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Human-relevant concentrations of the antifungal drug clotrimazole disrupt maternal and fetal steroid hormone profiles in rats

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ABSTRACT

Clotrimazole is a non-prescription and broad-spectrum antifungal drug sold under brand names such as Canesten® and Lotrimin®. It is used to treat different types of fungal infections, from oral thrush to athlete's foot and vaginal mycosis. The level of exposure to clotrimazole is uncertain, as the exact usage amongst self-medicating patients is unclear. Recent studies have raised potential concern about the unsupervised use of clotrimazole during pregnancy, especially since it is a potent inhibitor of CYP enzymes of the steroidogenesis pathway. To address some of these concerns, we have assessed the effects of intrauterine exposure to clotrimazole on developing rat fetuses. By exposing pregnant rats to clotrimazole 25 or 75 mg/kg bw/day during gestation days 7–21, we obtained internal fetal concentrations close to those observed in humans. These *in vivo* data are in strong agreement with our physiologically-based pharmacokinetic (PBK)-modelled levels. At these doses, we observed no obvious morphological changes to the reproductive system, nor shorter male anogenital distance; a well-established morphometric marker for anti-androgenic effects in male offspring. However, steroid hormone profiles were significantly affected in both maternal and fetal plasma, in particular pronounced suppression of estrogens was seen. In fetal testes, marked up-concentration of hydroxyprogesterone was observed, which indicates a specific action on steroidogenesis. Since systemic clotrimazole is rapidly metabolized in humans, relevant exposure levels may not in itself cause adverse changes to the reproductive systems. Its capacity to significantly alter steroid hormone concentrations, however, suggests that clotrimazole should be used with caution during pregnancy.

1. Introduction

Developmental exposures to endocrine disrupting chemicals (EDCs) are associated with a string of reproductive disorders in humans, from genital malformations at birth to reproductive cancers and infertility in adulthood (Johansson et al., 2017; Skakkebaek et al., 2016). Animal studies have confirmed these associations and established clear links between compromised hormone signaling and adverse reproductive outcomes. The list of suspected EDCs now comprises a large number of chemical substances, including industrial chemicals such as phthalates and parabens, pesticides including azole fungicides, and also pharmaceutical drugs.

Non-prescription medicines harboring endocrine disrupting properties may be of particular concern to the unborn child. This is because the use of non-prescription medicines reportedly is widespread and in some instances very substantial, prompting researchers to raise the alarm over for instance the indiscriminate use of mild-analgesics during pregnancy (Kristensen et al., 2016). Besides analgesics, non-prescription antifungal medications containing azoles are used indiscriminately during pregnancy. Since many azoles have endocrine disrupting properties (Draskau et al., 2019; Goetz et al., 2007; Hass et al., 2012; Kjærstad et al., 2010; Laier et al., 2006; Melching-Kollmuss et al., 2017; Roelofs

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et al., 2014; Taxvig et al., 2008, 2007; Vinggaard et al., 2005), it raises the questions if non-prescription azole fungicides themselves are endocrine disruptors and if they could pose a risk to a developing child at relevant exposure doses. One example is clotrimazole, a broad-spectrum antifungal azole drug that could potentially cause anti-androgenic effects in human male fetuses (Mogensen et al., 2017).

Clotrimazole is used to treat different fungal infections such as oral thrush, diaper rash, athlete's foot and vaginal mycosis (Crowley and Gallagher, 2014). It is sold over-the-counter under various trade names (*e.g.* Canesten® and Lotrimin®). Exact usage amongst self-medicating patients, and thus human exposure to clotrimazole is not clear. Its use is considered safe, however, with low absorption levels from topical applications and rapid metabolism once absorbed. For instance, internal blood concentrations in women after vaginal treatment with clotrimazole vaginal tablets is estimated to be only 3–10% of the dose concentration (Munkboel et al., 2019; Ritter et al., 1982). Worryingly though, a recent study puts this assumption of safety into question as it shows clotrimazole to be a very potent disruptor of human steroidogenesis *in vitro,* even at concentrations below those observed in women after vaginal application (Munkboel et al., 2019). These data, coupled with preliminary outcomes from an epidemiological study reporting a (non-statistically significant) association between clotrimazole exposure and a shorter anogenital distance (AGD) in human male offspring (Mogensen et al., 2017) warrants further investigations. Especially since a short AGD in male new-borns is an established biomarker for incomplete masculinization caused by suppressed androgen action during fetal life, an effect that can lead to various male reproductive disorders (Schwartz et al., 2019). In other words, is the relatively indiscriminate use of clotrimazole during pregnancy unproblematic, or should there be tighter regulations or warnings for pregnant women? To investigate this further, we designed an intrauterine exposure study in rats with a clear focus on characterizing potential endocrine disrupting effects in the developing fetus, including pharmacokinetic considerations; measured and modelled.

2. Materials and methods

2.1. Chemicals

The test substance used in this study was clotrimazole (≥98% pure, CAS no.: 23593–75-1, product no.: C6019, lot no.:039M4778V) purchased from Sigma-Aldrich (MO, US). Acetonitrile, methanol, formic acid, ethyl acetate and 25% ammonium hydroxide, all LC-MS grade, were purchased from Sigma Aldrich (Schneldorf, Germany). Isolute bulk C-18 sorbent was purchased from Biotage, (Uppsala, Sweden) and washed with acetonitrile and ethyl acetate and dried prior to use. A Milli Q system (Millipore Corporation, US) was used for water purification.

2.2. Androgen receptor (AR) reporter gene assay

Clotrimazole (Sigma Aldrich, MO, US, CAS no.: 23593–75-1) was used to prepare a stock solution of 100 mM prepared in DMSO. The stock was visually inspected to verify solubility.

