phase II metabolism of ZEN at the human placental barrier.

Methods: Human placentas were perfused with 1 μM (318 μg/L) ZEN for 6 h. Samples from the maternal and fetal compartment, placental tissue, and fetal plasma were analyzed by a highly sensitive UHPLC-MS/MS assay to detect ZEN as well as nine key metabolites (α-zearalenol, β-zearalenol, zearalanone, α-zearalanol, β-zearalanol, ZEN-14-glucuronide, α-zearalenol-14-glucuronide, β-zearalenol-14-glucuronide, ZEN-14-sulfate).

Results: The model revealed a fast maternofetal transfer of ZEN across the human placental barrier. We also unraveled phase I and phase II metabolism of the parent toxin ZEN into the approximately 70-times more estrogenic α-zearalenol and the less active ZEN-14-sulfate conjugate, which are effectively released into the maternal and fetal circulation in considerable amounts.

Conclusions: Our findings suggest that exposure to ZEN (such as through consumption of ZEN-contaminated cereal-based products) during pregnancy may result in *in utero* exposure of the fetus, not only to ZEN but also some of its highly estrogenically active metabolites. In the light of the known affinity of ZEN and potentially co-occurring xenoestrogens to the estrogen receptor, and our results demonstrating placental transfer of ZEN and its metabolites in an *ex vivo* model, we recommend further research and more comprehensive assessment of gestational exposures in women. https://doi.org/10.1289/EHP4860

Introduction

Zearalenone (ZEN) is a fungal toxin (mycotoxin) produced by *Fusarium* species and is regularly found in cereal-based food and feed in various countries (Maragos 2010; EFSA 2011; Mally et al. 2016). Other sources, such as legume-based food and vegetable oils, were also reported (Schollenberger et al. 2007; Maragos 2010; EFSA 2011). Because this mycotoxin possesses potent estrogenic activity, it is often referred to as xeno- or mycoestrogen (Bennett and Klich 2003; Kowalska et al. 2016; Warth et al. 2018). Humans are frequently exposed to low doses, either directly by ingestion of contaminated food or indirectly by the consumption of livestock that were fed with contaminated chow (Kowalska et al. 2016).

The most prevalent phase I metabolites are α- and β-zearalenol (α- and β-ZEL) and α- and β-zearalanol (α- and β-ZAL) (Miles et al. 1996; Pfeiffer et al. 2011), whereas zearalanone (ZAN) is a metabolite of α-ZAL (Migdalof et al. 1983). The favorably formed metabolite is highly dependent on the species [reviewed in Zinedine et al. (2007)]. Detoxification occurs via phase II metabolism by sulfation and glucuronidation (Migdalof et al. 1983; Miles et al. 1996; Pfeiffer et al. 2011).

The human metabolism of ZEN has been characterized in cell models (Pfeiffer et al. 2010, 2011) as well as *in vivo* in human intervention studies (Mirocha et al. 1981; Warth et al. 2013). It is mainly metabolized via phase I metabolism to α-ZEL and to a minor extent to β-ZEL and is thereafter glucuronidated. ZEL metabolites were also shown *in vitro* using Caco-2 cells to conjugate to sulfate metabolites (Pfeiffer et al. 2011). In line with this, Huuskonen et al. (2015) unraveled human placental phase I metabolism of ZEN to α-ZEL and to a minor extent to β-ZEL in chorion carcinoma JEG-3 cells and human term placental subcellular fractions.

ZEN is often referred to as an endocrine-disrupting chemical (EDC) (Kowalska et al. 2016). According to the European Commission, EDCs are "substances that alter the functions of the hormonal system and consequently cause adverse effects" (EC 2018). ZEN and several of its metabolites can bind to estrogen receptors and resemble, therefore, with different potency, the estrogenic properties of endogenous estrogens. α-ZEL was demonstrated to be the most potent structure, being about 70 times more potent than the parent toxin ZEN (Frizzell et al. 2011), followed by α-ZAL and ZEN (Arukwe et al. 1999; Malekinejad et al. 2005; Frizzell et al. 2011; Tatay et al. 2017). Phase II metabolism (glucuronidation and sulfation) reduced estrogenicity in cell models (Jard et al. 2010; Frizzell et al. 2015).

Environmental exposure to molecules mimicking endogenous estrogens, so-called xenoestrogens, may result in various adverse effects in different human organs, including the reproductive tract and the nervous system (Singleton and Khan 2003). Such adverse effects have been reported frequently in animal studies [reviewed in Xu et al. (2017)] but human data are difficult to generate due to the complexity of the endocrine system and the diversity of xenoestrogens, which usually appear at low doses in various mixtures. Moreover, there is growing evidence that exposure to

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xenoestrogens at early developmental stages can be related to chronic diseases later in life, such as breast cancer and the development of other tumors (Palmlund 1996; Fernandez and Russo 2010; Fucic et al. 2012).

Consequently, the investigation of the *in utero* and early life exposure to xenoestrogens is of high priority. Different approaches have been developed to study the permeability of the human placental barrier [reviewed in Muoth et al. (2016)]. Among these, the dually perfused *ex vivo* placental perfusion model provides highly predictive, human-relevant data on the transfer and metabolism of compounds due to the use of intact human placental tissue and dynamic exposure conditions (Panigel et al. 1967; Hutson et al. 2011; Grafmüller et al. 2013; Mathiesen et al. 2014).

The unborn child is particularly responsive to endocrine disruption, and a perturbation of the fragile hormone balance may lead to a maldevelopment of the reproductive system (Palmlund 1996; Saunders et al. 1997; Welshons et al. 1999; Delbès et al. 2006; Reed and Fenton 2013). It has been described that the xenoestrogens bisphenol A (BPA), 4-nonylphenol, and genistein can cross the human placental barrier in *ex vivo* perfusion studies (Balakrishnan et al. 2010a, 2010b, 2011). Moreover, the detection of estrogenic compounds, such as parabens in umbilical cord plasma (Kolatorova et al. 2018) or nonylphenol and BPA in amniotic fluid (Shekhar et al. 2017) further supports that there is a transfer of these chemicals across the placenta *in vivo*. Therefore, an unborn child might be at immediate risk of chronic low-dose exposure to a mixture of xenoestrogens. The combination of different estrogenic compounds, even at very low levels, has been demonstrated to potentially result in synergistic adverse effects in cell models (Rajapakse et al. 2002; Vejdovszky et al. 2017).

