Classical toxicity endpoints in female rats are insensitive to the human endocrine disruptors diethylstilbestrol and ketoconazole

Johansson, Hanna Katarina Lilith; Christiansen, Sofie; Draskau, Monica Kam; Svingen, Terje; Boberg, Julie

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1. Introduction

There has been a rise in reproductive disorders over the last few decades. This includes malformed genitals at birth, disrupted timing of puberty, increased incidences of reproductive cancers, as well as a growing number of people experiencing fertility problems as young adults [1–3]. The reasons for this increase in reproductive diseases are likely many and there is still a large gap in our knowledge as to exactly why they are on a rise. One likely contributing factor is increased exposure to environmental chemicals such as endocrine disrupting chemicals (EDCs). In particular, if exposure to EDCs occurs during early development, there is strong evidence to suggest that this may lead to reproductive disorders later in life, in men and women [4,5].

Based on our understanding of male and female reproductive development, we assume that males are more vulnerable to EDCs than females. This is because development of the male phenotype is dependent on high levels of circulating androgens, whereas development of the female phenotype appears less dependent on steroid hormones [6]. The real picture is likely more nuanced than this dichotomous classification, however, and there is increasing evidence to suggest that female reproductive development is more sensitive to EDCs than previously thought. An issue that perhaps has perpetuated the view that females are not as sensitive to endocrine disruption during development is the simple fact that we have not applied sufficiently sensitive tests in female reproductive toxicity studies.

Rodents remain the preferred model for reproductive toxicity testing for regulatory purposes [7]. This poses a challenge in that we still lack sensitive markers of endocrine disruptive effects in rodents, at least in females. In recent years, sensitive endocrine disruption effect endpoints for male reproductive development have been added to OECD test guidelines and include anogenital distance (AGD) and nipple retention (NR); both anti-androgenic effect measurements [8]. In females, on the other hand, we suspect that the endpoints that are included in current rodent test guidelines are not sensitive enough to reveal endocrine disrupting effects that could pose a risk to women’s reproductive health. This is not due to lack of observable effects in rodents per se, but rather that the used endpoints are not sensitive or specific enough.

Two classic EDCs that cause adverse reproductive outcomes in both
humans and rodents are diethylstilbestrol (DES) and ketoconazole (KTZ). In humans, prenatal exposure to DES increases the incidence of reproductive tract cancers, impairs fertility and causes early menopause in daughters of mothers who have taken DES during pregnancy [9–13]. Being a synthetic estrogen, it is likely that the harmful effects caused by fetal DES exposure are related to an estrogenic mode of action. KTZ, a pharmaceutical used to treat fungal infections, can perturb steroid hormone synthesis in humans and rodents by inhibiting various cytochrome P450 (CYP) enzymes of the steroidogenesis pathway [14–16] interfering with both androgen and estrogen synthesis. KTZ has also been used to reduce the rate of folliculogenesis in women during fertility treatment 17,18]. KTZ is thus a bona fide human EDC. However, the mechanisms by which either DES or KTZ cause these adverse reproductive effects are poorly understood.

To address the lack of sensitive markers of female reproductive toxicity, and to put female reproductive health concerns on par with that of male, we designed a rat reproductive toxicity study with DES and KTZ to answer some of the unknowns raised above. By using these well-known human-relevant EDCs, we surmised that we could investigate how well current OECD test guideline endpoints would detect effects on both the male and female reproductive system, with a particular focus on the sensitivity of female endpoints. In turn, results from these experiments will set the stage for follow-up studies designed specifically to identify better and more sensitive biomarkers for use in future test guidelines aimed at detecting female reproductive toxicants or the development of Adverse Outcome Pathways for regulatory use [19]. These are all goals of our current Horizon 2020 project FREIA [20].

2. Materials & methods

2.1. Chemicals

Diethylstilbestrol (DES), CAS no. 56-53-1, purity ≥ 99 %, was purchased from Sigma/Aldrich (cat.no. D4628). Ketoconazole (KTZ), CAS no. 65,277-42-1, purity 98 % was purchased from BOC Sciences Inc., USA. Corn oil was purchased from Sigma/Aldrich (cat.no. C8267 – 2.5L) and used as control and vehicle. Solutions used for dosing of animals were stored in glass bottles in the dark at room temperature and continuously stirred during the dosing period.

