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Primary endometrial 3D co-cultures: A comparison between human and rat endometrium

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ABSTRACT

Human and rat reproductive systems differ significantly with respect to hormonal cyclicity and endometrial cell behavior. However, species-differences in endometrial cell responses upon hormonal stimulation and exposure to potentially toxic compounds are poorly characterized. In this study, human and rat endometrial hormonal responses were assessed *in vitro* using a 3D co-culture model of primary human and rat endometrial cells. The models were exposed to the aryl hydrocarbon receptor (AHR) ligands 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), laquinimod, and its AHR active metabolite DELAQ.

In both the human and rat endometrial models, estrogen receptor and progesterone receptor gene expression was modulated by the hormonal treatments, comparable to the *in vivo* situation. AHR gene expression in the human endometrial model did not change when exposed to hormones. In contrast, AHR expression decreased 2-fold in the rat model when exposed to predominantly progesterone, which resulted in a 2.8-fold attenuation of gene expression induction of cytochrome P450 1A1 (CYP1A1) by TCDD. TCDD and DELAQ, but not laquinimod, concentration-dependently induced CYP1A1 gene expression in both human and rat endometrial models. Interestingly, the relative degree of DELAQ to induce CYP1A1 was higher than that of TCDD in the human model, while it was lower in the rat model.

These data clearly show species-differences in response to hormones and AHR ligands between human and rat endometrial cells *in vitro*, which might greatly affect the applicability of the rat as translational model for human endometrial effects. This warrants further development of human relevant, endometrium-specific test methods for risk assessment purposes.

1. Introduction

Uterine diseases and disorders like endometriosis, uterine tumors and infertility are conditions that seriously harm female reproductive health, with each disease affecting about 10% of women in their lifetime [1–3]. Despite the rising incidence of uterine diseases [4,5], the pathogenesis behind these diseases is still poorly understood but generally involve hormonal dysregulation in the reproductive organs. Increasing evidence shows that exposure to environmental contaminants may contribute to the development of reproductive diseases in various species, including humans [6–9]. For example, a role for dioxin-like substances has been suggested in the pathogenesis of endometriosis [10]. Dioxins and numerous other environmental contaminants have also been associated with reduced couple fecundity in epidemiological studies [11].

Exposure to dioxins, *e.g.* 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), can disrupt estrogen signaling *via* activation of the aryl hydrocarbon receptor (AHR) and consequently cause reproductive dysfunction [12]. AHR activation can result in the inhibition of estrogen receptor (ER) signaling by interfering with ER-DNA binding to specific estrogen responsive elements (EREs) that contain an overlapping dioxin response element sequence. This may lead to inhibition of selected estradiol (E2)-target genes, stimulating ER proteasomal degradation, increasing estrogen metabolism by AHR-inducible cytochrome P450 (CYP) enzymes and/or other as yet unidentified mechanisms [13–17]. Consequently, compounds that activate the AHR may have the ability to inhibit estrogen signaling pathways. Indeed, TCDD has been shown to inhibit ER-mediated chemical-activated luciferase reporter gene expression (ER-CALUX) in human breast cancer cell lines *in vitro* [17,18]. Although estrogenic regulation between breast and endometrial tissue

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sometimes differs, inhibition of E2 signaling *via* AHR activation has been associated with anti-cancer properties of TCDD as it can inhibit estrogen-induced proliferation in endometrial adenocarcinoma cell lines (ECC1 and HEC-1A) and in female breast cancer MCF-7 cells *in vitro* and in *in vivo* mice studies [19–22].

Studying the effects of chemical agents like TCDD on the female reproductive organs remains challenging because of the continuously changing hormone levels, and the interplay between various cell types in the endometrium throughout the menstrual cycle. The first phase of the menstrual cycle, the proliferative phase, is reflected by the thickening of uterine endometrium under the influence of E2. After ovulation, the endometrium is maintained under the influence of progesterone (P4) which is secreted by the corpus luteum. When fertilization does not occur, P4 levels drop in the secretory phase and the breakdown of the endometrial wall is promoted by an increase in *e.g.* matrix metalloproteases (MMPs) that degrade the extracellular matrix [23].

Currently, toxicological research predominantly uses *in vivo* rodent models to assess possible adverse effects of compounds on female reproductive health. Yet, the rodent estrous cycle differs significantly from the menstrual cycle, which is unique to humans and primates [24]. Not only do rodents not menstruate, the regulatory patterns and specific roles of certain hormones differ as well. For example, pituitary hormone prolactin stimulates the production of progesterone in rats, but not in humans [25]. In addition to these differences in the reproductive cycle, rodents differ also in sensitivity to certain classes of chemical compounds compared to humans. For example, rat liver cells show a higher sensitivity for cytochrome P450 1A1 (CYP1A1) induction than human liver cells in response to TCDD [26]. Thus, it is likely that present testing strategies in toxicological risk assessment do not adequately predict human endometrial effects. However, a clear comparison between responses of human and rat endometrial cells has never been made. Commercially available human (tumorigenic) endometrial cell lines like HEC-1A, KLE and Ishikawa are sometimes used to study endometrial effects *in vitro*. These studies are typically performed with mono-cultured cells that lack the interaction with surrounding extracellular matrix and stromal fibroblasts, which is crucial for epithelial cell behavior.