To date, the ability of mycotoxins to cross the placental barrier in the *ex vivo* placental perfusion model has been investigated only for the carcinogenic aflatoxin B_1 (Partanen et al. 2010), the nephrotoxic ochratoxin A (Woo et al. 2012), and the type-B trichothecene deoxynivalenol (Nielsen et al. 2011), which inhibits protein synthesis. Although aflatoxin B_1 and deoxynivalenol can pass through the human placental barrier, ochratoxin A could not in the described experiments. In a rat model, ZEN and its phase I metabolite α-ZEL were shown to be transferred to the fetus (Bernhoft et al. 2001) and cause adverse effects (Gao et al. 2017), but human data is still lacking to date. In addition, ZEN conversion to all of its potential metabolites has not yet been systematically investigated in an advanced human placenta model. Verification of ZEN translocation and metabolism in a predictive human placenta model is urgently needed, considering the significant species-specific differences in placental development, structure, function, and pathology (Enders and Blankenship 1999; Malassine et al. 2003; Schmidt et al. 2015). Important considerations are that the mouse and rat placenta does not produce estrogens (aromatase-negative), and the level of circulating estrogens is much lower than in human pregnancy, resulting in different pharmacokinetics and metabolic profiles (Witorsch 2002; Al-Bader 2006). Therefore, the primary aims of this study were to *a*) determine the permeability of the human placental barrier in respect to ZEN and *b*) to investigate the human placental metabolism of ZEN that might lead to biotransformation products exhibiting enhanced estrogenic activity.

Material and Methods

Chemicals and Reagents

Liquid chromatography–mass spectrometry (LC-MS)-grade acetonitrile (ACN) and methanol (MeOH) were obtained from Sigma-Aldrich, and water (H_2O, LC-MS grade) was purchased from VWR International GmbH. Ammonium fluoride (LC-MS grade) was obtained from Honeywell Fluka. ZEN, α-ZEL, β-ZEL, α-ZAL, β-ZAL, and $^{13}C_{18}$-ZEN were obtained from either Romerlabs or Sigma-Aldrich, whereas ZEN-14-GlcA, α-ZEL-GlcA, and β-ZEL-GlcA were synthesized at the Technical University of Vienna and kindly provided by Dr. Mikula (Mikula et al. 2012, 2013). ZEN-14-Sulf was a kind gift from Prof. Berthiller from the University of Natural Resources and Life Sciences, Vienna (Vendl et al. 2010). Antipyrine was obtained from Sigma-Aldrich. All reference standards were dissolved in ACN. ZEN and its metabolites were combined manually to a multianalyte calibration solution (each analyte at a concentration of 10 mg/L). All single and combined analytes were stored at −20°C. ZEN for transfusion experiments was obtained from Toronto Research Chemicals (purity 98.9%).

Ex Vivo Placental Perfusion

In total, six placentas were obtained from uncomplicated pregnancies after caesarean section from the Kantonsspital St. Gallen and Hirslanden Klinik Stephanshorn St. Gallen with written informed consent from the expecting mothers. The study was approved by the local ethics committee (EKOS 10/078; PB-2018-00,069) and performed according to the principles of the Declaration of Helsinki. The recirculating dually perfused *ex vivo* placental perfusion model was performed as previously described with some modifications (Figure 1) (Grafmüller et al. 2013; Grafmueller et al. 2015). Briefly, a fetal artery–vein pair of an intact cotyledon was cannulated, and the placenta was placed into a perfusion chamber with the maternal side up. To connect the maternal side, three blunt cannulas were gently inserted in the intervillous space, and a venous drain was introduced to return the fluid to the maternal circulation. Perfusion was achieved with two peristaltic pumps at 4 mL/min for fetal flow and 12 mL/min for maternal flow. Oxygenators were applied to maintain gas compositions of 95% N_2/5% CO_2 at the fetal side and 95% synthetic air/5% CO_2 at the maternal side. All components of the perfusion system were kept in perfusion chamber at 37°C. To flush out the blood and allow recovery of the tissue from the ischemic period after the delivery, the cotyledon was perfused for 20 min with perfusion medium. The perfusion medium was M199 tissue culture medium, which was diluted with Earl's buffer (1:2) and further supplemented with glucose (1 g/L), bovine serum albumin (BSA; 10 g/L), dextran 40 (10 g/L), sodium heparin [2,500 International Units (IU)/L], amoxicillin (250 mg/L), and sodium bicarbonate (2.2 g/L; medium and all supplements were obtained from Sigma-Aldrich). After the recovery phase, the medium was replaced by fresh medium in the presence or absence of ZEN. In total, six placentas from six different women were perfused. Three placentas were perfused with perfusion medium without addition of ZEN to obtain baseline data. In the other three perfusions, ZEN was added to the maternal circulation at a final concentration of 1 μM, corresponding to 318 μg/L. The concentration was chosen to be in a nontoxic range [nontoxic on human choriocarcinoma cells (BeWo)] up to 10 μM (Prouillac et al. 2009, 2012) but high enough to enable quantitative measurements also of minor biotransformation products. Concentrations of ZEN in maternal blood during pregnancy are not reported to date to the best of our knowledge. However, based on available exposure data and current legislation [in the EU EC (2007); EFSA (2011)] we expect chronic low-dose exposure to be frequent.

Criteria for a successful perfusion were: *a*) the pre-perfusion of the tissue showed no leakage; *b*) the leakage (fetal to maternal) was less than 4 mL/h during the translocation experiment; and *c*) the pH remained constant during the experiment (7.2–7.4). Moreover, the passive diffusion reference markers antipyrine and creatinine were added to all perfusions (final concentration of