2.2. Animals and exposure

Two studies were performed: a range-finding study to establish the optimal dose range for DES and KTZ (n = 4 dams per group) and a main toxicity study using three doses for each compound based on toxicity finding in the range-finding study (n = 16 dams per group), as listed in Table 1. Doses used in the range finding study were based on literature studies and were deliberately high, as they were used to assess upper-limit for maternal toxicity by perinatal loss and delayed birth/ dystocia. In the main study, offspring were investigated at several postnatal ages. The animal strain, housing conditions and general study design were the same in both studies (Fig. 1). Time-mated nulliparous Sprague-Dawley rats (CD IGS Rat, Crl:CD(SD), Charles River Laboratories, Sandhofer Weg 7, Sulzfeld, Germany) were supplied on gestation day (GD) 3 to the animal facilities at DTU Food, Lyngby, Denmark. The day of vaginal plug was designated GD 1 and the expected day of delivery (GD 23) designated pup day (PD) 1. The animals were housed in pairs until GD 17 and hereafter singularly in High Temperature Polysulfone (PSU) cages with Tapvei wooden shelves. The cages were placed in Scandainers (Ventilated Cabinets from Scanbur) with controlled environmental conditions: 12 h light (21.00–9.00 h): 12 h dark (9.00–21.00 h) cycle, humidity 55 % ± 5, temperature 22 °C ± 1 °C and ventilation changing air 50–60 times per hour. Animals were fed Altromin 1314 (soy and alfalfa free) and tap water (BPA free bottles 84-ACB70702SU; Polysulfone 700 mL w/ring square) ad libitum.

On GD 4, animals were pseudo-randomly distributed into seven groups with similar body weight distributions. Exposure was administered by oral gavage with either chemical or vehicle once daily from GD 7 until birth and from the day after birth until PD 22. The vehicle volume was 2 mL/kg and the individual doses were based on body weight of the animal assessed daily. Dams were inspected for general toxicity and changes in clinical appearance twice daily. Body weights were recorded on GD 4 and daily during the dosing period to monitor changes in weight gain, to follow pregnancy status and to adjust dose according to weight. The animal experiments were approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation, license number 2015–15-0201–00553) and monitored by our in-house (DTU Food) Animal Welfare Committee.

2.3. General litter data, anogenital distance, nipple retention and puberty onset

Dams and pups were weighed on the day of birth. Offspring were counted, sexed, and any anomalies noted. Litter size was not adjusted (all pups were kept during lactation period). AGD was measured using an ocular stereomicroscope with a micrometer eyepiece. All offspring were weighed on PD 6, PD 14, and PD 22. On PD 14, offspring were examined for NR defined as a dark focal area with or without a nipple bud, located where nipples are normally present in the female. Female rats normally have 12–13 nipples whereas male rats normally have none. The same experienced technician, blinded to exposure, conducted all AGD measurements and NR examinations. After weaning (PD 22), pups were divided into subgroups and paired up two by two for housing depending on their use for further studies. Puberty onset was registered in all weaned male and female offspring. In female offspring, puberty onset was assessed by determining the day of vaginal opening (VO). Females were examined daily from PD 30. In male offspring, puberty onset was assessed as time of preputial separation (PPS) (observed by full retraction of prepuce). Males were examined daily from PD 38. On the day of VO or PPS the age and weight of the animals were recorded.

2.4. Reproductive organs, genital malformations and semen quality in male offspring

One male per litter was weighed, anesthetized with CO2/02 and decapitated at PD 16 and PD 132. The following organs were excised and weighed: liver, left and right testis, ventral prostate, epididymis, seminal vesicle, levator ani plus bulbocavernous muscles with penis (LABC + penis), bulbourethral gland, and adrenal gland.

At PD 17, an observer blinded to animal exposure status inspected...
on PD 22; vaginal opening (VO) assessed from PD 30 (until positive); preputial separation (PPS) assessed from PD 38 (until positive); estrous cycling registered from PD 69-89; female organ weights on PD 90; male organ weights on PD 132; sperm count on PD 132. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the external genitals. The changes were scored on a scale from 0 to 3 in order to investigate if male external genitals were demasculinized (as described in [21]).

At PD 132, alternately the left or right cauda epididymis, including 1 cm of ductus deferens, was frozen in liquid nitrogen and stored at −80 °C for later sperm count analysis. On the day of analysis, the cauda epididymis was thawed, weighed and prepared as described previously [22] using computer-assisted sperm analysis (CASA). A DNA-specific stain and fluorescence illumination was used to identify sperm cells. The average value of the three counts is presented as number of sperm per gram cauda.

2.5. Reproductive organs and estrous cycling in female offspring

One female per litter was weighed, anesthetized in CO₂/O₂ and decapitated at PD 90. The weight of liver, ovary and adrenal gland was registered.