In this study, primary healthy human and rat endometrial cells were obtained and their response to hormones was investigated *in vitro*. For that, a three dimensional (3D) *in vitro* endometrial co-culture model was established using primary healthy human and rat endometrial epithelial and stromal cells, based on previously described models [27–30]. The endometrial cells were exposed to relevant hormonal concentrations to mimic the various stages of the reproductive cycle (E2 dominant and P4 dominant). Changes in gene expression of nuclear hormone receptors ERα, progesterone receptor (PR) and the AHR were determined, because of their important role in endocrine processes. Considering the ability of the pharmaceutical laquinimod to activate the AHR [31], the effects on AHR gene expression and activation of CYP1A1 by laquinimod, and its metabolite DELAQ, as well as TCDD were assessed in these human and rat *in vitro* endometrial models.

2. Material and methods

2.1. Human endometrial cell isolation

Human endometrial tissue was obtained from healthy premenopausal women undergoing hysterectomy for non-malignant conditions at the St. Antonius Hospital (Nieuwegein, the Netherlands). The study was approved by the hospital's Medical Ethical committee (Study number Z15.038) and written informed consent for the use of the endometrial tissue was obtained from all patients. None of the donors were using hormonal medication prior to or at time of surgery. Immediately after surgery, the endometrium was placed in HBSS (Thermo Fisher Scientific, the Netherlands) + 1% 100 U/ml penicillin/100 µg/ml streptomycin (p/s; Thermo Fisher Scientific) buffer and kept at 4 °C until further processing. The protocol for isolating and separating the endometrial cells was based on a combination of previous work of several laboratories [27–30]. Upon arrival in the lab, the endometrial tissue was incubated in 0.25% trypsin (Thermo Fisher Scientific) for 1 h at 37 °C in a shaking water bath. Then, the endometrial tissue was minced into smaller pieces and incubated in 0.05% DNAse type I (Sigma-Aldrich, the Netherlands), 0.1% collagenase type II (Sigma-Aldrich) and 0.1% trypsin in HBSS for 1 h and 15 min in a 37 °C shaking water bath. The suspension was flushed over a 40 µm nylon mesh cell strainer (Corning, the Netherlands) to allow stromal cells to pass through the filter, whereas it retained the epithelial cells. All cellular fractions were placed separately into culture flasks. After 1 h and 20 min, the supernatant of the cultures was transferred to a new culture flask to further separate stromal and epithelial cells by selective adherence. Human endometrial cell fractions were cultured in RMPI-1640 medium (Thermo Fisher Scientific) + 1% p/s + 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and maintained in an incubator at 37 °C in 5% CO_2.

2.2. Rat endometrial cell isolation

Animal experiments were carried out in accordance with the Dutch law for animal welfare, and protocols were approved by the Ethical Committee of Animal Research of Utrecht University (AVD108002015135) and the Institutional Animal Care Committee of Utrecht University (WP800-15-135-01-002). For the rat endometrial cell isolations, 15 female Sprague-Dawley rats aged 7 weeks (Envigo, the Netherlands) were euthanized by using O_2/CO_2 asphyxiation upon arrival in the animal facility. The uteri were dissected and placed in cold HBSS + 1% p/s buffer. Uterine horns were subsequently separated and slit longitudinally. The horns were incubated in 0.25% trypsin for 1 h in a shaking water bath at 37 °C, the supernatant was seeded in a culture flask and transferred to a new flask after 1 h. The uterine horns were subsequently incubated in HBSS containing 0.01% DNAse type I, 0.05% collagenase type II, and 0.08% trypsin. After 1 h and 15 min in a shaking water bath at 37 °C, supernatant was collected and the cells were flushed over 40 µm nylon mesh cell strainer to separate epithelial and stromal cells. Both fractions were collected and seeded in separate culture flasks. After 1 h, the supernatant was transferred to new culture flasks. Rat endometrial cells were cultured similarly to the human endometrial cells.

2.3. Experimental setup

Approximately 5 days after isolation, culture media of the endometrial cultures was replaced with culture media containing charcoal-stripped FBS (Hyclone, GE Health Care, the Netherlands). Two days later, cells were detached from the culture flasks with Accutase (Thermo Fisher Scientific). Stromal cells were plated at a density of 70.000 cells/well in 120 µl extra cellular matrix (ECM, Sigma-Aldrich) gel:medium (1:1) in a 24-well plate. After 30 min at 37 °C, 1 ml of 70.000 patient-paired epithelial cells/mL was added to each well on top of the ECM containing stromal cells. Both stromal and epithelial cells were seeded in medium that contained hormones to mimic the different phases of the reproductive cycle. For human cells this included a vehicle control (0.2% EtOH), an E2 dominant situation (100 nM E2 (Sigma-Aldrich) and 1 nM P4 (Sigma-Aldrich)), and a P4 dominant situation (1 nM E2 and 100 nM P4) [29,30]. For the rat cells, a vehicle control (0.2% EtOH) and a P4 dominant treatment (1 nM E2 and 100 nM P4) were used. No E2 dominant phase was included, as the rat estrous cycle lacks an apparent E2 dominant phase. After 3 days, half of the medium was removed and replaced with fresh medium with corresponding hormone and compound concentrations (Fig. 1). Experiments were performed in triplicate. After 2 days of exposure, culture media was collected and RNA Instapure (Eurogentec, Belgium) was added to the cells. Samples were stored at −80 °C for further analysis.