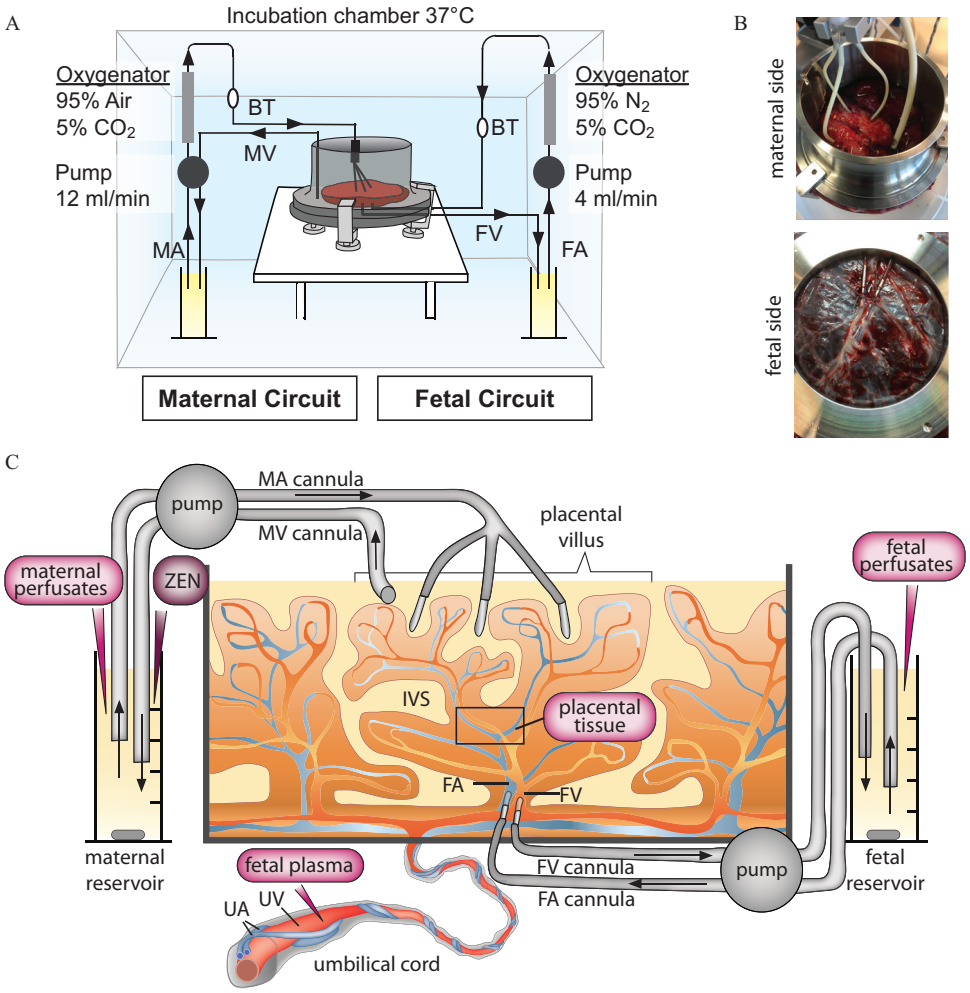

Figure 1. *Ex vivo* dually perfused human placenta perfusion model. (A) Scheme of the perfusion system. (B) Photograph showing cannulation from the maternal and fetal side, respectively. (C) Scheme with details of maternal and fetal side cannulation of an intact cotyledon and sampling sites (magenta). Fetal plasma is isolated from the umbilical vein blood before perfusion. ZEN is introduced to the maternal reservoir at the start of perfusion. Maternal and fetal perfusates are sampled from the corresponding reservoirs at different time points during perfusion. Placental tissue (black quadrant) is taken from the intervillous region of a perfused cotyledon at the end of perfusion. Note: BT, bubble trap; FA, fetal artery; FV, fetal vein; IVS, intervillous space (maternal blood space); MA, maternal artery; MV, maternal vein; UA, umbilical artery; UV, umbilical vein.

100 mg/L in maternal reservoir) to ensure sufficient overlap between maternal and fetal circulation.

Samples (5 mL) from the maternal and fetal circulations (referred to as maternal and fetal perfusates) were collected at 0, 0.25, 0.5, 1, 2, 3, 4, 5, and 6 h of perfusion and analyzed immediately for pH, creatinine, glucose, and lactate using a blood gas analyzer (epoc® Blood Analysis system with BGEM test cards; Epocal Inc.). One of the control perfusions (medium without addition of ZEN) showed a slight leakage after 5 h of perfusion and was therefore stopped at that time point. Afterwards, supernatants were stored at −80°C for further UHPLC-MS/MS analysis. Placental tissue samples from the intervillous region were taken before (from a nonperfused neighboring cotyledon) and after perfusion and stored at −80°C for UHPLC-MS/MS analysis. Fetal plasma was isolated from umbilical vein blood by centrifugation [2,000 × *g*, 10 min, in S-Monovette with Li-Heparin (Sarstedt)] and stored at −80°C for later UHPLC-MS/MS analysis.

Sample Preparation

A volume of 200 µL of maternal and fetal perfusates and fetal plasma, respectively, was spiked with 10 µL of internal standard (IS) solution (100 µg/L $^{13}C_{18}$-ZEN), vortexed, mixed with 800 µL MeOH/ACN (1/1), and thereafter sonicated for 10 min in an ice bath. To assist protein precipitation, the samples were stored at −20°C for 1 h and subsequently centrifuged at 18,000 × *g* at 4°C for 15 min. The supernatant was evaporated at 4°C with a CentriVap Vacuum concentrator (Labconco) and resolved with 200 µL ACN/H_2O (1/9). The samples were centrifuged for 5 min (18,000 × *g*, 4°C), and the supernatant was transferred into a glass vial containing an insert. All steps were performed on ice.

Placental tissue samples were cut into pieces of approximately 500 mg. In addition, 20 times the volume ACN/MeOH/H_2O (4/4/2) was added as extraction solvent. Ceramic beads were added, and homogenization was performed with a Fast Prep Homogenizer (MP Biomedicals). Four cycles (4 m/s for 20 s; 120 s break) were conducted. After 10 min of sonication, the samples were centrifuged at 4,000 × *g* at 4°C. 1,000 µL of supernatant was spiked with 10 µL of IS solution (100 µg/L $^{13}C_{18}$-ZEN), vortexed and put at −20°C for one hour. After 15 min of centrifugation at 18,000 × g and 4°C, the supernatant was evaporated at 4°C with a CentriVap Vacuum concentrator and resolved in 200 µL ACN/H_2O (1/9). The samples were centrifuged for 5 min (18,000 × *g*, 4°C) and the supernatant transferred into a glass vial containing an insert. All steps were performed on ice.

LC-MS/MS Measurements

Measurements were performed utilizing a Dionex Ultimate 3000 UHPLC coupled to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific) as described in detail by Preindl et al. (2019). In brief, an Acquity HSS T3 column (1.8 µm, 2.1 × 100 mm) equipped with a VanGuard precolumn (1.8 µm; Waters Corporation) served for chromatographic separation. As mobile phases, water with 0.3 mM ammonium fluoride as additive (A) and acetonitrile (B) were used at a flow rate of 0.4 mL/min. Aqueous ammonium fluoride solutions have to be handled with care, because hydrogen fluoride may outgas. A stock solution of 0.3 M was prepared and stored at 4°C. The final concentration in the eluent was very low; however, contact with acid (e.g., in the waste bottle) was prevented for safety reasons. Separation was achieved by the following gradient: 0–1 min: 5% B; rise to 18% B until min 1.8; rise to 35% B until min 4.2; rise to 48% B until min 13; rise to 90% B until min 14; flush with 98% B from min 15.8 to min 17.6; re-equilibrate with 5% B from min 17.7 to min 20. The column compartment was operated at 40°C and the sample tray cooled to 10°C. The multiple reaction monitoring (MRM) experiments were performed in negative electrospray ionization (ESI) mode, using fast polarity switching. Electrospray settings were set as follows: spray voltage −3,100 V, vaporizer temperature 340°C, sheath gas pressure 50 au, ion sweep gas pressure 2.5 au, aux gas pressure 30 au and capillary temperature 315°C.