Estrous cycling was assessed with vaginal smears for 21 consecutive days from PD 69–89 in 28–35 females per exposure group (3–5 females from the same litter). Vaginal smears were obtained by gently rotating a cotton swab saturated in 0.9 % saline solution in the vaginal entrance and smearing the cell sample on a glass slide. The samples air dried for approximately ten minutes and were hereafter fixed in 96 % ethanol for five minutes. The glass slides were kept at 4 °C until staining with Gill’s hematoxylin, Orange G6 and eosinazure 50 according to the adapted Papanicolaou (PAP stain) procedure [23]. The stained smears were mounted with eukit and cover glass, scored using light microscope, and classified as estrous, metestrous, diestrous or proestrous [24,25].

Regularity of estrous cycling was evaluated according to criteria based on Goldman et al. [24]: regular cycling: 3–5 days cycle length with either 1–2 days of estrus or/and 2–3 days of diestrus, irregular cycling: < 3 days cycle length or ≥ 6 days cycle length, or/and with 3–4 days of estrus or/and 4–5 days of diestrus, or prolonged cycling: > 4 consecutive days of estrus or/and > 6 days of diestrus. The person conducting scoring of estrous stage and evaluation of cycling regularity was blinded to animal exposure status.

2.6. Statistical analysis

P-values < 0.05 were considered statistically significant and litter was used as the statistical unit. When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor. Maternal parameters and litter data were assessed for normal distribution by residual statistics. Data not normally distributed were log-transformed and assessed again to confirm normality. Normally distributed data were analyzed using one-way ANOVA followed by Dunnett’s multiple comparison test using GraphPad Prism 8 (GraphPad Software, San Diego, CA, US, version 8.0). Non-normally distributed data (such as day of birth) was analyzed by Kruskal-Wallis followed by Dunn’s multiple comparison test using GraphPad Prism 8. Time-to-Event data endpoints (day of VO) were analyzed by Kaplan-Meier methods, with general dose-related pattern identified by a trend test and paired mean differences between controls and dose group by the nonparametric Wilcoxon test under the control of p-value adjustments according to Dunnett-Hsu [26].

Organ weights (liver, left and right testis, prostate, epididymis, seminal vesicle, LABC + penis, bulbourethral gland, ovary, and adrenal gland), body weights and birth weights, AGD and AGDindex (AGDI) were analyzed using one-way ANOVA followed by Dunnett’s post hoc test in SAS (SAS Institute Inc., Cary, NC, version 8.2). AGD and birth weights were analyzed using birth weight or the number of offspring per litter as a covariate.

The number of nipple/areolas (or NR) was assumed to follow a Binomial distribution with a response range between 0 and 12, with the latter assumed to reflect the biologically possible maximal number of nipples in rats. Litter effects on NR and over-dispersion in the data were accounted for by using Generalized Estimating Equations (GEE) as reported in [27]. The analyses were carried out using SAS® software (SAS Institute Inc., Cary, NC, version 8.2) and GraphPad Prism (GraphPad Software, San Diego, CA, US, version 8.0).

The quantal distribution of sperm counts was considered as sufficiently close to the continuous Gaussian distribution, and therefore this endpoint was analyzed by ANOVA.

Estrous cycle data were analyzed in Graphpad Prism version 8.3.1 using Fisher’s exact test. The two categories ‘irregular cycling’ and ‘prolonged cycling’ were pooled and compared to the category ‘regular’ cycling. Each exposure group was compared with control group.

3. Results

3.1. Pregnancy data and postnatal survival

3.1.1. Range finding study

We conducted a range-finding study to establish the doses where DES and KTZ induce maternal toxicity. Maternal body weight gain was significantly reduced to 43 % of control in DES-0.014 and 30 % of control in DES-0.021 from GD 7-GD 21, but from GD 7 – PD 1 no effect was seen (Table 2). Gestation length was prolonged (recorded as birth later than GD 23) in 25 % of the animals (one out of four) in the two highest doses of DES and the middle dose of KTZ. Importantly, two dams in the highest KTZ group (KTZ-21) were unable to give birth and had to be euthanized on GD 23 or GD 24 due to dystocia. In this group, one out of the remaining two animals had prolonged gestation (Table 2). The incidence of post-implantation loss was increased in DES-0.014 and DES-0.021 exposure groups, but the effect was not statistically significant. No effect was seen in KTZ-21, but may in part be explained by the two animals euthanized due to dystocia in this dose group. The number of live born pups was decreased and the perinatal pup loss was increased in the DES-0.021 and KTZ-14 and KTZ-21 groups when compared to controls (Fig. 2). No effect on birth weight or weight at PD 6, 10 or 22
Table 2
Pregnancy and litter data from range-finding study. Pregnancy and litter data for dams and offspring exposed to 0, 0.007, 0.014, 0.021 mg/kg bw/day DES or 7, 14, or 21 mg/kg bw/day KTZ from gestation day (GD) 7 to pup day (PD) 22. Data represent group means based on litter means ± SD. N = 2–4 litters (4 time-mated Sprague Dawley rats in each group). Statistically significant findings (p < 0.05) are highlighted in bold.