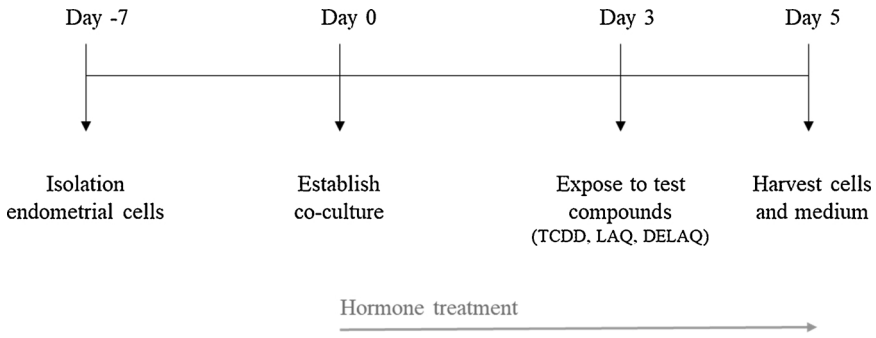

Fig. 1. Timeline of the experimental setup in this study.

2.4. Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR)

The RNA of the endometrial cultures was isolated using the phenol–chloroform extraction method and RNA Instapure to lysate the cells as described previously [32]. The purity and concentration of the isolated RNA were determined spectrophotometrically at an absorbance wavelength of 260 and 280 nm. Complementary DNA (cDNA) was synthesized using the iScript cDNA Synthesis Kit (BioRad, the Netherlands), according to the manufacturer's instructions. The obtained cDNA was diluted 10 times and stored at 4 °C until further analysis. Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) was performed using the CFX96 cycler (BioRad). A PCR mastermix was made containing IQ SYBR Green Supermix (BioRad), a forward and a reverse primer, after which cDNA of the sample was added. Oligonucleotide sequences and primer-specific annealing temperatures of the primers are listed in Table 1. Primers were obtained from literature and subsequently confirmed in the National Center for Biotechnology Information Primer-BLAST database. Primer efficiencies were between 90% and 110%. Gene expression was determined using the ΔΔCq method of relative quantification where gene expression in the cells was normalized to GAPDH (human) or acidic ribosomal phosphoprotein P0 (Arbp) (rat). None of the hormonal or test compound treatments affected gene expression of the reference genes and coefficients of gene expression variation was below 5%.

2.5. Aromatase activity

CYP19 enzyme (aromatase) activity was measured in the endometrial cells after exposure to the compounds using the tritiated water-release method, as described previously [33]. CYP19 activity was measured as the amount of tritiated water formed after the conversion of the CYP19 substrate, radiolabeled [1β-3 H]-androstenedione to estrone. Endometrial stromal and epithelial cells were exposed to laquinimod (0.1 μM–10 μM), DELAQ (0.1 nM–10 nM) and known inducers of CYP19 enzyme activity (100 nM phorbol 12-mystrate 13-acetate, 100 nM prostaglandin E2, 100 nM dexamethasone) or letrozole (3 μM) as control for inhibition of CYP19 enzymatic activity. After 2 days, medium was discarded and cells were incubated with 54 nM [1β-3 H]-androstenedione for 6 h. After chloroform extraction and dextran-charcoal treatment, radioactivity was determined in the samples in disintegration per minute (dpm).

2.6. AHR CALUX assay

The human hepatoma HG2L7.5C1 CALUX cell line was created by a

Table 1
Primer sequences and primer-specific annealing temperatures used in this study.

	Encoding gene		5' 3'	Annealing Temperature (°C)
Human	GAPDH	FP	GAAGGTGAAGGTCGGAGTCAAC	60
		RP	CAGAGTTAAAAGCAGCCCTGGT	
	CYP1A1	FP	CAGAAGATGGTCAAGGAGCA	60
		RP	GACATTGGCGTTCTCATCC	
	AHR	FP	ACATCACCTACGCCAGTCGC	60
		RP	TCTATGCCGCTTGGAAGGAT	
	ERα	FP	CCACCAACCAGTGCACCATT	60
		RP	GGTCTTTTCGTATCCCACCTTTC	
	PR	FP	CGCGCTCTACCCTGCACTC	60
		RP	TGAATCCGGCCTCAGGTAGTT	
	IL-6	FP	GGTACATCCTCGACGGCATCT	60
		RP	GTGCCTCTTTGCTGCTTTCAC	
	IL-8	FP	TATGCACTGACATCTAAGTTCTTTAGCA	60
		RP	CTCTTGGCAGCCTTCCTGATT	
	MMP-9	FP	GGCTCCTGGCACACGCCTTT	57
		RP	TGGAACCACGACGCCCTTGC	
		RP	GACATGCCTGCGCTCTCATACTTA	
Rat	Arbp	FP	CCTAGAGGGTGTCCGCAATGTG	63
		RP	CAGTGGGAAGGTGTAGTCAGTCTC	
	Cyp1a1	FP	ATGTCCAGCTCTCAGATGATAAGGTC	60
		RP	ATCCCTGCCAATCACTGTGTCTAAC	
	Ahr	FP	TGGCTGTGATGCCAAAGGGCAG	59
		RP	AGCATGTCAGCGGCGTGGAT	
	ERα	FP	GGCTGCGCAAGTGTTACGAA	60
		RP	CATTTCGGCCTTCCAAGTCAT	
	PR	FP	TGGTTCCGCCACTCATCA	60
		RP	TGGTCAGCAAAGAGCTGGAAG	

stable transfection of an AHR responsive firefly luciferase reporter gene plasmid pGudLuc7.5 under the control of dioxin-response elements [34], and was a kind gift of Prof. M. Denison (University of California, Davis, CA). The HG2L7.5C1 cells were cultured in MEMα medium (Invitrogen), supplemented with 10% FBS and 1% p/s. The cells were maintained at 37 °C in 5% CO_2 and sub-cultured twice a week. The cells were seeded in 96-wells luciferase plates at a density of 40.000 cells/well. Cells were exposed to ranges of the AHR ligands or 0.1% v/v DMSO as vehicle control. After 24 h, the cells were lysed and mixed with luciferin reagent (pH 7.8) and luminescence was measured as a proxy for AHR activation. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays were performed to identify cytotoxicity. The AHR CALUX assay was performed in three independent experiments in triplicate.