To account for matrix effects and potential retention time deviations in the different analyzed matrices, matrix-matched reference standards were prepared by resolving evaporated blank extractions of perfusion medium [pooled aliquots of the three control perfusions without ZEN (min 0, 120, and end of perfusion)], fetal plasma (pooled fetal plasma from six distinct experiments) and placental tissue (pooled tissue from six distinct placentas) with 200 µL of standard level solutions (nine-point calibration, 0.05 µg/L to 500 µg/L in 1/9 ACN/H_2O). ZEN was quantified using the $^{13}C_{18}$-ZEN IS. To determine extraction recoveries, blank extractions of perfusion medium [pooled aliquots of the three control perfusions without ZEN (min 0, 120, and end of perfusion)] and fetal plasma (pooled fetal plasma from six distinct experiments) were spiked in triplicate at 15 µg/L before extraction and sample preparation. Placental tissue (pooled tissue from six distinct placentas) was spiked in triplicate at a concentration of 15 µg/L post homogenization. For control measurements of antipyrine, a separate reference standard was dissolved in pure solvent at different concentrations for external calibration purpose (10 to 100 mg/L in 1/9 ACN/H_2O).

Data Processing and Statistics

Data evaluation and quantification was performed with the Xcalibur and TraceFinder software packages (version 4.1, Thermo Scientific). Limit of detection (LOD) and limit of quantification (LOQ) were estimated for each matrix in spiked blank matrix samples by a signal-to-noise ratio of three and ten, respectively. All results from medium-derived samples were corrected by the calculated analyte-specific recovery values.

Data regarding ZEN and its metabolites represent mean ± standard deviation (SD) of three independent placentas, perfused with medium containing ZEN. Perfusion data comparing maternal and fetal perfusate concentrations were analyzed by unpaired Student's *t*-test. Differences were considered statistically significant at $p < 0.05$. The percentages of ZEN metabolized to α-ZEL and ZEN-14-Sulf were calculated by the following formula: $100 \times (M + F + T + S)/M_0$, where M and F is the amount of the metabolite (α-ZEL or ZEN-14-Sulf) in the maternal and fetal circulation at the end of perfusion, T is the amount of metabolite in the placental tissue after perfusion, S is the amount of metabolites present in the samples being collected during perfusion, and M_0 is the amount of ZEN added to the maternal circulation at the beginning of perfusion. The percentages of ZEN released into the fetal or maternal circulation or present in placental tissue were calculated similarly as described above (% maternal: $100 \times (M + S)/M_0$; (% fetal: $100 \times (F + S)/M_0$; (% tissue: $100 \times T/M_0$).

Results

Analytical Performance

A highly sensitive and specific UHPLC-MS/MS multimethod for ZEN and nine of its key metabolites (chemical structures are depicted in Figure 2) was established for quantification. As demonstrated in Figure 3 and S1, we were able to achieve baseline chromatographic separations and desirable peak shapes despite the high structural similarity. MRM transitions and retention times of the measured analytes are summarized in Table 1. Estimated LOD values in perfusion medium ranged from 0.05 to 1.50 µg/L, whereas in fetal plasma and placental tissue they ranged from 0.05 to 5.0 µg/L and from 0.6 to 6.0 ng/g, respectively (Table 1). Extraction recoveries as obtained from the spiking experiments were above 82% for placental tissue and plasma samples and 74% for the perfusion medium, respectively (Table S1).

ZEN and Metabolite Levels in Placental Tissue and Fetal Plasma

To understand potential background levels of ZEN and its metabolites present in the placental tissue or introduced during the

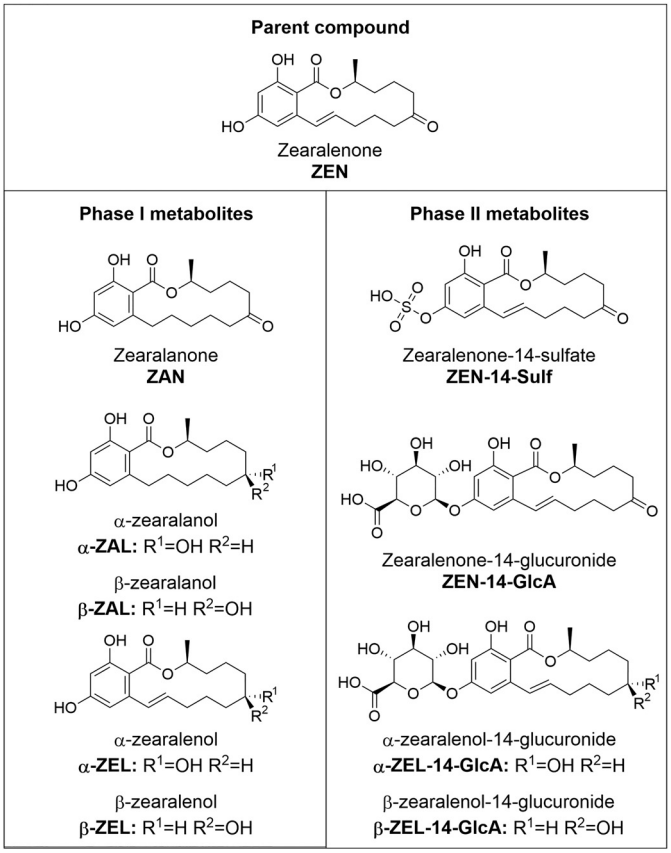

Figure 2. Chemical structures of ZEN and its metabolites investigated in this study.

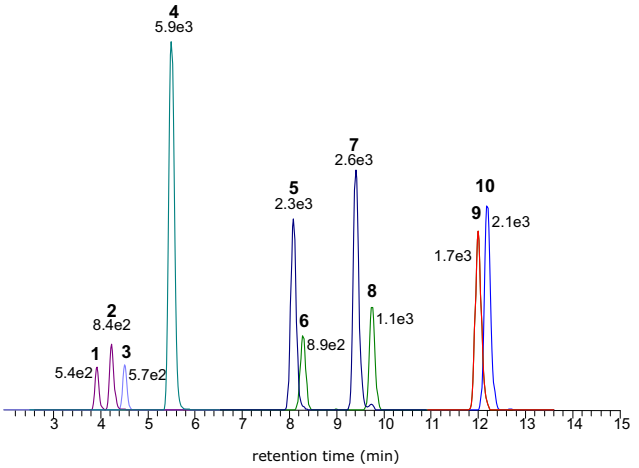

Figure 3. Multiple reaction monitoring (MRM) chromatograms of reference standards spiked into blank medium. β-zearalenol-14-glucuronide (β-ZEL-14-GlcA) m/z 495 → 319.3 (1); α-zearalenol-14-glucuronide (α-ZEL-14-GlcA) m/z 495.2 → 319.3 (2); zearalenone-14-glucuronide (ZEN-14-GlcA) m/z 493.2 → 317.2 (3); zearalenone-14-sulfate (ZEN-14-Sulf) m/z 397.1 → 317.2 (4); β-zearalanol (β-ZAL) m/z 321.2 → 277.2 (5); β-zearalenol (β-ZEL) m/z 319.2 → 275.2 (6); α-zearalanol (α-ZAL) m/z 321.2 → 277.2 (7); α-zearalenol (α-ZEL) m/z 319.2 → 275.2 (8); zearalanone (ZAN) m/z 319.2 → 275.2 (9); zearalenone (ZEN) m/z 317 → 175 (10); stated numbers represent the intensity arbitrary units (a.u.).

perfusion process, we determined their concentrations in three control perfusion experiments (placentas perfused with perfusion medium without addition of ZEN) from maternal and fetal perfusate as well as placental tissue samples taken before and after 6 h of perfusion (Table S1). In each experiment (all six perfused placentas), placental tissues and perfusates were assayed for ZEN and nine metabolites before the experimental addition of ZEN. In all these measurements, ZEN and the nine metabolites were not detected. Moreover, these compounds were also absent in fetal vein plasma from any of the six placentas investigated.