The AR reporter gene assay was performed as previously described (Draskau et al., 2019) using the AR-EcoScreen cell line from the JCRB Cell Bank (cat. no. JCRB1328). These cells are stably transfected with the human AR, as well as an androgen-dependent reporter plasmid and a constitutive reporter plasmid as described in the OECD guideline no. 458 (OECD, 2016). We measured both agonistic and antagonistic activity, as well as effects on cell viability. Cell culture was performed in DMEM-F12 medium without phenol red (Life Technologies, CA, US) supplemented with 5% FBS (Life Technologies, CA, US), 50 units/mL penicillin, 50 μg/mL streptomycin (Life Technologies, CA, US), 200 μg/mL Zeocin (Invivogen, CA, US), and 100 μg/mL Hygromycin (Invitrogen, CA, US) in a humidified atmosphere at 37 °C with 5% CO_2. During experiments, the same medium was used; however, Zeocin and Hygromycin was removed and FBS exchanged for 5% DCC-FBS (Sigma Aldrich, MO, US). Cells were seeded at 9000 cells/well in clear-bottomed 96-well plates (Costar, Corning, NY, US). After 24 h, clotrimazole was added at concentrations ranging from 0.01–3.1 μM, based on a range finding study showing compromised cell viability at higher concentrations (data not shown). In the antagonist mode, cells were co-treated with 0.1 nM R1881 (PerkinElmer, MA, US) - a synthetic AR agonist – in a concentration approximately corresponding to the EC_{50}-value based on historical data (Rosenmai et al., 2021). Finally, R1881 and the AR antagonist hydroxyflutamide (OHF, Toronto Research Chemicals, ON, Canada) were tested as positive controls in concentrations ranging from 0.01–1 nM and 4–9000 nM, respectively. The vehicle concentration was kept constant across the plate and never exceeded 0.1%. The assay was terminated with the Dual-Glo® Luciferase Assay System (Promega, WI, US) to measure both Firefly and Renilla luciferase activity on a luminometer (BioOrbit, Galaxy). Chemicals were tested across three independent experiments, each run with three technical replicates. Means of technical replicates from independent biological experiments were pooled. All treatment concentrations leading to >20% decreased cell viability compared to vehicle control were perceived as cytotoxic and omitted from the dataset before further analysis.

2.3. Animal study

The animal experiment had ethical approval from the Danish Animal Experiments Inspectorate (license number 2015-15-0201-00553). Two blocks of 12 time-mated Crl:CD(SD) rats (Charles River, Germany, *via* SCANBUR, Karlslunde, Denmark), 9 weeks old, weighing 200–250 g, were delivered to the animal facility at the Technical University of Denmark on gestational day (GD) 3 (the day following overnight mating was denoted GD1). Dams were divided into three groups of four animals with similar body weight (bw) distribution on GD4. The animals were housed in pairs until GD17, and thereafter single-housed in High Temperature polysulfone cages with wood chip bedding, nesting material and a wooden shelter (Tapvei, Denmark). The polysulfone bottles and cages as well as the aspen wood shelters (instead of plastic shelters) were used to reduce the risk of migration of BPA that potentially could confound the study results. Animals were kept under standard conditions with 12 h light/dark cycles, in ScanTainers (Ventilated Cabinets from SCANBUR) with 50–60 air changes per hour at 22 °C ± 10 and humidity kept at 55% ± 5. Animals were fed Altromin 1314 (Altromin GmbH, Lage, Germany, soy and alfalfa-free) and tap water in Bisphenol A-free bottles (Polysulfone 700 mL, 84-ACBT0702SU Tecniplast, VA, Italy) *ad libitum*.

Dams were acclimatized for four days before starting exposure on GD7. Dosing was initiated first at GD7 to avoid implantation loss, yet early enough to cover early fetal developmental stages and throughout the sensitive periods for reproductive development. The rats were weighed and gavaged each morning with vehicle (corn oil, CAS no.: 8001-30-7, Sigma-Aldrich, Søborg, Denmark, product no.: C8267–2.5 L, lot-nr.: MKCG3257) or clotrimazole (25 or 75 mg/kg bw/day) until GD21. Doses of 25 and 75 mg/kg bw/day were selected as these were the highest achievable doses not expected to induce maternal toxicity (Bayer HealthCare, 2006). The dams were given a dosing volume of 2 mL/kg bw.

2.4. Necropsy

On GD21, dams were gavaged 1 h ± 15 min before decapitation under CO_2/O_2-anesthesia. Maternal trunk blood was collected in heparin-coated vials and the liver was taken out and weighed. Uteri were collected, weighed, and the number of resorptions, implantations, live fetuses, and the location in uterus, were registered. Any anomalies and the body weight of the fetuses were recorded and the anogenital distance (AGD) was measured using a stereomicroscope with a micrometer eyepiece by an experienced technician blinded with respect to

treatment groups. Fetal trunk blood was collected in heparin-coated vials, pooled for each sex within each litter. All blood samples were kept on ice before centrifugation at 4000 rpm at 4 °C for 10 min after which the plasma was transferred to new tubes and stored at −80 °C.

Fetal rat testes were harvested under a stereomicroscope and immediately snap frozen in liquid nitrogen, or placed in Bouin's fixative, formalin, or RNA-later. Tissue in Bouin's fixative was transferred to 50% ethanol the following day (day 2), 70% on day 3 and run on an Excelsior AS Tissue Processor (Thermo Scientific™) on day 4 before paraffin embedding. Tissue in formalin was run on the tissue processor on day 4 before paraffin embedding. Tissue in RNA-later was stored at −80 °C.

2.5. Chemical analysis of clotrimazole in fetal plasma

Clotrimazole concentration in fetal plasma was measured by Liquid Chromatography-Mass Spectrometry (LC-MS) as previously described (Draskau et al., 2019). External calibration standards were used for quantification. This means that the concentrations determined in plasma samples are semi-quantitative since correction for loss during extraction and analysis are not fully corrected. Plasma samples were mixed with acetonitrile and Isolute C18 sorbent, vortexed and incubated at 4 °C for 30 min before centrifugation at 10,000 x*g* for 10 min at 4 °C. Prior to analysis supernatants were mixed with 75% acetonitrile.

LC was performed on a Dionex Ultimate 3000 RS (Thermo Scientific™) with a Poroshell SB C-8 (100 × 2.1 mm, 2.7 μm particle size) column held at 40 °C (Agilent technologies, Walbron, Germany). The solvent system (ammonium hydroxide, formic acid, and acetonitrile), programming, and flow rate were setup as described in (Draskau et al., 2019).

The LC system was connected to a Bruker Daltonics, maXis qTOF MS equipped with an electrospray ion source operated in positive ion mode (Bruker Daltonics, Bremen, Germany). Sodium formate dissolved in 50% 2-propanol was introduced in the ion source in a 0.2–0.4 min time segment and used for internal calibration of the data files. Hexakisperflouroetoxyphophazene was used as lock mass calibrant to compensate for drift in the mass axis during analysis.

External standard samples were prepared by diluting standard solutions with milli Q water at six different levels in the concentration range of 0 to 1000 ng/mL clotrimazole. Standards and blanks were analyzed in the beginning of a sequence and after each set of samples. The ion source settings were: dry gas temperature 200 °C, nebulizer pressure 2 bars, capillary voltage 2500 V, drying gas flow 8 L/min. The scan range was from 80 to 700 *m/z* with an acquisition rate of 2 Hz.

Data files were processed using QuantAnalysis (Bruker Daltonics, Bremen, Germany). Extracted ion chromatograms of m/z 277.078 ± 0.005 Da were constructed and integrated. Plasma concentrations were calculated based on linear calibration curves constructed using 1/x weighing.