<table>
<thead>
<tr>
<th>Exposure group</th>
<th>No. of litters</th>
<th>Dam BW gain GD-21’ (g)</th>
<th>Dam BW gain GD 7–PD 1’ (g)</th>
<th>Gestation length (days)</th>
<th>% Postimplantation loss</th>
<th>Litter size</th>
<th>Birth weight males (g)</th>
<th>Birth weight females (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>119.7 ± 23.6</td>
<td>29.7 ± 10.8</td>
<td>23.0 ± 0.0</td>
<td>6.6 ± 5.9</td>
<td>14.8 ± 1.9</td>
<td>7.0 ± 0.3</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>DES-0.007</td>
<td>4</td>
<td>88.9 ± 9.8</td>
<td>6.6 ± 3.2</td>
<td>23.0 ± 0.0</td>
<td>5.0 ± 3.4</td>
<td>14.0 ± 0.8</td>
<td>7.0 ± 0.2</td>
<td>6.5 ± 0.2</td>
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<td>DES-0.014</td>
<td>4</td>
<td>79.9 ± 14.7</td>
<td>9.3 ± 16.9</td>
<td>23.1 ± 0.3</td>
<td>16.3 ± 12.5</td>
<td>11.8 ± 0.5</td>
<td>7.2 ± 0.4</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>DES-0.021</td>
<td>4</td>
<td>84.4 ± 17.2</td>
<td>7.9 ± 5.2</td>
<td>23.3 ± 0.5</td>
<td>17.4 ± 9.7</td>
<td>11.8 ± 1.7</td>
<td>7.1 ± 0.9</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>KTZ-7</td>
<td>4</td>
<td>121.1 ± 15.4</td>
<td>31.2 ± 8.3</td>
<td>23.0 ± 0.0</td>
<td>2.1 ± 4.2</td>
<td>14.3 ± 2.8</td>
<td>7.3 ± 0.4</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>KTZ-14</td>
<td>4</td>
<td>116.0 ± 19.0</td>
<td>21.8 ± 22.98</td>
<td>23.4 ± 0.75</td>
<td>5.1 ± 6.4</td>
<td>12.8 ± 4.2</td>
<td>7.1 ± 0.5</td>
<td>6.6 ± 0.4</td>
</tr>
<tr>
<td>KTZ-21</td>
<td>2(*)</td>
<td>110.7 ± 15.9</td>
<td>29.7 ± 13.8</td>
<td>23.5 ± 0.71</td>
<td>#</td>
<td>13.5 ± 0.7</td>
<td>7.4 ± 0.2</td>
<td>6.8 ± 0.1</td>
</tr>
</tbody>
</table>

Values in bold are statistically different from Control.

*Only pregnant dams included, # two dams in KTZ-21 group were euthanized due to dystocia (on gestation day 23 or 24).

Diethylstilbestrol (DES), ketoconazole (KTZ), bodyweight (BW).

Fig. 2. Implantation loss in range-finding study. In the range-finding study an increased implantation loss was seen in DES-0.014, DES-0.021, and KTZ-21, albeit not statistically significant. The lack of statistical significance in KTZ-21 may be explained by a low group size as to two animals were killed due to dystocia. Perinatal pup loss was increased in DES-0.021, KTZ-14 and KTZ-21 groups. (Diethylstilbestrol (DES), ketoconazole (KTZ)).

was observed in male or female offspring compared to controls (birth weight presented in Table 2, other weight data not shown). Based on these data we reduced the highest dose of DES to 0.012 mg/kg bw/day and KTZ to 12 mg/kg bw/day in the main study to avoid severe maternal toxicity.

3.1.2. Main study

Maternal body weight gain was reduced from GD 7-GD 21 in all three DES groups, but no effect was seen after KTZ exposure (Table 3). However, the body weight gain from GD 7 - PD 1 in dams was significantly decreased in all DES groups and in the highest dose of KTZ (KTZ-12) (Table 3). Gestation length and the incidence of post-implantation loss was not significantly affected in any of the dose groups, but one dam in the KTZ-12 group died at GD 24, probably due to dystocia (Table 3).