Human Placental Metabolism of ZEN

The human placenta expresses a wealth of enzymatic machinery responsible for both phase I and phase II reactions (Prouillac and Lecoeur 2010). Therefore, we explored the biotransformation of ZEN into previously described metabolites in the human *ex vivo* placental perfusion model as well as the bi-directional release of the metabolites into the maternal and fetal circulation.

After 6 h of perfusion with 318 μg/L ZEN, only the parent compound ZEN and the metabolites α-ZEL and ZEN-14-Sulf were detected in the placental tissue at concentrations of 205 ± 31 ng/g tissue, 13 ± 2 ng/g tissue, and 14 ± 7 ng/g tissue, respectively. Although ZEN standard material of highest quality was purchased (98.9% purity), our highly sensitive UHPLC-MS/MS method revealed that this material contained trace amounts of the metabolites ZAN and β-ZEL (each about 1% of the applied ZEN concentration) (Table S2). Although placental tissue concentrations of ZAN were below LOQ, a small amount of 4.6 ± 1.6 ng/g tissue β-ZEL was detected after 6 h of perfusion with ZEN. Release of the newly formed metabolites α-ZEL and ZEN-14-Sulf into the maternal and fetal circulation was observed already within the first 15 min of perfusion (Figure 4, Table S1). For α-ZEL, a plateau was reached after 3 h of perfusion with approximately double the concentrations in the maternal in comparison with the fetal compartment (4.8 ± 0.6 μg/L and 2.8 ± 1.4 μg/L α-ZEL at 6 h of perfusion, respectively) (Figure 4). In contrast, ZEN-14-Sulf concentrations showed a linear increase to 14.9 ± 4.5 μg/L in the maternal and 8.2 ± 3.5 μg/L in the fetal circulation at 6 h of perfusion (Figure 4, Table S1). Overall, ∼4 and 8% respectively of the introduced ZEN was metabolized to α-ZEL and ZEN-14-Sulf and released into the maternal and fetal circulation at the end of the perfusion studies, whereas no significant metabolism to ZAN, β-ZEL, α/β-ZAL, ZEN-14-GlcA, or α/β-ZEL-14-GlcA was observed.

Human Placental Transfer of ZEN

To investigate if ZEN can cross the human placental barrier and reach the fetal circulation, we applied the dually perfused *ex vivo* placental perfusion model that is extensively used to assess the transplacental transfer of compounds and delivers highly predictive results (Hutson et al. 2011).

In all placental perfusion experiments (three control perfusions without addition of ZEN and three perfusions with addition of ZEN), two reference compounds with fast or slow passive diffusion kinetics, namely antipyrine and creatinine, were added to ensure sufficient overlap between the maternal and fetal circulation and to normalize the translocation data. Antipyrine showed a rapid translocation to the fetal circulation similar to those reported in previous publications (Wick et al. 2010; Nielsen et al. 2011; Woo et al. 2012; Grafmueller et al. 2015) (Figure 5). In contrast, transplacental transfer of creatinine was slower and did not reach an equilibrium within 6 h of perfusion (Figure 5). Addition of ZEN

Table 1. Chromatographic and mass spectrometric parameters of the assessed analytes.

Analyte	Retention time (min)	Parent ion	Ion species	Quantifier ion	Qualifier ion	Ionization mode	Placental tissue (ng/g) LOD	Placental tissue (ng/g) LOQ	Fetal plasma (μg/L) LOD	Fetal plasma (μg/L) LOQ	Perfusion medium (μg/L) LOD	Perfusion medium (μg/L) LOQ
β-ZEL-14-GlcA	3.82–4.03[a]	495	$[M-H]^-$	319.3	175	Negative	2	6	5	15	1	1.5
α-ZEL-14-GlcA	4.10–4.35[a]	495	$[M-H]^-$	319.3	175	Negative	6	6	5	5	0.5	1.5
ZEN-14-GlcA	4.39–4.64[a]	493.2	$[M-H]^-$	317.2	175.1	Negative	6	20	5	15	1.5	5
ZEN-14-Sulf	5.36–5.57[a]	397.1	$[M-H]^-$	317.2	175.1	Negative	2	6	0.15	0.5	0.15	0.5
β-ZAL	8.08	321.2	$[M-H]^-$	277.2	303.2	Negative	2	2	0.15	0.5	0.5	1.5
β-ZEL	8.32	319.2	$[M-H]^-$	275.2	160.1	Negative	2	2	0.5	1.5	0.5	1.5
α-ZAL	9.40	321.2	$[M-H]^-$	277.2	303.2	Negative	2	2	0.15	0.5	0.5	0.5
α-ZEL	9.74	319.2	$[M-H]^-$	275.2	160.1	Negative	2	6	0.5	1.5	0.5	1.5
ZAN	12.00	319.2	$[M-H]^-$	275.2	205.2	Negative	2	2	0.15	0.5	0.15	0.5
ZEN	12.18	317	$[M-H]^-$	175.0	131.0	Negative	0.6	2	0.05	0.15	0.05	0.15
$^{13}C_{18}$-ZEN (IS)	12.18	335	$[M-H]^-$	185.0	—	Negative						

Note: —, indicate data not available, Multiple reaction monitoring (MRM) transitions, retention times and limit of detections (LOD), limit of quantifications (LOQ) of tested matrices. Zearalenone (ZEN), ZEN-14-glucuronide (ZEN-14-GlcA), ZEN-14-sulfate (ZEN-14-Sulf), α-/β-zearalenol (α-/β-ZEL); α-/β-ZEL-14-glucuronide (α-/β-ZEL-GlcA), α-/β-zearalanol (α-/β-ZAL), zearalanone (ZAN); IS, Internal Standard.
[a]Minor deviations in different matrices.