2.6. Plasma and testis steroid hormone analysis by HPLC-MS/MS

Plasma and testis samples were analyzed for steroid hormone levels by LC-MS/MS as previously described (Hadrup et al., 2016). The extraction procedure and LC-MS/MS setup were performed as in (Draskau et al., 2019).

Plasma samples were vortexed with formic acid in acetonitrile with 3.33 ng/mL internal standard (deoxycortisol-d8 and cortisol-d4 from Cerilliant (TX, US), methyltestosterone-d3, beta-testosterone-d2, beta-estradiol-d3 and progesterone-c2 from Rikilt (Wageningen, Netherlands). Vials were incubated at −20 °C for 20 min and centrifuged at 10,000 x*g* for 7 min at 4 °C before supernatants were mixed with Supel™ QuE *Z*-Sep powder (Supelco, PA, USA, #55418-U), shaken for 60 s, and centrifuged for 3 min at 3500 x*g*. Supernatants were then dried by nitrogen evaporation before reconstitution with acetonitrile and water.

For testes samples, one testicle was transferred to a beetBeater tube (Fischer Scientific, MA, USA) and 1 mL of 80% acetonitrile (with internal standards, 1 ng/mL) was added. The sample was homogenized for 1 min and 50 mg of Que. Z-Sep (Sigma-Aldrich, MO, US) was added followed by whirlmix for 1 min. The sample was centrifuged at 3000 x*g*, 4 °C for 10 min and placed at −18 °C for 1 h. The sample was then centrifuged at 10,000 x*g* at 4 °C for 10 min, followed by filtration through a SPE column (Waters Corp., MA, US) (HLB, 30 mg), conditioned with 2 mL 80% acetonitrile. The sample was evaporated to dryness (50 °C, N2) and dissolved in 500 μL 10% acetonitrile. On-line-SPE LC-MS/MS was used for steroid hormone separation, detection, and quantification exactly as described in (Draskau et al., 2019).

We detected 8 (bold) out of 14 hormones assayed for: **testosterone**, **epi-testosterone**, **androstenedione**, dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), **corticosterone**, cortisol, hydroxycortisol, deoxycortisol, pregnenolone, **progesterone**, **hydroxyprogesterone**, **estradiol**, and **estrone**.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated as the concentrations corresponding to three and 10 times signal-to-noise, respectively. LOQs were estimated to be 0.1 ng/mL for androstenedione, progesterone, testosterone, estradiol, and estrone, 0.2 ng/mL for epi-testosterone, and 0.3 ng/mL for corticosterone and hydroxyprogesterone. For quantification, external calibration standards were run before and after the samples at concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ng/mL, with 2.0 ng/mL internal standard. Blank plasma samples were spiked with analyte at three concentration levels: 0.0 (blank sample), 0.5, and 2.0 ng/mL and run for quality control. However, some measured values in the groups with high clotrimazole-exposure were below the LOQ or LOD. Values below the detection limit were set as LOD divided by the square root of 2. In fetal testes, the level of hydroxy-progesterone was below LOD in controls but increased dramatically with azole-exposure. Values below LOD in fetal testes were set as zero.

The mass spectrometer was an EVOQ Elite Triple Quadropole Instrument from Bruker (Bremen, Germany) and the UPLC system was an Ultimate 3000 system with a DGP-3600RS dual-gradient pump. Data handling was performed in MS Workstation v. 8.2.1 software.

2.7. Histopathology

One Bouin's fixed, paraffin embedded testis from each litter in all dose groups (n = 7–8, 23 testes in total), was randomly selected for histopathological examination. Two sections per testis were evaluated (dose group blinded to observer) by light microscopy after Meyer's hematoxylin and eosin (H&E) staining. Testes were assessed for the presence of Leydig cell clusters, edemas, multinucleated gonocytes, and testis cord dysmorphology.

2.8. PBK model

A generic physiologically-based pharmacokinetic (PBK) model was used to simulate age-dependent physiological and biochemical changes in rodents associated with ongoing pregnancy after repeated daily oral dosing (gavage) of clotrimazole. This basic flow-limited model was previously developed as part of a quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) approach for predicting *in vivo* anti-androgenicity in male rats arising from gestational exposures (Scholze et al., 2020). In this model, the focus is on the simulation of internal exposure concentrations in the blood plasma of the fetus at GD15–18, which corresponds to the developmental stage considered most critical for male sexual differentiation in rats (Welsh et al., 2008). The PBK model includes the most relevant pharmacokinetic/dynamic compartments such as blood/plasma, gut, liver, kidney, fat, placenta, fetus, and two remaining maternal compartments, which include all other well- or poorly-perfused organs and tissues lumped together. All clotrimazole-specific kinetic parameters were determined from public sources: i) the overall metabolic clearance rate was scaled from an *in vitro* intrinsic hepatic

clearance of 77.1 μL/min/million hepatocytes (U.S. Environmental Protection Agency, 2020; Williams et al., 2017) to 18.5 L/h for the simulation; ii) the unbound fraction in blood plasma was set to 0.01 (Pearce et al., 2017); iii) the enterohepatic circulation from liver back to intestine was neglected by setting the biliary excretion rate to zero; iv) renal elimination was considered negligible due to high metabolic elimination and thus the urinary excretion rate fixed to zero; v) the blood-to-serum partition ratios were set to 1; vi) the tissue:plasma partition coefficients were derived from tissue-composition-based equations according to (Poulin and Theil, 2002). Quantitative details about the absorption from the gastrointestinal (GI) tract into the liver in rodents could not be retrieved from public literature. Pharmacokinetic studies in dogs show that only 10% of the orally administered dose reached the systemic circulation, but most of the substance is detectable in the feces (Conte et al., 1992). By use of these data, the uncertainty of the dog-to-rat extrapolation was operationalized by assuming that 5–20% of the oral administered dose reached the internal system of the pregnant rat, with each value in the range considered as equally likely. In order to comply with the PBK model structure, the fecal excretion rate was fixed to zero and all intake doses were reduced by 80–95% in the PBK simulations.

The plasma exposure concentration-time curve after the daily gavage is expected to be of short life, with 98% mass of the total integrated area under this curve (AUC) located within the first 12 h after each gavage. A fetal plasma concentration was approximated as the average concentration over the time between GD15–18 and estimated as the area under the curve (AUC) divided by 36 h (*i.e.* the sum of all three 12-h periods after gavage).