2.7. Data analysis

Human gene expression data were collected from patient-paired co-cultures that were established from five independent endometrial isolations (donors). Rat experiments were performed four times. Within each experiment, exposures were performed in triplicate. The normalized gene expression data was calculated relatively to the vehicle control treatments within each experiment. The differences of the relative means for each treatment were analyzed using a Student's t-test. Differences of the means were considered to be statistically significant at $P \leq 0.05$.

3. Results

3.1. Physiological relevance of human endometrial model

Co-cultures of endometrial epithelial and stromal cells were exposed to different hormonal conditions to mimic the phases of the menstrual cycle. The physiological relevance of the human endometrial model was assessed at gene expression level of several marker genes. Chemokines play an important role in the endometrium to facilitate embryo implantation. IL-6 and IL-8 are chemokines that are higher expressed in the secretory phase of the menstrual cycle, compared to the proliferative phase [35,36]. Indeed, in our human endometrial model gene expression of IL-6 was on average 1.5-fold higher in the P4-dominant phase, compared to the E2-dominant phase (Fig. 2). Gene expression of IL-8 was on average 1.9-fold higher in the P4-dominant phase, compared to the E2-dominant phase.

MMPs are involved in the degradation of the ECM surrounding the endometrial cells. Therefore, the expression of MMPs is highest at the onset of menstruation and low during the secretory phase of the menstrual cycle when P4 levels are highest [23]. Indeed, gene expression of MMP9 was 1.4 fold lower in the P4-dominant phase compared to the E2-dominant phase. Compared to the no hormone control phase, MMP9 expression decreased with an average of 1.4-fold in the E2-dominant phase, and an average of 2-fold in the P4 dominant phase.

Ki-67 is considered a proliferation marker and its expression increases when endometrial cells are in the proliferative phase of the menstrual cycle [37]. As endometrial cells are stimulated to grow under the influence of E2, it is expected that gene expression of Ki-67 will be highest in the endometrial cells that are in the E2-dominant phase. In our endometrial model, gene expression of Ki-67 increased on average 1.4-fold in the E2 dominant phase and on average 1.7-fold in the P4 dominant phase compared to the no hormone control.

Aromatase activity was assessed in three independent endometrial stromal cultures by the tritiated-water release assay. None of the tested cultures displayed aromatase activity (data not shown), even after treatment with known aromatase activity inducers prostaglandin E2 (100 nM), phorbol 12-myristate 13-acetate (100 nM) and dexamethasone (100 nM). Similar to the human endometrial stromal cells, no aromatase activity could be detected in rat endometrial stromal cells. Incubation with known inducers for aromatase activity did also not lead to detectable aromatase activity in the rat endometrial cells (data not shown).

3.2. Human vs rat endometrial responses to hormones

In the human endometrial model, gene expression of ERα increased 2.0-fold when the endometrial model was exposed to the E2-dominant phase, and increased 1.5-fold when exposed to the P4-dominant phase although the expression was not statistically significantly different from vehicle- treated control cells (Fig. 3A). While ERα expression tends to increase in human endometrial cells upon the exposure to hormones, the gene expression decreased statistically significantly by 2-fold in the rat endometrial cells in the P4 dominant phase (Fig. 3B).

The change in PR gene expression in response to the hormonal phases was similar in both the human and rat endometrial models. In the human model, gene expression of PR statistically significantly increased 2.1-fold in the E2-dominant phase, and increased 1.7-fold in the P4-dominant phase (Fig. 3A). In the rat endometrial model, PR gene expression statistically significantly increased 2.4-fold in the P4-dominant phase compared to the control (Fig. 3B).

AHR gene expression changes differed between human and rat endometrial models following exposure to the different hormonal phases. Gene expression of the AHR did not change when the human endometrial model was exposed to the different hormonal phases compared to the vehicle-treated control (Fig. 3A). In contrast, AHR gene expression statistically significantly decreased 2-fold when the rat model was exposed to the P4-dominant phase compared to the vehicle-treated control cells (Fig. 3B).

3.3. Effects of TCDD, laquinimod and DELAQ on human and rat endometrial cells

To gain insight into the relative potencies, the ability of TCDD,

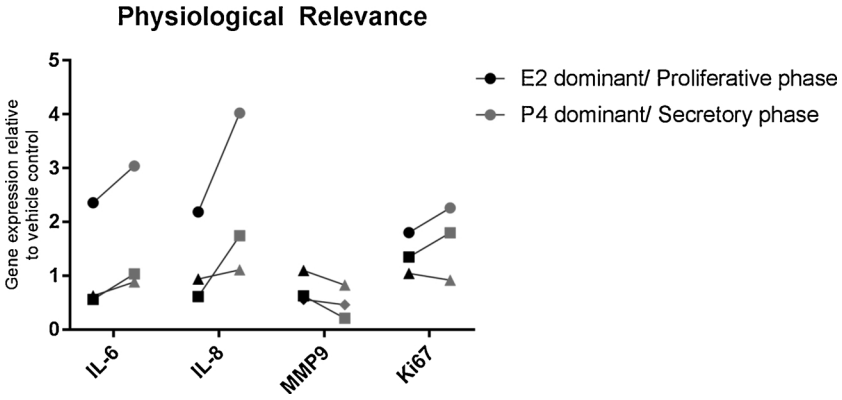

Fig. 2. Gene expression of IL-6, IL-8, MMP9 and Ki67 in human co-cultures. Gene expression changes in individual human endometrial co-cultures in E2-dominant phase (black) and P4-dominant phase (gray) compared with no hormone treated cultures (set to 1). Gene expression was normalized using GAPDH as a reference gene. Individual co-cultures (N = 3) are indicated with different symbols for every donor in each hormonal phase for all genes.