No significant effects were seen on birth weight or on body weights at PD 6, 14 or 22 in male and female offspring compared to controls, apart from a lower body weight at PD 6 in male DES-0.003 offspring (birth weight presented in Table 3, other weight data not shown).

3.2. Endocrine markers in developing offspring affected by DES and KTZ

AGD index was shorter in all males exposed to KTZ, whereas only the low dose of DES (DES-0.003) significantly reduced male AGD index (2%, Table 3 and Fig. 3A). Females exposed to the highest dose of DES (DES-0.012) displayed significantly longer AGD (mm and index) than controls (6%, Table 3). NR was increased in males from all dose groups of both DES and KTZ, and even though the effect was subtle, it was statistically significant and displayed an increasing trend with increasing doses (Table 3 and Fig. 3B).

3.3. Female puberty onset was delayed by DES and KTZ exposure

Although the average age at vaginal opening (VO) was not significantly different between exposed groups and control group, a time-curve analysis revealed that VO was delayed in all DES exposed groups as well as the KTZ-6 group (Table 3 and Fig. 4). No difference in the average body weight at VO was observed (Table 3).

3.4. No clear dose-related effects on male reproductive organ weights or sperm count

In prepubertal and adult male offspring, no effects on reproductive organ weights were seen except for a statistically significant reduction in LABC + penis weight at PD 132 in the highest KTZ group (KTZ-12; Table 4). No external genital malformations were observed at PD 17 in males. Neither DES nor KTZ affected age at PPS (Table 3). No effects were seen on sperm count (Table 4). However, one animal in each of three dose groups (control, DES-0.006 and KTZ-12 group) had very few sperm cells compared to all other animals in the study. This pattern, with a few animals showing much lower sperm count, has been seen in previous studies from our laboratory and has been reported irrespective of dose [28]. Exclusion of these outliers did not affect the results (data excluding outliers not shown).

3.5. No change in reproductive organ weights or estrous cycle irregularities in adult females

No effects on female reproductive organ weights (Table 5) or the incidence of irregular estrous cycles were seen at PD 90 (data not shown).
Table 3: Pregnancy and litter data from the main study. Pregnancy and litter data from dams and offspring exposed to 0, 0.003, 0.006, 0.012 mg/kg bw/day DES or 3, 6, or 12 mg/kg bw/day KTZ from gestation day (GD) 7 to pup day (PD) 22. Data represent group means based on litter means ± SD. *Indicates significant findings (p < 0.05) are highlighted in bold.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>No. of litters</th>
<th>Dams</th>
<th>Gestation length (days)</th>
<th>Litter size</th>
<th>% Postimplantation loss</th>
<th>Body weight at GD14 (g)</th>
<th>AGD at PD 1 (mm)</th>
<th>Age at VO (days)</th>
<th>Body weight at VO (g)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>122.4 ± 23.1</td>
<td>23.0 ± 0.0</td>
<td>5.9 ± 7.4</td>
<td>14.6 ± 0.5</td>
<td>28.0 ± 1.6</td>
<td>71.4 ± 0.5</td>
<td>7.4 ± 0.4</td>
<td>124 ± 10.8</td>
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<tr>
<td>DES</td>
<td>0.003</td>
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<td>23.0 ± 0.4</td>
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<td>7.3 ± 0.3</td>
<td>124 ± 10.8</td>
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<tr>
<td>DMS</td>
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<td>4.7 ± 6.9</td>
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<td>28.3 ± 0.9</td>
<td>72.9 ± 0.2</td>
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<td>124 ± 10.8</td>
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<tr>
<td>DES-97</td>
<td>0.012</td>
<td>97.1 ± 25.5</td>
<td>23.0 ± 0.0</td>
<td>5.9 ± 7.4</td>
<td>10.9 ± 1.4</td>
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<td>7.4 ± 0.4</td>
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</tr>
<tr>
<td>KTZ-3</td>
<td>16</td>
<td>122.3 ± 26.4</td>
<td>23.0 ± 0.0</td>
<td>5.9 ± 7.4</td>
<td>13.5 ± 0.3</td>
<td>28.3 ± 0.9</td>
<td>72.9 ± 0.2</td>
<td>7.3 ± 0.2</td>
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<td>KTZ-6</td>
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<tr>
<td>KTZ-12</td>
<td>12</td>
<td>108.4 ± 25.5</td>
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<td>5.9 ± 7.4</td>
<td>13.5 ± 0.3</td>
<td>28.3 ± 0.9</td>
<td>72.9 ± 0.2</td>
<td>7.3 ± 0.2</td>
<td>124 ± 10.8</td>
</tr>
</tbody>
</table>

Values in bold are statistically different from Control P < 0.05.