2.9. RNA extraction, cDNA synthesis and RT-qPCR analysis

Total RNA was isolated using RNeasy Microkit (Qiagen, Hilden, Germany) and quantified on a Nanodrop-1000 Spectrophotometer. cDNA was synthesized from 500 ng RNA using the Omniscript RT kit (Qiagen, Hilden, Germany) according to manufacturer's description. RT-qPCR was performed in duplicates on a QuantStudio 7 Flex Real-Time PCR System (Applied biosystems, CA, US) in 20 μL reaction volumes containing TaqMan Fast Universal Master mix (Life Technologies, CA, US), 3 μL diluted (1:20) cDNA and gene specific TaqMan assays (Life Technologies, CA, US): *Ddx4* (Rn01489814_m1), *Sox9* (Rn01751069_mH), *Cyp11a1* (Rn00568733_m1) and *Bcl-2* (Rn99999125_m1). Data were analyzed with the comparative Ct method normalized with the geometric mean of two verified reference genes *Sdha* (Rn00590475_m1) and *Rps18* (Rn01428913_gH). The suitability of these reference genes for the tissue in question was previously verified (Svingen et al., 2015) and were monitored for cross-sample stability (Ct-values) in the present study.

2.10. Statistical analyses

Data from the *in vitro* AR assay, analysis of *in vivo* internal clotrimazole concentrations, analysis of hormone levels in fetal plasma and testis, maternal parameters and litter data, as well as RT-qPCR data were assessed for normal distribution and homogeneity of variance by residual statistics. Data that were not normally distributed were log-transformed and assessed again. Data that were normally distributed were analyzed using one-way ANOVA followed by Dunnett's *post hoc* test using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Non-normally distributed data were analyzed by Kruskal-Wallis followed by Dunn's multiple comparison test using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

In vivo data on AGD, AGD index (AGDi; AGD/cube root of bw), and fetal body weight at GD21, were analyzed using one-way ANOVA followed by Dunnett's *post hoc* test using SAS® (SAS Enterprise Guide 8.2, SAS Institute, USA). Statistical analyses of AGD and AGDi were adjusted using litter as an independent, random and nested factor and analyzed using body weight as a covariate. Fetal body weights were analyzed using the number of offspring per litter as covariate. For these analyses, the litter was the statistical unit.

3. Results

3.1. Clotrimazole below cytotoxic levels does not affect AR activity in vitro

Clotrimazole did not agonize or antagonize AR activity at non-cytotoxic concentrations (Fig. 1). Clotrimazole compromised cell viability from 0.4 μM, thus treatment groups above 0.2 μM were omitted from statistical analysis.

3.2. Measured and modelled clotrimazole concentrations in fetal plasma

Measured and PBK-simulated plasma levels of clotrimazole in male fetuses after gestational exposure (GD7–21) are shown in Fig. 2. Simulations from the PBK-modelled relationship between fetal plasma concentrations of clotrimazole and doses administered to dams indicate that the fetal exposure levels at doses up to 100 mg/kg are expected to be well below 1 μM. If we assume that only a small amount of the dose reaches the systemic circulation (5% absorption), then the PBK model simulated fetal plasma levels around 10 nM, and at a higher absorption (20%) around 100 nM (illustrated in Fig. 2 by the red shaded areas reflecting least- and worst-case kinetic model assumptions for describing 5% and 20% absorption).

We could detect and quantify clotrimazole in all plasma samples from exposed GD21 male fetuses, with values ranging from 0.01 to 0.4 μM. The median clotrimazole concentration in the 25 and 75 mg/kg bw/day dose groups were 0.031 μM and 0.057 μM, respectively. Plasma concentrations varied greatly between fetuses (Fig. 2), with both the lowest (0.01 μM) and highest concentrations (0.40 μM) detected in the high-dose group. The measurements agreed relatively well with the PBK simulations, at least when a higher absorption was assumed in the kinetic model. It should be noted that samples were taken at a time where we expected the internal maternal levels to be around its peak of the concentration-time curve (1 h ± 15 min after gavage), meaning that inter-individual exposure measurements can vary significantly, even in the fetal compartment as observed at both doses. Furthermore, simulated concentrations are expressed as average within the first 12 h after gavage, and therefore well below the peak levels of the concentration-time curve.

Clotrimazole can inhibit both CYP17A1 and CYP19A1 *in vitro* within nanomolar concentration ranges (Munkboel et al., 2019). We used IC_{50} estimates for both enzymes (obtained from the H295R cell assay) and compared them with the fetal levels of clotrimazole measured at GD21. Both the simulated and the measured fetal compartment concentrations in the 25 mg/kg bw/day and 75 mg/kg bw/day exposure groups exceeded the concentration associated with *in vitro* CYP17A1 inhibition (lower horizontal line depicting *in vitro* IC_{50} value in Fig. 2). Additionally, for a few animals the measured fetal concentrations exceeded the concentration associated with CYP19A1 inhibition (upper horizontal line in Fig. 2).

3.3. High orally-administered doses of clotrimazole induce moderate maternal toxicity

We observed no external signs of maternal toxicity during the animal study, which was terminated at GD21. The body weights of pregnant dams in the high dose group were 11% lower than the control group at termination. The dams displayed a significantly lower bw gain from GD7–21 and the adjusted maternal bw (bw after subtraction of uterus weight) was significantly lower in both dose groups relative to controls (Table 1). Dam liver weights, and liver weights relative to bw, were significantly increased in the high dose group (Table 1) as compared to

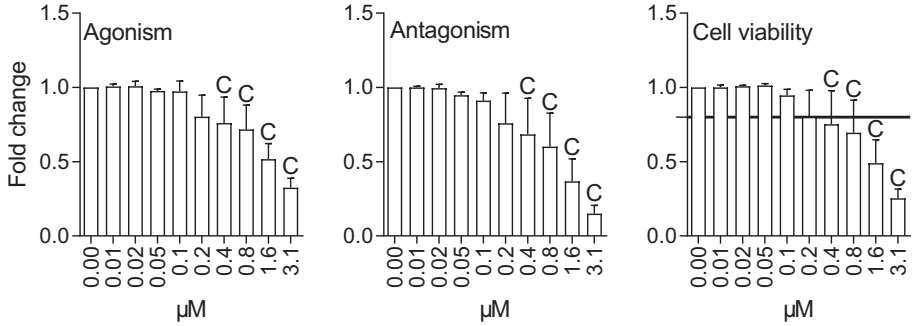

Fig. 1. Clotrimazole is not an *in vitro* AR antagonist. Clotrimazole was tested in the AR-Eco reporter gene assay for both agonistic and antagonistic activity, as well as cell viability. Bars represent pooled means from three independent experiments ($N = 3$; mean ± SEM) normalized to the vehicle control, set to 1. Treatment concentrations that compromised cell viability (C) with a threshold of 20% (horizontal line) were omitted from the datasets before statistical analysis.