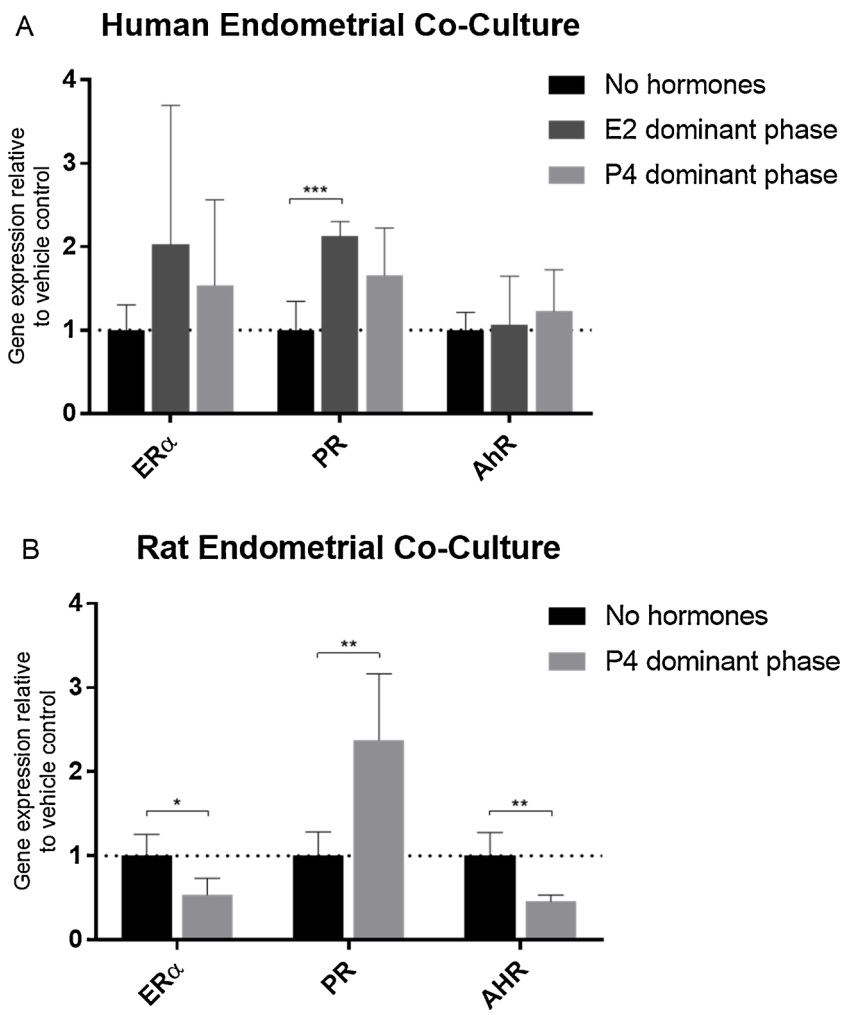

Fig. 3. Nuclear receptor gene expression in human and rat endometrial co-cultures. Average gene expression of ERα, PR and AHR in human (A) and rat (B) endometrial co-cultures after exposure to an E2 dominant phase (human) and P4 dominant phase (human and rat). Gene expression was normalized with GAPDH (human) or Arbp (rat) as reference gene and expressed relative to the no hormone phase (set to 1). Bars represent 5 (human) or 4 (rat) individual donors/independent experiments ± SD. Statistically significantly different from vehicle treated control with *P ≤ 0.05, **P ≤ 0.01 and *** P ≤ 0.001.

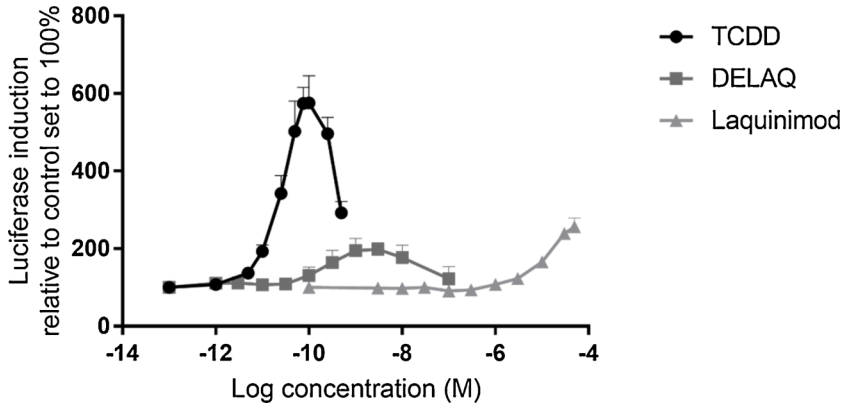

Fig. 4. TCDD, DELAQ and laquinimod activate the AHR in human HG2L7.5C1 cells. TCDD (1 pM-0.5 nM), DELAQ (1 pM-100 nM) and laquinimod (3 nM-0.1 mM) induce luciferase after 24 h of exposure to the human AHR CALUX HG2L7.5C1 cells. Data is expressed as mean ± SD.