4. Discussion

Although we have sensitive markers for endocrine disruption pertaining to male reproductive development, current endpoints applied for female reproduction seem inadequate. They simply seem unable to systematically catch endocrine disrupting effects that are potentially harmful to humans. To start addressing this shortcoming, we have evaluated the sensitivity of a series of endocrine related endpoints used in OECD guidelines to test for reproductive toxicity in male and female rodents following exposure to well-known human endocrine disrupting chemicals.

4.1. Expected endocrine disrupting effects on pregnancy outcome and male offspring

Endocrine disrupting effects were evident for both DES and KTZ, with the range-finding study showing post-implantation loss, prolonged pregnancy and perinatal death, thus confirming previous studies [29–31]. Labor complications have also been reported for otherazole fungicides [32,33], and it is likely that dystocia after exposure to these azoles is caused by dysregulated temporal control of estrogen and progesterone levels during pregnancy [34,35].

We expected to see effects on the endocrine sensitive marker AGD, and possibly NR in male offspring for both DES and KTZ. Previous studies with DES [36] and KTZ [37], as well as several other azoles [32,33,37-41] have reported shorter male AGD after intrauterine exposure. Effects on NR is not as frequently reported, but seen in some studies with azoles [32,40-43]. To our knowledge, effects on NR in males is not reported with DES or other potent estrogenic compounds such as EE2 [44]. We did see effects on both endpoints, albeit relatively small changes compared to controls. No effects were seen on PPS and sperm count. This shows that, for male reproductive endpoints, the rat model can provide early and relatively sensitive measurements for endocrine disruption.

4.2. Subtle, or lack of, effects on endocrine sensitive endpoints in female offspring

In female offspring only a few, and rather subtle, effects were observed with the endpoints that were examined. Female AGD was slightly longer in offspring exposed to the highest dose of DES. A longer AGD in females is considered a masculinization effect caused by excess androgen action [6]. How compounds considered to have estrogenic properties induce longer female AGD remains unclear, but it has also been reported to occur in several studies with the synthetic estrogen ethinyl estradiol and phytoestrogens [44–46]. One possible explanation is that high concentrations of estrogens – as used in these studies – could potentially agonize the androgen receptor, as seen in vitro [47]. We also expected KTZ to affect the female AGD, since previous studies withazole fungicides have shown both longer [41,43] and shorter [37] female AGD after exposure. Contrary to expectations, however, we did not see any effects on AGD in the KTZ-exposed female offspring. When considering our results on female AGD after DES and KTZ exposure, together with several studies reporting on female AGD (reviewed by Schwartz et al. [6]) any effect pattern for female AGD in response to chemical exposure is inconsistent. AGD is clearly not a sensitive endpoint for endocrine disrupting effects in females when looking beyond strong masculinization effects in response to excess androgen action. The prospect of developing in vitro or in silico assays thus remains low as the mechanism behind the effects seen is unclear and, for that reason, unpredictable.

We also assessed the sensitivity of ‘timing of puberty onset’ in female offspring, defined as day of vaginal opening (VO). In humans, fetal DES exposure is associated with early menarche [48] and in rats the literature reporting on puberty onset after perinatal exposure to DES is mixed. One study reports no change in age at VO [49], a second study reports delayed VO at low dose exposure and early VO at high dose exposure.
[50], and a third study reports early VO despite low bodyweight [51]. Although some of the varied effects can be attributed to different study designs, not least timing of exposure, these disparate finding on the same endpoint highlight some of the challenges we are faced with when testing for endocrine disruption and potential effects on female reproduction. This is further complicated with the fact that downstream data interpretation can also yield different effect outcomes depending on, for instance, what statistical tests are applied. With respect to our data on day of VO, applying ANOVA do not reveal any effect on day of VO, whereas applying a so-called ‘time-to-event’ analysis reveals a slight delay in puberty onset in all groups exposed to DES, as well as the group exposed to the middle dose of KTZ. These results support the mixed effect pattern in the literature and again highlight the fact that endpoints used to measure effects in females in reproductive toxicity studies are not very sensitive. The individual variation is large and the measure of adversity is quantitatively close to the control group. This underlines the importance of improved understanding of factors influencing puberty onset as well as optimization and choice of statistical methods when evaluating “classical” toxicity endpoints such as puberty onset. Such improvements can, hopefully, increase the assay sensitivity enabling use of fewer animals than currently required.