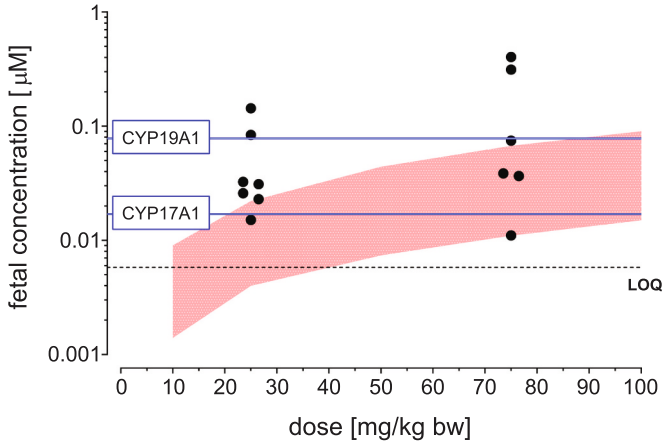

Fig. 2. Measured (GD21) and PBK simulated (GD15-GD18) plasma concentrations of clotrimazole in male fetuses after gestational exposure (GD7–21). PBK simulated fetal concentrations are shown as a red shaded area reflecting least- and worst-case kinetic model assumptions for describing 5% and 20% absorption (see Materials and Methods). Symbols (●) show the measured fetal plasma levels for each litter taken from male fetuses 1 h ± 15 min. After gavage (pooled from 4 to 11 fetuses per litter) with mean levels at 0.051 ± 0.018 μM after 25 mg/kg bw/day and 0.150 ± 0.069 μM after 75 mg/kg bw/day. Horizontal solid lines show the concentrations associated with strong *in vitro* CYP19A1 and CYP17A1 activities (IC_{50}), and the dashed line indicates the analytical limit of quantification (LOQ) for the plasma concentration measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controls. These livers displayed pale and speckled patches consistent with an increased induction of hepatic enzymes and hepatotoxicity in the dams from the high dose group. Effects on litter sizes and number of resorptions were not statistically significant in any exposure group. One occurrence of a very late resorption (around GD19), as well as one litter with a very high number of early resorptions (79% post-implantation loss), were observed in the highest exposure group. We observed no obvious signs of toxicological effects in the live fetuses, with both males and females having similar size and weight between dose groups (Table 1). AGD was not significantly different in clotrimazole-exposed fetuses compared to controls (Table 1).

3.4. Clotrimazole exposure decreases maternal blood concentrations of progesterone and estrone

In dam's plasma, progesterone and estrone levels were lower at GD21 in the 75 mg/kg bw/day clotrimazole exposure group relative to controls. Estrone levels were also significantly lower in the 25 mg/kg bw exposure group (Fig. 3) when compared to control group. No statistically

Table 1
Pregnancy and litter data. Data represent group means ± SD, based on litter means.

	Control	Clotrimazole 25 mg/kg bw/day	Clotrimazole 75 mg/kg bw/day
GD21 Caesarean section			
No. of litters	N = 8	N = 8	N = 8
Maternal bw (g), GD7	257.3 ± 19.1	256.4 ± 13.3	253.8 ± 20.9
Maternal bw (g), GD21	385.0 ± 24.0	366.1 ± 22.8	**343.3 ± 35.9**
Maternal bw gain (g), GD7–21	127.7 ± 11.8	109.7 ± 13.2	**89.5 ± 27.4**
Adjusted maternal bw (g), GD21[a]	297.4 ± 23.3	**272.4 ± 11.3**	**264.4 ± 17.4**
Maternal liver weight, GD21	14.1 ± 1.3	14.7 ± 1.2	**18.8 ± 2.7**
Maternal relative liver weight, GD21	0.04 ± 0.002	0.04 ± 0.002	**0.06 ± 0.003**
No. of fetuses	14.1 ± 1.5	13.6 ± 2.6	11.9 ± 4.2
No. of resorptions	0.8 ± 1.0	0.8 ± 1.0	2.0 ± 3.7
% postimplantation loss	4.8 ± 6.6	5.3 ± 6.8	14.6 ± 26.7
Fetal bw male (g)	4.1 ± 0.3	4.4 ± 0.3	3.99 ± 0.3
Fetal bw female (g)	3.8 ± 0.3	4.2 ± 0.3	3.8 ± 0.4
Male AGD (mm)	3.89 ± 0.1	3.95 ± 0.2	3.91 ± 0.1
Male AGD index	2.44 ± 0.05	2.41 ± 0.07	2.50 ± 0.08
Female AGD (mm)	2.15 ± 0.1	2.19 ± 0.1	2.23 ± 0.2
Female AGD index	1.38 ± 0.04	1.36 ± 0.08	1.44 ± 0.14

Values in **bold** are statistically significantly different from Control.
AGD (mm) was analyzed with fetal weight as covariate.
AGD index = AGD divided by cube root of the bodyweight.
[a] Adjusted maternal bw (g) is bw at GD 21 after subtracting the uterus weight.

significant changes to dam androstenedione, corticosterone, testosterone, or estradiol were observed (Fig. 3).

3.5. Intrauterine clotrimazole exposure decreases fetal blood estrogen and progesterone levels

Estrone and progesterone levels were significantly lower in GD21 male and female fetuses exposed to 75 mg/kg bw/day clotrimazole relative to control fetuses. In the male fetuses, estradiol levels were also lower in the high dose group and decreased in a linear dose-dependent manner (Fig. 4) relative to controls. We observed no changes for androstenedione, corticosterone, or testosterone levels in fetal plasma (Fig. 4).

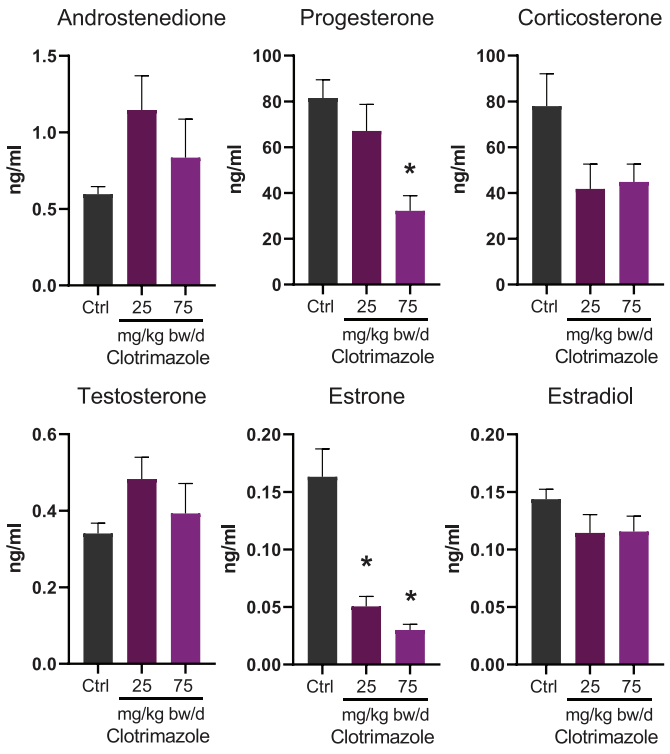

Fig. 3. Clotrimazole exposure affects steroid hormone concentrations in pregnant rats. Maternal blood plasma steroid hormone levels at gestational day (GD) 21 (ng/mL), following daily exposure from GD7. *$p < 0.05$. Dams were exposed (GD7–21) to vehicle (Ctrl), 25, or 75 mg/kg bw/day of clotrimazole. Results are shown as mean ± SEM ($n = 8$).