laquinimod and DELAQ to activate the AHR was assessed in an AHR reporter gene assay, using luciferase signal as a proxy for AHR activation. TCDD was the most potent AHR ligand in the human AHR luciferase reporter gene assay, followed by DELAQ and laquinimod (Fig. 4). TCDD concentration-dependently induced the luciferase signal, with a statistically significant increase from 10 pM TCDD up. The concentration DELAQ needed to induce a similar response as 10 pM TCDD was approximately 100-fold higher, suggesting a relative potency of 0.01. Moreover, the efficacy of DELAQ to induce a luciferase signal was 2.9-fold lower than that of TCDD. Laquinimod only statistically significantly induced the luciferase signal at the concentration of 10 μM, which makes it ~10^6 less potent than TCDD in AHR activation.

Table 2
Human and rat TCDD-induced CYP1A1 gene expression relative to the no hormone situation. Average human and rat CYP1A1 gene expression induced by 1 nM TCDD relative to the no hormone situation per individual experiment N = 5 (human) and N = 4 (rat).

	HUMAN		RAT	
	AVR	SD	AVR	SD
No hormones	1	0.3	1	0.3
E2-dominant	1.01	0.4	NA	NA
P4-dominant	0.96	0.2	0.36	0.1

NA = Not Applicable.

Next, the effects of the AHR ligands on the primary human and rat endometrial models under the different hormonal conditions were assessed using CYP1A1 gene expression as a proxy for AHR activation. Upon exposure to hormones, the AHR was differentially expressed in the human and rat endometrial cells (Fig. 3). However, AHR gene expression did not change after exposure to TCDD, laquinimod, or DELAQ in the human and rat endometrial models (data not shown) in any of the hormonal conditions.

No statistically significant difference was observed between the hormonal conditions on TCDD-mediated induction of CYP1A1 gene expression in the human endometrial model (Table 2). Here, maximum induction of CYP1A1 gene expression by 1 nM TCDD was approximately 9.8-fold compared with vehicle-treated control cells (Table 3).

In the rat endometrial model however, the induction of CYP1A1 gene expression by 1 nM TCDD ranged between approximately 950-fold and 1500-fold with an average of 1191-fold compared to the vehicle-treated control cells under hormone-free conditions (Table 3). However when rat endometrial cells were exposed to TCDD under P4-dominant conditions, CYP1A1 gene expression was markedly attenuated and ranged between approximately 360-fold and 580-fold with an average of 429-fold compared to the vehicle-treated control cells. This statistically significant attenuation in CYP1A1 gene expression in the P4-dominant phase was on average 36 percent of that induced by TCDD with no hormones, *i.e.* an average of a 2.8-fold reduction (Table 2).

Laquinimod did not affect CYP1A1 gene expression in human or rat endometrial models up to 1 μM at any of the hormonal conditions in human (Fig. 5A) or up to 10 μM in rat (data not shown). In contrast, DELAQ statistically significantly induced CYP1A1 gene expression in a concentration-dependent manner in both human and rat endometrial models at concentrations higher than 10 nM (Fig. 5A).

DELAQ concentration-dependently induced CYP1A1 gene expression, which was already statistically significant at 0.01 nM in human endometrial cells under all hormonal conditions (Fig. 5A). The average induction of CYP1A1 gene expression was similar for 0.1 nM DELAQ and 1 nM TCDD (Table 3), suggesting a roughly estimated relative potency of 10 for DELAQ in the induction of CYP1A1 gene expression in the human model. In the rat model, DELAQ also statistically significantly induced CYP1A1 gene expression in a concentration-dependent manner starting at 0.01 nM under hormone-free conditions (Fig. 5B). However, this induction was ~3-times less potent compared to TCDD, suggesting a relative potency of 0.3 for DELAQ in induction of CYP1A1 (Table 3). Under P4-dominant conditions in rat endometrial cells, all DELAQ concentrations tested caused a statistically significant upregulation of CYP1A1 gene expression, but without clear concentration-dependency. The maximal induction of CYP1A1 gene expression by 1 nM TCDD was 122-fold lower in human than in the rat endometrial model. The maximal induction of CYP1A1 gene expression by 1 nM DELAQ was 51-fold lower in the human model compared to the rat model (Table 3).

4. Discussion

Clear differences were observed between primary human and rat endometrial 3D co-culture models in response to hormones alone and in combination with TCDD and DELAQ. AHR gene expression was not changed by hormones in the human model, while it was reduced in the rat model in the presence of hormones. This decrease in AHR gene expression coincided with an attenuated CYP1A1 induction by TCDD. Interestingly, DELAQ was a more potent AHR activator than TCDD in the human endometrial model, while DELAQ was less potent than TCDD in the rat endometrial model.