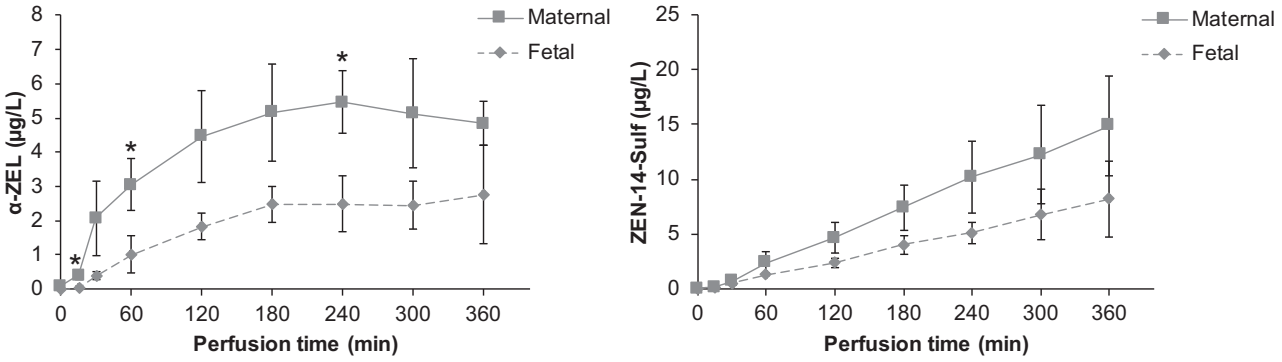

Figure 4. Perfusion profiles of α-zearalenol (α-ZEL) and zearalenone-14-sulfate (ZEN-14-Sulf). Release of α-ZEL and ZEN-14-Sulf to the maternal and fetal circulation during human placental perfusion with 318 µg/L zearalenone (ZEN) over 6 h. Data represent mean ± SD of three independent placentas perfused with medium containing ZEN. Note: $p < 0.05$ is considered statistically significant (* denotes differences between maternal and fetal concentrations in α-ZEL perfusions). Perfusion data comparing maternal and fetal concentrations were analyzed by unpaired Student's *t*-test.

did not alter the transplacental transfer kinetics of creatinine but slightly increased the transfer of antipyrine (Figure 5).

After addition of ZEN (318 µg/L) to the maternal circulation, its levels rapidly decreased in the maternal compartment and increased in the fetal compartment to a concentration of 53 ± 18.5 µg/L after 6 h of perfusion (Figure 6, Table S1). Translocation of ZEN across the placental barrier was only slightly slower than the reference compound antipyrine but substantially faster than creatinine as indicated by the fetal/maternal (FM) ratios (Figure 6). At the end of perfusion, 15.3 ± 3.8% of the initially added ZEN was present in the fetal circulation, 30.8 ± 7% in the maternal circulation, and 24.6 ± 5.8% in the cotyledon. The rest of ZEN was metabolized to α-ZEL and ZEN-14-Sulf, and usually a small amount of the perfused substance will diffuse into the tissue surrounding the perfused cotyledon (Frederiksen et al. 2010) or bind to the perfusion system (Mose et al. 2008; Aengenheister et al. 2018).

Due to the above-mentioned minimal contamination of ZEN with ZAN and β-ZEL, we could additionally obtain placental transfer data of these two compounds. Transfer of ZAN and β-ZEL across the placental barrier was observed, but due to the low initial amount in the maternal circulation (~3 µg/L), the deviations of the individual measurements were relatively large (Figure S2).

Discussion

To the best of our knowledge, we report the first examination of the human placental transfer and metabolism of ZEN in an *ex vivo* human placental perfusion model and determined a placental transfer of ZEN at only a slightly slower pace than the highly diffusible reference compound antipyrine. Additionally, we showed that the human placenta is capable of phase I and II metabolism of ZEN to α-ZEL and ZEN-14-Sulf. The metabolites were quickly released into the fetal and maternal circulation with a preferential release toward the maternal circulation. Our findings are in good agreement with previous results from pregnant rats fed with ZEN in mid and end gestation (Bernhoft et al. 2001). In contrast, no placental transfer of ZEN was observed upon exposure of mice in early pregnancy (Appelgren et al. 1982). Therefore, placental metabolism and transfer of ZEN may be different in early human pregnancy as well, when the placental barrier is much thicker to protect the developing fetus. However, this possibility is difficult to investigate because human placental perfusion studies are limited to term placentas.

For our studies, we applied a high but noncytotoxic concentration of ZEN (1 µM; 318 µg/L) based on previous *in vitro* studies showing that ZEN as well as α- and β-ZEL were not cytotoxic up to a concentration of 10 µM to BeWo cells after 48 h of exposure (Prouillac et al. 2009, 2012). However, ZEN and its metabolites might induce subtoxic effects to the placental tissue. For instance, ZEN (10 µM) has been shown to induce trophoblast differentiation and alter ABC transporter gene expression in BeWo trophoblast cells (Prouillac et al. 2009). In contrast, its reduced metabolites were not able to induce trophoblast differentiation but did again modulate ABC transporter gene expression (Prouillac et al. 2012). Additionally, ABC transporters were altered in placentas of pregnant rats exposed to ZEN (Gao et al. 2017). An interesting aspect is that we also observed a potential impact of ZEN on human placental permeability in our *ex vivo* placental perfusion experiments. Translocation of the passive transcellular diffusion marker antipyrine was increased in the presence of ZEN, suggesting that ZEN may interfere with placental transfer of essential endogenous compounds or decrease barrier function toward xenobiotic substances. However, ZEN did not affect paracellular transport because it did not alter the translocation of the passive paracellular diffusion marker creatinine. Another explanation for how small amounts of xenoestrogens could affect fetal health is by disturbance of endocrine signaling pathways. In pregnancy, endogenous estrogen levels are very high in the maternal circulation as a consequence of placental estrogen production and preferential release toward the maternal circulation [reviewed in Kaludjerovic and Ward (2012)]. However, the high maternal estrogen plasma levels appear to be shielded from the embryo by the placenta as shown in rhesus monkeys (Slikker et al. 1982) and through fetal sequestering by alpha-fetoprotein, protecting the developing embryo from the bulk of maternal circulating estrogens [reviewed in Bondesson et al. (2015)]. Therefore, in light of the lower fetal estrogen levels, maternofetal translocation of xenoestrogens may significantly contribute to fetal estrogen levels and interfere with the fetomaternal endocrine system that regulates estrogen production during development. Evidence exists that a decrease in placental estrogen production during sensitive stages of development may have the potential to alter gene transcription and DNA methylation patterns of cells, suggesting that environmental estrogens may be able to induce epigenetic changes in the developing fetus [reviewed in Kaludjerovic and Ward (2012)].