3.6. Intrauterine clotrimazole exposure increases intratesticular levels of hydroxy-progesterone

In utero exposure to 75 mg/kg bw/day of clotrimazole elevated fetal testis hydroxy-progesterone at GD21 (Fig. 5) when compared to control group. No significant changes to testis progesterone, androstenedione, corticosterone, testosterone, or epi-testosterone were observed (Fig. 5).

3.7. Intrauterine clotrimazole exposure at human-relevant concentrations do not adversely affect testis histology or marker gene expression

Histopathological examination of GD21 testes revealed no gross changes to testis histology after *in utero* clotrimazole exposure. We detected no obvious signs of edemas, testis cord dysmorphology, Leydig cell hyperplasia or multinucleated gonocytes (Fig. 6). In general, the exposed testes displayed same histology as control testes.

To further support the observation that testis integrity was maintained, we tested a small number of gene transcripts for potential dysregulation. We included the germ cell marker *Ddx4*, the Sertoli cell marker *Sox9*, the Leydig cell marker *Cyp11a1* and the apoptosis-related marker *Bcl-2*. Neither of the four genes were affected by clotrimazole exposure relative to controls (Fig. 7).

4. Discussion

A recent study reported that the widely used, non-prescription antifungal drug clotrimazole can inhibit steroidogenesis *in vitro* at doses relevant for human exposure (Munkboel et al., 2019). Furthermore, a human study indicated possible anti-androgenic effects in boys born to mothers using clotrimazole during pregnancy (Mogensen et al., 2017). Together, these two studies warrant concern about potential indiscriminate use of antifungal medicines containing clotrimazole during pregnancy. To start addressing this concern, we performed an intrauterine exposure study in rats to specifically look at potential effects on steroid hormone profiles and the anti-androgenic effect biomarker AGD. We show that clotrimazole can significantly alter the steroid hormone profile in rat fetuses exposed *in utero* to human-relevant doses, whereas AGD was not significantly affected in the same male fetuses.

Many azoles are potent disruptors of human steroidogenesis *in vitro* (Draskau et al., 2019; Karmaus et al., 2016; Kjærstad et al., 2010; Munkboel et al., 2019; Taxvig et al., 2013). Many can also inhibit androgen receptor (AR) activity *in vitro* (Draskau et al., 2019; Kjærstad et al., 2010). Since effects of clotrimazole have already been determined in the H295R steroidogenesis assay (Karmaus et al., 2016; Munkboel et al., 2019), we supplemented the *in vitro* profiling by testing for potential effects on the AR using the human AR-EcoScreen™ assay. As we observed neither agonistic nor antagonistic effects at concentrations below cytotoxic levels, it is likely that the main modality by which clotrimazole exert endocrine disrupting activities is as a steroidogenesis disruptor.

Based on a previous study showing potential, albeit non-significant, short male AGD in infant boys after intrauterine exposure (Mogensen et al., 2017), we specifically aimed to see if human-relevant exposure to clotrimazole could induce shorter male AGD in male rat fetuses. In our study, intrauterine exposure to clotrimazole did not induce short male AGD. This lack of response is in agreement with the observation that testosterone levels were unchanged and that clotrimazole does not inhibit AR activity, corresponding with what we know about effects of anti-androgenic chemicals on AGD (Schwartz et al., 2019).

Significantly decreased estrone concentrations were seen in plasma of both dams and fetuses. In male fetuses, this decrease was accompanied by a linear dose-dependent decrease in estradiol concentrations. These observations are in line with a potent inhibitory effect on aromatase (CYP19A1), the enzyme responsible for androgen to estrogen conversion. Even though our PBK model did not predict fetal clotrimazole concentrations reaching concentrations corresponding to the IC_{50} for CYP19A1 inhibition, we did observe fetal plasma concentrations exceeding this value. This mode of action – aromatase inhibition – is seen for many azole fungicides (Andersen et al., 2002; Trösken et al., 2006; Vinggaard et al., 2000; Zarn et al., 2003) and has also been observed previously for clotrimazole *in vitro* (Mason et al., 1985; Munkboel et al., 2019). Our study indicates that maternal exposure to doses of 75 mg/kg bw/day can induce fetal clotrimazole concentrations reaching and exceeding the IC_{50} for aromatase inhibition leading to significantly lower plasma estrogen concentrations in rats.

Concomitant with decreased estrogen concentrations, progesterone concentrations were also decreased in plasma of dams and fetuses in response to clotrimazole exposure. Both estrogen and progesterone are important regulators of pregnancy and parturition, and chemical-induced disruptions of estrogen and progesterone levels can induce post-implantation loss, including late resorptions in both rodents and primates (Albrecht et al., 2000; Stinchcombe et al., 2013; Taxvig et al., 2008, 2007). We also observed a few cases of post-implantation loss and a very late resorption in dams in the high dose group. Estrogen and progesterone disruption in women would also be of concern with clotrimazole use during pregnancy.

The observed changes to steroid hormone concentrations following exposure to clotrimazole is not only relevant for pregnancy outcomes. The balance between androgen and estrogen levels during fetal life is also important for reproductive development, at least for the external genitalia (Cripps et al., 2019; Govers et al., 2019; Hess et al., 2021; Mattiske and Pask, 2021; Sathyanarayana et al., 2012; Zheng et al., 2015). Limited by our study design, we could not assess postnatal male pups for gross genital abnormalities. General assessments of GD21 male fetuses, however, did not reveal any obvious genital malformations. Thus, although we may speculate that the hormonal changes induced by our administered doses of clotrimazole are not sufficient to induce gross malformations to the genitals, we cannot exclude the possibility that