4.1. Physiological relevance of the endometrial model

A human 3D endometrial co-culture model was established by employing co-cultures of patient-paired primary epithelial and stromal endometrial cells with different hormonal conditions to mimic the proliferative (E2-dominant) and secretory (P4-dominant) phases of the human menstrual cycle. To assess the physiological relevance of the model, gene expression of several marker genes was determined. In the human model, higher gene expression of chemokines IL-6 and IL-8 was observed in the P4-dominant phase compared to the vehicle-treated control. This is in line with literature describing that gene expression of IL-6 and IL-8 is highest at the end of the secretory phase of the menstrual cycle [35,36]. In addition, Marbaix and coworkers [23] reported that P4 blocks the secretion and activation of MMPs in human endometrial explants, which corresponds to the findings in our study and is in line with the concept that P4 controls *in vivo* endometrial breakdown. Gene expression of Ki-67 was also induced by hormones in the endometrial model, but did not differ between the experimental hormonal phases. Finally, it is generally acknowledged that aromatase expression and activity in endometrium is restricted to women with endometriosis, leiomyomas and adenomyosis [38]. The lack of aromatase activity in the human endometrial cells further supports the

Table 3
CYP1A1 gene expression induction in human and rat endometrial co-cultures by TCDD and DELAQ. Data is expressed as average fold change (AVR) with standard deviation (SD) and biological replicates (N) relative to vehicle treated co-cultures.

Treatment		HUMAN			RAT					
		Hormonal conditions combined			No hormones			P4- dominant		
		AVR	*SD*	*N*	*AVR*	*SD*	*N*	*AVR*	*SD*	*N*
TCDD	0.1 nM	1.3	0.3	6	609	243	4	100	61	4
	1 nM	9.8	9.1	15	1191	212	4	429	103	4
DELAQ	0.01 nM	4.4	1.7	6	19	18	3	128	136	3
	0.1 nM	8.8	1.9	12	159	246	4	83	63	4
	1 nM	18.3	6.3	12	926	775	4	501	498	4
	10 nM	25.8	9.2	6	859	735	3	303	254	3

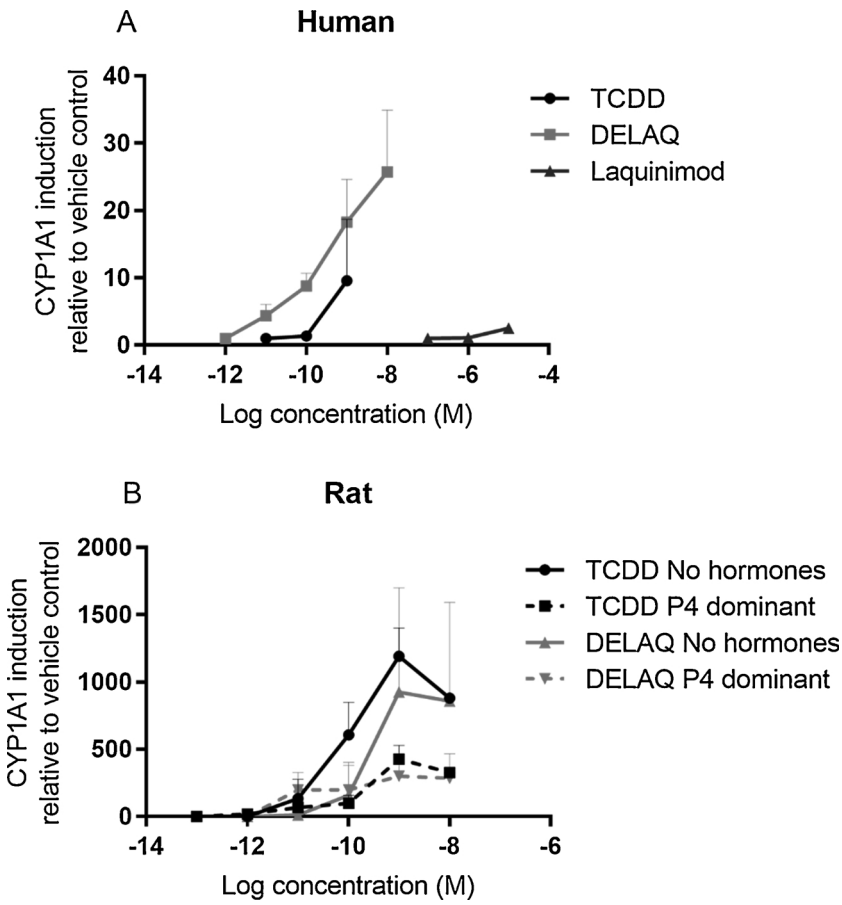

Fig. 5. CYP1A1 gene expression in human and rat endometrial co-cultures by TCDD, laquinimod and DELAQ. A: mean gene expression values and standard deviations for CYP1A1 gene expression relative to vehicle treated controls after exposure to 0.1–1 nM TCDD, 0.01–10 nM DELAQ, and 0.1–10 μM laquinimod in human endometrial co-cultures. Data for all the hormonal phases are pooled with a total N = 15 as hormones did not affect CYP1A1 expression. Gene expression was normalized with GAPDH as reference gene. B: mean gene expression values and standard deviations for CYP1A1 gene expression relative to vehicle treated controls after exposure to 1 pM-10 nM TCDD and 0.01–10 nM DELAQ in rat endometrial co-cultures. Data is expressed for every hormonal phase with total N = 4. Gene expression was normalized with Arbp as reference gene.

notion that our model is representative for the healthy human endometrium.

ER expression is a classical marker of E2-responsiveness in healthy endometrium. This expression is highest when circulating levels of E2 are high, *i.e.* during the proliferative phase of the menstrual cycle [39,40]. Indeed, increased ERα gene expression was observed in the human endometrial model in the E2-dominant phase. This is also in line with results from Prange-Kiel et al. who showed that E2 increased ER receptor expression in human endometrial cultures, while co-treatment with P4 reduced this expression [41]. The increased ERα expression in the P4-dominant phase in our study may therefore be explained by the relatively low concentration of E2 still present, and possibly a P4 concentration that was not high enough to inhibit the E2-induced ERα increase. In rats, it has also been reported that E2 induces ERα gene expression in the endometrium [42]. The rat model in our study was not exposed to an E2-dominant phase. In contrast to the human model, ERα expression was reduced in the rat model in the P4-dominant phase. The results from our study may indicate that P4 exerts a stronger inhibitory effect in the rat endometrial cells compared to the human cells. Similar findings have been reported by Medlock et al. who found that progesterone down-regulates the ER in rat uterine tissue even when *in vivo* estrogen levels are high [43].