In humans, ZEN has been shown to be predominately metabolized into α-ZEL (major phase I metabolite) and to a minor extent also to β-ZEL and thereafter glucuronidated. These forms are then, together with free and glucuronidated ZEN, excreted via urine (Mirocha et al. 1981; Warth et al. 2013). Until now, to the best of our knowledge, human sulfation pathways have been

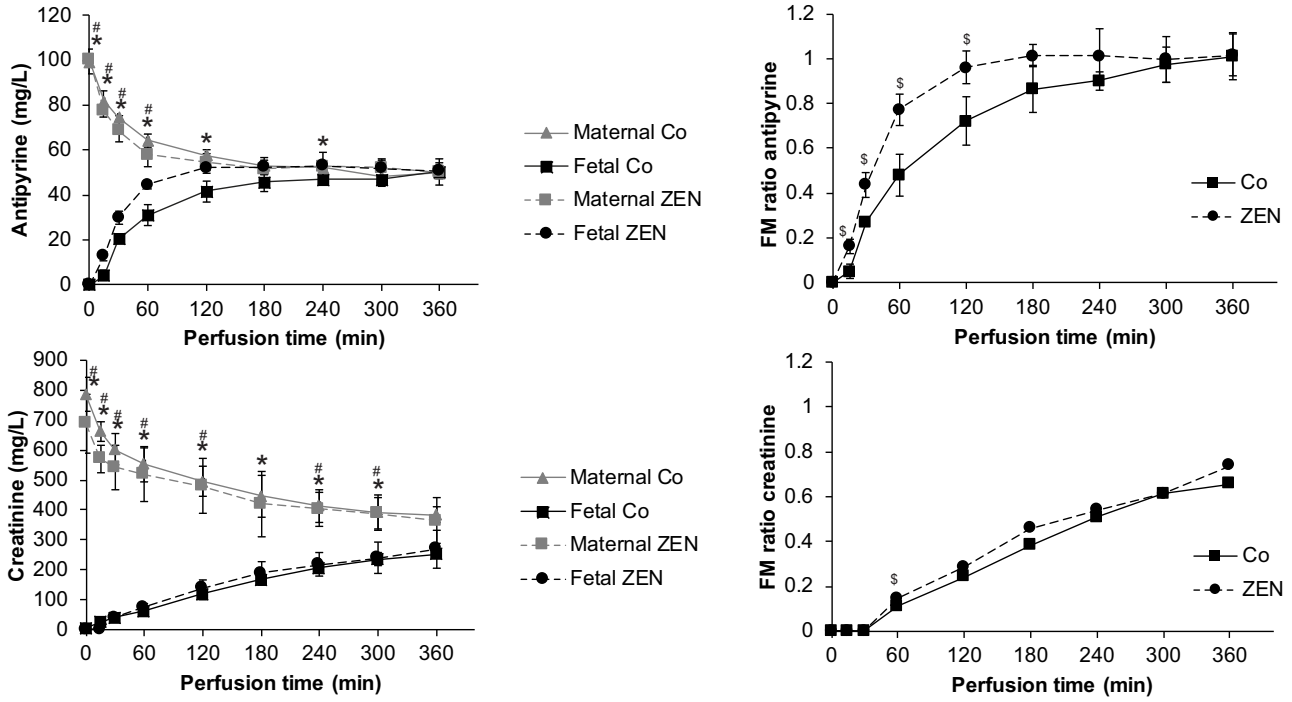

Figure 5. Perfusion profiles and fetal–maternal (FM) ratio of the reference compounds antipyrine and creatinine. Antipyrine (100 mg/L) and creatinine (100 mg/L) were added to the maternal circulation in six independently perfused placentas from six different mothers (three placentas were perfused with medium without zearalenone to obtain baseline data (Co) and three placentas were perfused with medium containing ZEN). The concentrations of both reference compounds were measured from the maternal and fetal perfusates by UHPLC-MS/MS at several time points during 6 h of perfusion. Data represent mean ± SD of three independently perfused placentas [three control perfusions without ZEN (Co) and three perfusions with addition of ZEN (ZEN)]. $p < 0.05$ is considered statistically significant (* and $^{\#}$ denote differences between maternal and fetal concentrations in Co and ZEN perfusions, respectively; $^{\$}$ denotes differences in FM ratio between Co and ZEN). Perfusion data comparing maternal and fetal concentrations were analyzed by unpaired Student's *t*-test.

reported only in epithelial colorectal adenocarcinoma cells (Caco-2) (Pfeiffer et al. 2011).

Researchers have proposed that hydroxylation of ZEN is mediated through 3 α- and 3 β- hydroxysteroid dehydrogenases (HSD) (Olsen et al. 1981; Malekinejad et al. 2005). The enzyme 3 β-HSD has been shown to be highly expressed in the human placenta (Mason et al. 1993). Previous studies in cell models revealed that the human placenta is likely capable of metabolizing ZEN and ZAN via phase I metabolism into their reduced forms (Huuskonen et al. 2015). We could partly confirm this data in the *ex vivo* perfusion model. Although ∼4% of the introduced ZEN was reduced to α-ZEL after 360 min of perfusion, a significant metabolism to β-ZEL was not observed. The placental metabolism of ZEN into α-ZEL is of particular relevance due to its far higher relative estrogenic potency in comparison with the parent compound (Arukwe et al. 1999; Frizzell et al. 2011; Tatay et al. 2017).

We further found phase II metabolism of ZEN to ZEN-14-Sulf, to the best of our knowledge for the first time in human placenta; ∼8% of ZEN introduced to the test system was converted to this metabolite after 360 min of perfusion. ZEN-14-Sulf has been described as fungal ZEN conjugate (Binder et al. 2017); however, as mentioned before, it can also be produced by human epithelial colorectal adenocarcinoma cells (Pfeiffer et al. 2011) *in vitro*. Because α-ZEL, β–ZEL, ZAN, α-ZAL, and β-ZAL carry the same 14-hydroxyl group as ZEN, we also expected possible

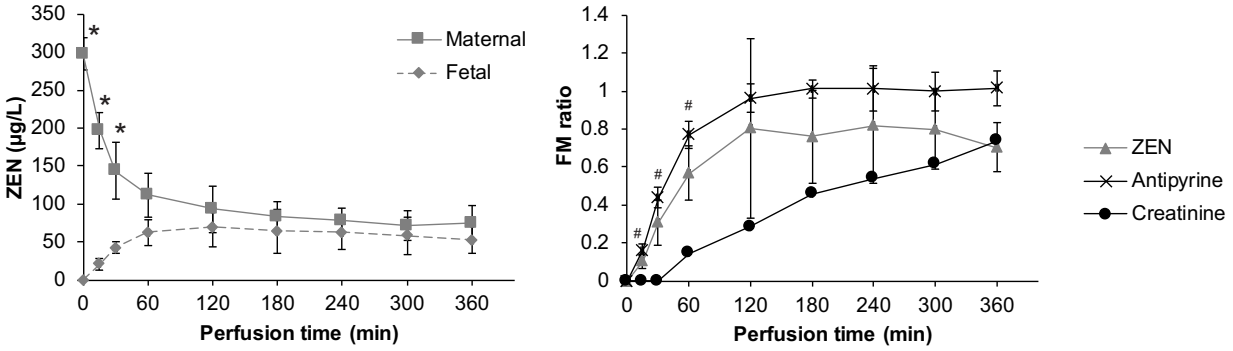

Figure 6. Perfusion profile and fetal–maternal (FM) ratio of zearalenone (ZEN). ZEN (318 µg/L) was added to the maternal circulation and its concentration was measured from the maternal and fetal perfusates by LC-MS/MS at several time points during 6 h of perfusion. FM ratios were calculated for each time point and FM ratios of antipyrine and creatinine were added for comparison. Data represent mean ± SD of three independent placentas, perfused with medium containing ZEN. $p < 0.05$ is considered statistically significant (* denotes differences between maternal and fetal concentrations in ZEN perfusions; $^{\#}$ denotes differences in FM ratio between ZEN and creatinine). Perfusion data comparing maternal and fetal concentrations were analyzed by unpaired Student's *t*-test.