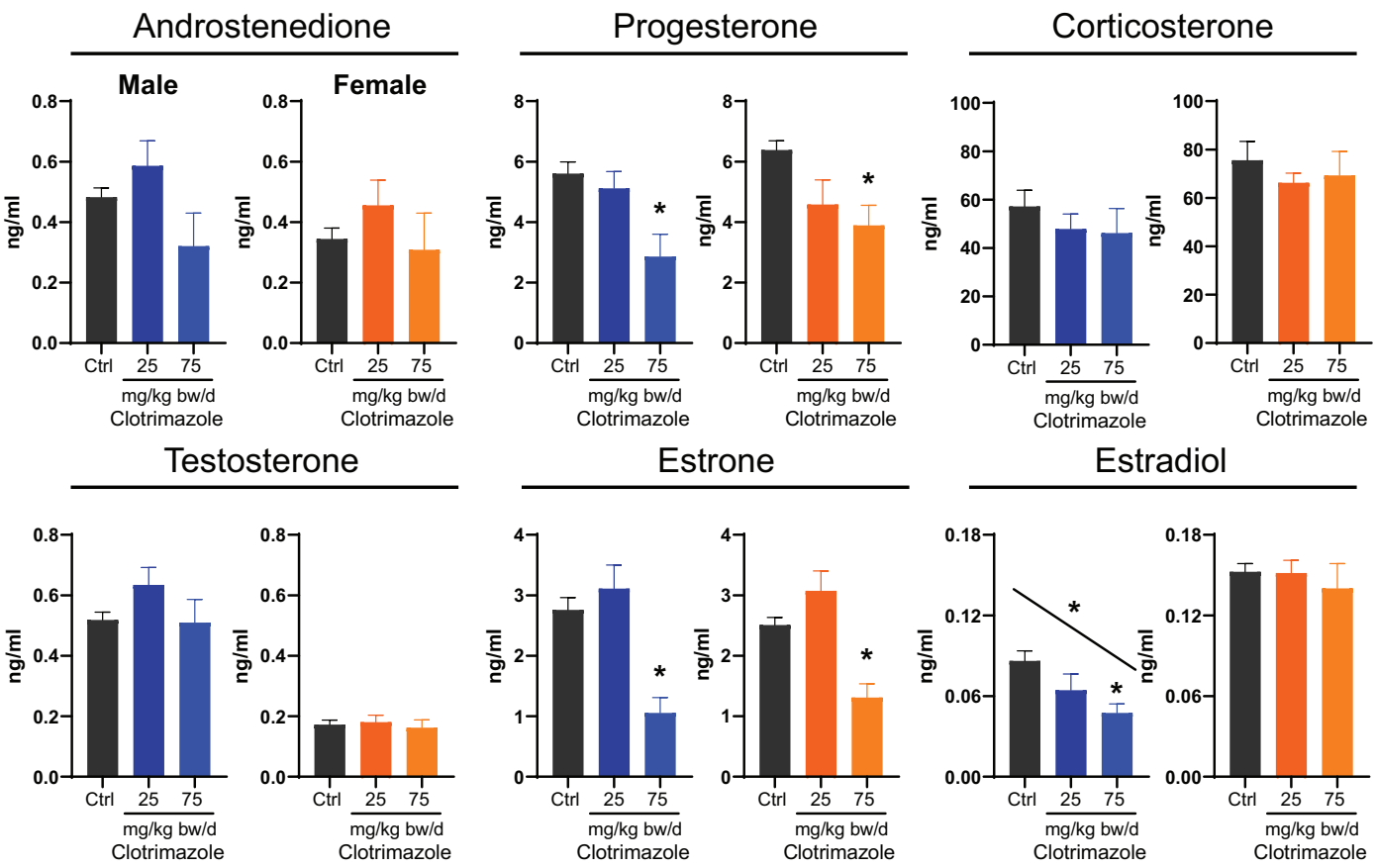

Fig. 4. Maternal clotrimazole exposure affects steroid hormone concentrations in male and female rat fetuses. Pregnant rats were exposed from gestational day (GD) 7–21, and steroid hormone levels measured in blood plasma (ng/mL) at GD21. Rats were exposed to vehicle (Ctrl), 25, or 75 mg/kg bw/day of clotrimazole. Results are shown as mean ± SEM of pooled samples from 6 to 8 litters per group (n = 6–8); *p < 0.05.

similar hormone disruption can contribute to milder effects in humans.

We also profiled intratesticular steroid hormones in the GD21 male fetuses. At this developmental stage, estrogen concentrations were too low to detect, which is consistent with the very low aromatase levels observed at this stage. This suggest that changes to estrone levels in male plasma is caused by disruption to adrenal steroidogenesis, a point that should be addressed in future studies. Notably, we also observed a large increase in hydroxy-progesterone, which is barely detectable in control testes. This effect is in contrast to what we observed in blood and could be the result of a systemic feedback mechanism – *e.g.* low maternal and fetal estrogen levels causing increased gonadotropin production. Regardless, hydroxy-progesterone is significantly affected in clotrimazole-exposed male fetuses, but any potential adverse consequences of this are difficult to surmise since there is limited background knowledge available. Prenatal exposure to progesterone can increase expression of genes important for Leydig and Sertoli cell function in sheep (Siemienowicz et al., 2020), affect sperm quality in rodents (Ahmed et al., 2020; Harini et al., 2009), and induce morphological and histological changes in rat testes (Ahmed et al., 2016) but we observed no testicular histopathological changes, nor changes to expression of selected marker genes at GD21.

In this study, we detected clotrimazole in fetal plasma at GD21. Since exposure route was *via* dams, clotrimazole can clearly cross the rat placental barrier and directly expose the fetus. In humans, plasma concentrations of clotrimazole following therapeutic dosing of 50–500 mg/day as vaginal tablets have been reported in the range of 0.03–0.09 μM (Kragie et al., 2002; Munkboel et al., 2019; Ritter et al., 1982). In our study, the mean male fetal plasma concentration in the high dose group (75 mg/kg bw/day) was 0.15 μM, which is only 1.7-fold higher than maximum plasma concentration measured in the human subjects of 0.09 μM (Ritter et al., 1982). Notably, the fetal rat plasma clotrimazole concentrations measured in our study were within the 0.03–0.09 μM range. Thus, we observed significant disruption to both maternal and fetal steroid hormone profiles at human therapeutically relevant concentrations, albeit these human ranges relates to women and not necessarily human fetal plasma levels. Indeed, even if assuming that clotrimazole levels in human fetuses would approach those observed in women, it has been established that clotrimazole is rapidly metabolized (Ritter et al., 1982).