Endometrial PR expression in humans and rats is predominantly regulated by E2, and E2-induced PR expression can be attenuated by P4 in both species [42,44]. In the human endometrial model, a higher PR induction was indeed observed in the E2-dominant phase. The PR induction in the P4-dominant phase in both models can be explained by the low concentration E2 present in that phase. It seems that the concentration P4 in our study counteracts the E2-induced PR expression. This suggests that in our model, PR expression is predominantly regulated by E2, while ERα expression is regulated by E2 but also *via* an inhibitory mechanism by P4 in rats, but not in humans.

A previous study described that AHR gene expression in human endometrial explants does not differ between the proliferative and secretory phases [45]. These findings are similar to our finding that AHR gene expression does not change in human endometrial cells when exposed to hormones. In contrast, reduced AHR gene expression levels were found in female Wistar rats that were treated with P4 *in vivo* [46], which is also in line with our *in vitro* findings.

Our results show a large human inter-individual variability in response to hormones, especially in the E2-dominant phase. It is possible that the phase of the menstrual cycle of the individual donors affected the observed quantitative responses, despite the wash out period that could be expected through *in vitro* culture of the cells. Unfortunately, no information was available regarding the donor's phase of the menstrual cycle at the time of surgery. However, the increase of both ERα and PR gene expression and the response to other physiological relevant marker genes in our endometrial model indicates an *in vivo*-like response for both human and rat endometrial cells. Combined with the lack of aromatase activity in the endometrial model, this indicates that the human and rat endometrial models may indeed be considered as relevant endometrial *in vitro* models.

4.2. Species differences in AHR response

In the human endometrial model, large inter-individual variation was observed in the induction of CYP1A1 gene expression by TCDD. This was in line with what was expected, as individual variation in sensitivity to AHR activation can be caused by *e.g.* genetic differences and/or lifestyle, such as smoking [32,47,48]. This variation may also be explained by different menstrual status of the donors. Nonetheless, similar trends with respect to the induction of CYP1A1 gene expression by TCDD and DELAQ were observed for every donor.

The induction of CYP1A1 gene expression by TCDD was similar to

that observed by others using endometrial explants or primary human immortalized endometrial cells [49,50]. However, in contrast to Willing and coworkers [49], we did not observe an attenuation of TCDD-mediated CYP1A1 gene induction by E2. This difference might be explained by our use of a co-culture with human epithelial and stromal cells *versus* immortalized human epithelial cells, or different hormonal exposure scenarios. In the rat endometrial model, an attenuated induction of CYP1A1 gene expression by TCDD was observed in the P4-dominant phase. Most likely, this was due to the observed decrease in AHR gene expression in the P4-dominant phase in these cells.

We observed that the relative potency of CYP1A1 induction by DELAQ was higher than TCDD in the human model, while it was lower than TCDD in rat. This may result from significant amino acid variations in the ligand binding domains of the AHRs from these species that contribute to significant differences in ligand binding specificity and species-differences in ligand response [51,52]. Additionally, in contrast to the human endometrial model, TCDD was more effective than DELAQ in the human liver CALUX cell line. Differential ligand binding by both compounds could lead to significant differences in the overall structure of each ligand:AHR complex that allow differential recruitment of co-activators and interaction with nuclear factors present in each cell type, leading to the observed differences in potency and efficacy of the induction response responsiveness [13]. Furthermore, a lower fold induction of CYP1A1 in human endometrial cells was observed compared to those of the rat. This observation is in agreement with other studies that have reported that rats are more sensitive to AHR activation of gene expression than humans, and this has been suggested to result from differential expression and recruitment of co-activators [53,54]. The species-difference in AHR activation and CYP1A1 induction may also be explained by the higher basal CYP1A1 expression in human endometrial cells. However, absolute AHR and CYP1A1 basal gene expression data in human and rat endometrial cells was not determined in this study and the interpretation of results should therefore be considered with caution.

Persistent activation of the AHR has long been believed to account for the adverse effects of dioxins. A variety of AHR agonists have however demonstrated to induce AHR-dependent genes without causing the toxicity that dioxins are known for [55]. It has been reported that AHR activation can lead to significant anti-estrogenicity *via* multiple mechanisms, including CYP1A1-mediated breakdown of E2 and downregulation of ER signaling *via* AHR-ER cross-talk and interaction with selected ER responsive elements on the DNA [9,13]. Our observation that DELAQ causes significant AHR activation in human endometrial cells may imply that this compound may exhibit anti-estrogenic properties in these cells, an effect that is considered to be associated with anti-carcinogenic properties.

5. Conclusion

The differences in regulation and activation of the AHR between human and rat endometrial cells can significantly impact the translation of toxicological findings from *in vivo* rat models to the human situation. This can have great consequences when performing risk assessment of chemicals, including pharmaceuticals, focused on female reproductive health. Our findings therefore underline the importance of using a human relevant *in vitro* model to determine potential effects of compounds on endometrial health.

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Declarations of interest

None.

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