sulfation of these compounds (in our system especially sulfation of α-ZEL) but could not verify this hypothesis due to a lack of appropriate standard material. The placenta is known to require sulfate for sulfation of endogenous estrogens and thyroid hormones. The sulfate supply for the fetus is also mediated through the placenta. Reduced availability of sulfate due to detoxification processes may therefore result in a placental phenotype and disturbed fetal development (Dawson et al. 2017).

Human glucuronidation of ZEN and its reduced metabolites is mediated by UDP-glucuronosyltransferases (UGTs), mainly by UGT1A1, 1A3, and 1A8 in the liver, intestine, and eventually also in other organs. These UGTs preferably glucuronidate the unhindered 14-hydroxyl moiety of ZEN and its phase I metabolites (Pfeiffer et al. 2010). It has been shown before, that the human placenta expresses UGT2B isoforms (Collier et al. 2002). For placental UGT1A isoform expression conflicting results are reported, but evidence is growing that UGT1A1, 1A4, and 1A6 are expressed in healthy placentas (Collier et al. 2002; Reimers et al. 2011; Collier et al. 2015). From the principal potential of the placenta to express UGT1A1, it can be assumed that ZEN and its metabolites might be glucuronidated also in our placental perfusion model. However, this assumption was not confirmed in this study. A possible explanation could be that the glucuronidation potential of the placenta may be far lower than its sulfation potential, and consequently, the glucuronides are produced in such a low concentration that detection by means of the applied analytical method was not feasible.

ZEN and its nine analyzed metabolites were detected neither in placental tissue previous to the perfusion nor in fetal plasma obtained from a fetal placental vein, indicating that ZEN did not accumulate in the fetal circulation during the pregnancies of these mothers. However, we point out that a relatively low number of placentas and corresponding fetal plasma samples ($n=6$) were examined, and the mothers were probably fasting due to planned caesarian sections. Therefore, previously ingested ZEN might have been cleared already because ZEN elimination has been shown to be nearly completed within 3 d in various mammals (Ueno et al. 1977; Mirocha et al. 1981).

Despite some obvious strengths of the *ex vivo* perfusion model, such as the use of a complex and highly functional human tissue in dynamic conditions and its accessibility for mechanistic studies, there are certain limitations to this approach. One limitation is the restriction of the perfusion duration to approximately 6 h. However, this duration was sufficient to detect placental metabolism of ZEN to α-ZEL and ZEN-14-sulfate as well as maternofetal transfer of ZEN with a plateau already at 3 h of perfusion. Another drawback is the use of term placenta, which does not provide insights on placental transfer and metabolism at earlier stages of pregnancy. Moreover, the throughput of this method is limited, yet we obtained highly consistent results from three independent perfusion studies. Finally, due to the absence of maternal and fetal tissues, kinetic and metabolic data reflect those of the isolated human placental tissue only and may not be fully representative of the physiological situation in pregnant women. An analytical limitation of the study is the targeted approach applied for the identification of ZEN metabolites which takes only known biotransformation products into account. Future work may exploit an isotope-based metabolomic workflow (Bueschl et al. 2017) to understand the full extent of ZEN metabolism in an untargeted manner. Finally, researchers need to confirm whether similar findings can be achieved with lower ZEN doses, although this will likely result in a lower number of identified metabolites due to limitations in sensitivity even when applying highly sensitive tandem mass-spectrometry–based assays.

A nonbalanced diet among pregnant women may increase the chance that the fetus is exposed to higher levels of these mycoestrogens, and more emphasis should be placed on accurate exposure assessment via human biomonitoring of ZEN and its key metabolites throughout pregnancy.

ZEN and some of its metabolites have been frequently detected in human urine from various countries at low doses. For example, ZEN, α-ZEL, and β-ZEL were found in urine samples from Germany (Ali and Degen 2018), southern Italy (Solfrizzo et al. 2014), and Nigeria (Šarkanj et al. 2018) at median concentrations of 0.04, 0.056, and 0.2 μg/L (ZEN), 0.23, 0.074, and 0.87 μg/L (α-ZEL), 0.03, 0.088, and 0.33 μg/L (β-ZEL), respectively. A study on New Jersey girls revealed urinary ZEN (median 0.32 μg/L), α-ZEL (median 0.06 μg/L), β-ZEL (median 0.16 μg/L), and α-ZAL (median 0.17 μg/L) (Bandera et al. 2011). Further urinary biomonitoring studies were summarized by Mally et al. (2016). The few biomonitoring studies investigating blood revealed ZEN levels typically below 1.0 μg/L (Massart et al. 2008; Sun et al. 2019).

In addition, these natural contaminants likely contribute significantly to the overall xenoestrogen exposure that unborn fetuses may face. Elevated *in utero* exposure to endogenous estrogens or xenoestrogens has been shown to disturb the development of the reproductive system of the fetus in human and animals (Palmlund 1996; Saunders et al. 1997; Welshons et al. 1999; Delbès et al. 2006). In pigs, a decreased fetus and uterus weight (Etienne and Jemmali 1982) as well as a decreased number of fetuses were observed upon maternal ingestion of zearalenone (Young et al. 1990). In mice, exposure to ZEN in early pregnancy caused delayed implantation, loss of conceptuses, and a decreased growth of the fetuses (Kunishige et al. 2017). In addition, environmental exposure to xenoestrogens like BPA may be related to the carcinogenesis of breast cancer and various other tumors (Fernandez and Russo 2010; Fucic et al. 2012).

Conclusions

We revealed in an *ex vivo* human placental perfusion model the fast transfer of ZEN across the human placental barrier as well as phase I and phase II metabolism of the parent toxin ZEN into the highly estrogenic α-ZEL and the less active ZEN-14-sulfate. Our study will help to further evaluate the risks of an *in utero* exposure to xenoestrogens and highlights the need for a more comprehensive assessment of exposure and combinatory toxicological effects in the context of the exposome paradigm (Dennis et al. 2017; Warth et al. 2017).

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