5. Concluding remarks

Clotrimazole is a widely used, non-prescription azole fungicide used to treat various fungal infections, including vaginal mycosis. Since many azoles are known to be endocrine disruptors, there is some concern than indiscriminate use by pregnant women could disrupt fetal hormone signaling and cause adverse health effects in the offspring. There are some epidemiological data indicating potential anti-androgenic effects of clotrimazole, with a trend towards shorter AGD in male boys associated with intrauterine exposure. In our rat study, we did not observe a significant effect on AGD in male rat offspring at exposure concentrations observed in exposed humans. Another study has shown that clotrimazole can act as a strong steroidogenesis inhibitor *in vitro* at the same human-relevant concentrations. We also observed significant changes to steroid hormones in the rat fetuses at similar exposures, thus demonstrating that clotrimazole can disrupt sex hormone levels *in vivo* at these dose levels. Considering these human and animal data together, there is some concern about sex hormone disruption in fetuses of pregnant women self-medicating with clotrimazole. However, since systemic maternal clotrimazole is rapidly metabolized, it is unclear whether

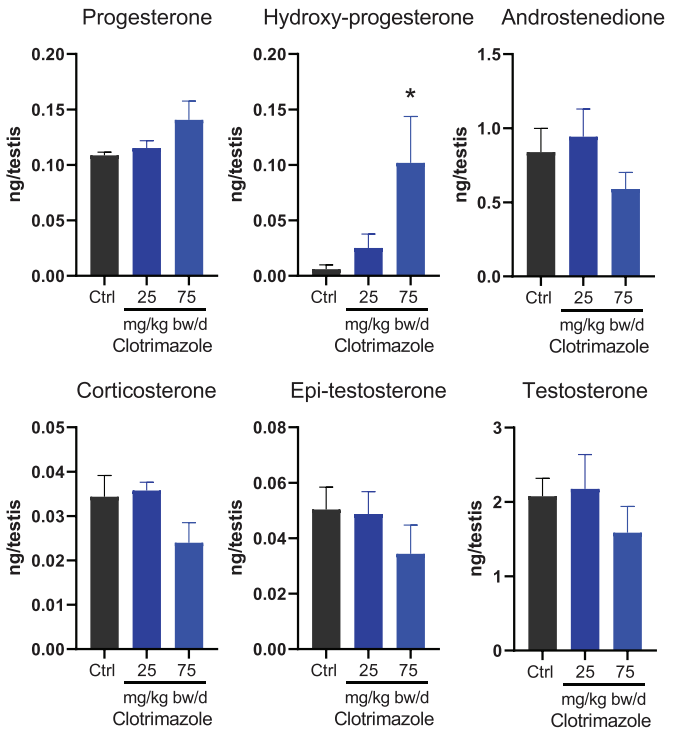

Fig. 5. Maternal clotrimazole exposure affects intratesticular steroid hormone concentrations in male fetuses (ng/testis). Fetuses were exposed *via* mothers from GD7–21 to vehicle (Ctrl), 25, or 75 mg/kg bw/day of clotrimazole. Results are shown as mean ± SEM (n = 7–8 testes per group, each from different litters). *p < 0.05.

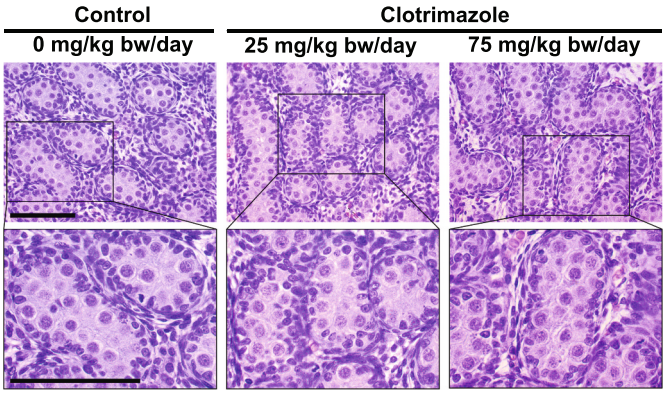

Fig. 6. Maternal exposure to clotrimazole does not adversely affect fetal testis histology. Fetuses were exposed *via* mothers from GD7–21 to vehicle (Control), 25, or 75 mg/kg bw/day of clotrimazole. GD21 testes were stained with H&E and analyzed by brightfield. Histopathological evaluation did not reveal any gross signs of histological aberrations compared to control testes. N = 6–8 testes per treatment group; scale bars = 50 μm.

human fetal concentrations are high enough to pose a risk of developing adverse reproductive defects. If applying a more cautionary viewpoint, the use of clotrimazole during pregnancy should probably be limited, as altered hormone levels could be problematic for both mother and fetus, not least if combined with exposure to other EDCs.

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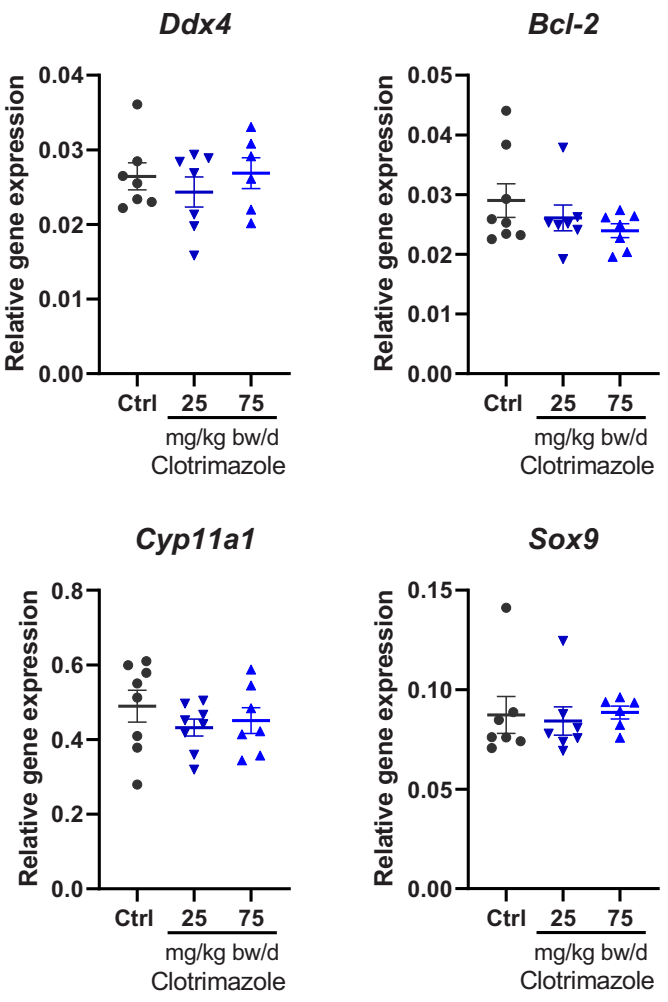

Fig. 7. Maternal exposure to clotrimazole does not affect expression of selected fetal testis marker genes. Fetuses were exposed *via* mothers from GD7–21 to vehicle (Ctrl), 25, or 75 mg/kg bw/day of clotrimazole. RT-qPCR analyses of the germ cell marker *Ddx4*, the Sertoli cell marker *Sox9*, the Leydig cell marker *Cyp11a1* and the apoptosis-related gene *Bcl-2* were unchanged in exposed GD21 testes relative to controls. N = 6–8 testes per treatment group.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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