Women exposed to DES during fetal life show compromised fertility [9] and advanced menopause [10,11]. In rats, irregular estrous cycling can indicate sub-optimal ovary function and, by proxy, point to fertility problems. Herein, we investigated estrous cycling in young adult offspring, but did not observe any adverse effect of DES or KTZ. This confirms a previous study with perinatal exposure to DES [51]; however, two other rat studies reported abnormal estrous cycling in young adults after perinatal exposure to DES [49,52]. In particular, the study by Ohmukai et al. [52] indicates earlier onset of age-related abnormal cycling after neonatal exposure to DES, which supports the relevance of examining regularity of estrous cycling several times during the animals life span to identify possible acceleration of reproductive senescence. However, rather than extending reproductive toxicity studies to evaluate late-life effects, we see a need for useful early-life markers for use in toxicity studies. One problem is that the rat estrous cycle may not be as sensitive to hormonal changes. Our own results, which we consider robust as we had a large group size for this endpoint, did not reveal effects on estrous cycling despite the fact that prenatal exposure to DES is known to affect human female fertility. This renders evaluation of estrous cycling regularity in young adult rats a non-sensitive measure of female reproductive toxicity and endocrine disruptive effects.

4.3. The necessity of sensitive endpoints for female reproductive effects

Female reproductive development is, to a large degree, considered sex hormone-independent during fetal development. However, we still see endocrine disrupting effects in females after fetal and developmental exposure during sensitive windows [4]. In this study, we have used two chemicals, DES and KTZ, to evaluate the sensitivity of commonly applied endpoints for female reproductive effects in rats, since both compounds are well-known endocrine disruptors in girls and women [9–13]. As discussed, the included female endpoints showed very subtle, or no, effects in response to exposure. Importantly, these endpoints are
Table 4
Male organ weights and sperm count in the main study. Organ weights in male offspring on pup day 16 and 132, sperm count in male offspring on pup day 132. Data represent means ± SD, statistically significant findings (p < 0.05) are highlighted in bold.

<table>
<thead>
<tr>
<th>Pup day 16</th>
<th>Exposure group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Liver (g)</th>
<th>Testis left (mg)</th>
<th>Testis right (mg)</th>
<th>Prostate (mg)</th>
<th>Epididymis (mg)</th>
<th>Seminal vesicle (mg)</th>
<th>LABC + penis (mg)</th>
<th>Bulbourethral gland (mg)</th>
<th>Adrenal (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>38.82 ± 5.01</td>
<td>57.00 ± 6.52</td>
<td>10.84 ± 2.55</td>
<td>22.53 ± 3.20</td>
<td>12.84 ± 3.23</td>
<td>82.45 ± 8.46</td>
<td>1.55 ± 0.52</td>
<td>8.77 ± 1.89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DES-0.003</td>
<td>12</td>
<td>35.68 ± 4.49</td>
<td>54.09 ± 6.09</td>
<td>10.00 ± 2.58</td>
<td>22.87 ± 4.09</td>
<td>12.45 ± 7.42</td>
<td>75.57 ± 1.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DES-0.006</td>
<td>12</td>
<td>39.50 ± 4.18</td>
<td>57.85 ± 5.45</td>
<td>10.48 ± 2.68</td>
<td>22.81 ± 3.80</td>
<td>12.75 ± 6.72</td>
<td>80.06 ± 1.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KTZ-3</td>
<td>12</td>
<td>39.76 ± 3.61</td>
<td>57.94 ± 5.45</td>
<td>10.48 ± 2.68</td>
<td>22.81 ± 3.80</td>
<td>12.75 ± 6.72</td>
<td>80.06 ± 1.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KTZ-6</td>
<td>12</td>
<td>36.34 ± 4.87</td>
<td>52.82 ± 4.87</td>
<td>10.48 ± 2.68</td>
<td>22.81 ± 3.80</td>
<td>12.75 ± 6.72</td>
<td>80.06 ± 1.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KTZ-12</td>
<td>12</td>
<td>36.34 ± 4.87</td>
<td>52.82 ± 4.87</td>
<td>10.48 ± 2.68</td>
<td>22.81 ± 3.80</td>
<td>12.75 ± 6.72</td>
<td>80.06 ± 1.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values in bold are statistically different from Control (p < 0.05).

Table 5
Female organ weights: Main study. Organ weights in female offspring on pup day 16. Data represent means ± SD.

<table>
<thead>
<tr>
<th>Exposure group</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Liver (g)</th>
<th>Ovary (mg)</th>
<th>Adrenal (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>271.8 ± 28.3</td>
<td>9.72 ± 1.49</td>
<td>75.3 ± 18.0</td>
<td>59.1 ± 10.0</td>
</tr>
<tr>
<td>DES-0.003</td>
<td>16</td>
<td>267.3 ± 26.8</td>
<td>9.27 ± 1.10</td>
<td>80.2 ± 16.7</td>
<td>62.2 ± 9.1</td>
</tr>
<tr>
<td>DES-0.006</td>
<td>16</td>
<td>269.6 ± 23.1</td>
<td>9.50 ± 1.65</td>
<td>81.2 ± 64.8</td>
<td>64.8 ± 8.1</td>
</tr>
<tr>
<td>DES-0.012</td>
<td>14</td>
<td>270.7 ± 20.7</td>
<td>9.32 ± 8.08</td>
<td>78.4 ± 14.0</td>
<td>59.8 ± 6.0</td>
</tr>
<tr>
<td>KTZ-3</td>
<td>14</td>
<td>277.7 ± 22.8</td>
<td>9.88 ± 1.02</td>
<td>82.2 ± 15.4</td>
<td>59.4 ± 7.0</td>
</tr>
<tr>
<td>KTZ-6</td>
<td>16</td>
<td>269.6 ± 21.7</td>
<td>9.40 ± 1.19</td>
<td>77.6 ± 15.7</td>
<td>58.8 ± 7.2</td>
</tr>
<tr>
<td>KTZ-12</td>
<td>16</td>
<td>258 ± 21.7</td>
<td>8.88 ± 0.70</td>
<td>72.6 ± 12.8</td>
<td>55.6 ± 9.2</td>
</tr>
</tbody>
</table>

Values in bold are statistically different from Control (p < 0.05).

Currently included in OECD test guidelines for reproductive toxicity testing [8] and the used rat strain (Sprague-Dawley) as well as exposure via gavage are commonly applied in regulatory toxicity studies. Several of the OECD test guidelines are not specifically designed to detect endocrine active substances (EASs) [8]. Even the most recently developed reproductive toxicity study; Extended One-Generation Reproductive Toxicity Study (OECD TG 443) needs further refinement with a number of additional endpoints before being sensitive to chemical interference with female reproductive health. In a workshop organized by the European Commission on “Setting priorities for further development of test methods and testing approach for endocrine disruptors” (Paris, 31 May – 2 June, 2017) [55], experts from academia and regulatory authorities agreed that there is a huge gap in regulatory processes. In addition, they mentioned that good assays and models are lacking that identify adverse outcomes for female reproductive health from endocrine disruptors. This raises concern about the ability of current OECD test guidelines to detect chemicals that may pose a risk to women’s reproductive health. Although current tests can detect some
degree of endocrine disruption, the applied tests may not be the most relevant for its purposes. Furthermore, since the endocrine disrupting effects in women for both DES and KTZ are well characterized, we surmised that both compounds could be used as positive controls in our study, but also for the results to be reproducible between studies. However, there is a challenge in finding good positive controls in the study of endocrine disruptors as has been discussed in relation to for instance ethinyl estradiol where different guideline studies showed different results [54]. This underlines the importance of having sensitive endpoints that are reproducible both within and between laboratories.

From a scientific standpoint, it would probably be better to target analyses towards effects on ovarian development, as regularly done in the open literature. Previous studies have shown that postpartum exposure to DES affects ovary development in both rats and mice by interfering with follicle assembly [55,56], as well as follicle development [57]. These endpoints thus seem more robust, in that they detect adverse effects on the female reproductive system induced by DES exposure. The challenge is, however, that assessment of follicle assembly and follicle development are not standardized, nor included in any OECD guidelines. Hence, there is a need for new sensitive and reproducible endpoints for investigating female reproductive health in general, and a need for biomarkers of altered ovary development in particular, as the ovary controls both reproductive and somatic health in women. Hopefully, the results presented in this study can serve as a baseline for further studies on biomarkers of ovary toxicity and pubertal timing and, by so doing, stimulate the development of more robust effect endpoints to be included in OECD test guidelines. One obvious avenue is to elaborate adverse outcome pathways (AOPs) and a test strategy in which improved in vivo biomarkers and newly developed in vitro tests can provide efficient toxicity testing for the future, as we recently proposed [19].

5. Conclusion

Using two well-known human endocrine disruptors, DES and KTZ, we have shown that in vivo reproductive toxicity studies with rats can detect expected endocrine disrupting effects in pregnant dams and male offspring, but not necessarily in female offspring. The lack of, or only subtle, effects in female offspring was not due to the test compounds not being female endocrine disruptors; rather, the endpoints used for female reproductive toxicity testing in current test guidelines are not sensitive enough. We thus conclude that new and improved test methods that are more sensitive are needed if we are to protect the reproductive health of girls and women from harmful chemicals.

Declaration of Competing Interest

The authors declare no conflict of interest.

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