**Original Article**

**Combined exposure to endocrine disrupting pesticides impairs parturition, causes pup mortality and affects sexual differentiation in rats**

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**Introduction**

Animal laboratory experiments have shown that in utero exposure to endocrine disrupting chemicals (EDCs) including some pesticides can cause adverse effects on male reproductive development (Foster, 2006; Gray et al., 2006; Hass et al., 2007; Metzdorff et al., 2007; Christiansen et al., 2008). Individual pesticides alone have so far not been shown to contribute to adverse human effects at relevant exposure levels. However, some studies indicate increased prevalence of cryptorchidism and decreased penile length in sons of women working as gardeners or living on farms where pesticides have been used (Kristensen et al., 1997; Weidner et al., 1998; Carbone et al., 2007; Andersen et al., 2008).

Many EDCs have been found as mixtures in humans (Blount et al., 2000; Swan et al., 2005), including children (Brock et al., 2002; Swan et al., 2005; Main et al., 2006). Damgaard et al. (2006) observed an association between congenital cryptorchidism and the levels of certain organochlorine pesticides in mothers’ milk (Damgaard et al., 2006). Earlier, Pierik et al. (2004) identified paternal exposures to pesticides and smoking as factors associated with these congenital malformations. These initial
observations in epidemiological studies points in the same direction as laboratory experiments with oestrogenic or anti-androgenic chemicals in which substantial mixture effects occurred even though each individual chemical was present at low, ineffective doses (Rajapakse et al., 2002; Silva et al., 2002; Hass et al., 2007; Metzdorf et al., 2007; Christiansen et al., 2008, 2009).

Some pesticides such as vinclozolin and procymidone antagonize competitively the androgen receptor (AR) binding of androgens and affect mainly the reproductive development in male offspring (Kelce et al., 1997; Ostby et al., 1999). Other pesticides such as mancozeb and propiconazole, indicates that these pesticides have the ability to react through several endocrine disrupting mechanisms, and to induce various endocrine disrupting effects (Vinggaard et al., 2005a; Vinggaard et al., 2005b; Taxvig et al., 2007). We have shown that prochloraz induced anti-androgenic effects in rats in vivo in a Hershberger test as well as in a developmental toxicity study (Vinggaard et al., 2002, 2005a). In addition, our studies show that prochloraz increases gestation length and indicate that prochloraz may also affect thyroid hormone levels and cause effects on the sexually dimorphic development of the brain (Vinggaard et al., 2002, 2005a). Both tebuconazole and epoxyconazole increase gestation length and pup mortality and furthermore, these pesticides virilise female pups, and affect steroid hormone levels in foetuses and/or dams (Taxvig et al., 2007).

In this article, we present data from two range-finding studies on the effects of a mixture of five endocrine disrupting pesticides. We selected procymidone, prochloraz, tebuconazole, epoxyconazole and mancozeb for our experiments. The choice of these pesticides was motivated by their common use as pesticides and their multiple mechanisms. The main aim of our range-finding studies was to assess whether there would be joint effects on pregnancy length and pup survival when every mixture component was present at doses that individually did not, in our earlier studies, produce observable effects on these endpoints. In addition, the aim was to obtain preliminary data on effects on the sexual development of the offspring to plan a large dose-response study.

Materials and methods

Animals and exposure

Two range finding studies, hereafter referred to as study 1 and study 2, were performed 2 months apart. In both studies, time-mated nulliparous, young adult Wistar rats (HanTac : WH, Taconic Europe, Ejby, Denmark) were supplied at gestation day 3 (GD3) of pregnancy.

The animals were housed in pairs until GD18 and alone thereafter under standard conditions in semi-transparent polycarbonate cages (15 × 27 × 43 cm) with Aspen bedding (Tapvei, Denmark) situated in an animal room with controlled environmental conditions (12 h light-dark cycles with light starting at 21:00 PM, light intensity 500 lux, temperature 21 ± 2 °C, humidity 50 ± 5%, ventilation 8 air changes per hour). A complete rodent diet for growing animals ALTROMIN 1314 (Soy- and alfalfa-free ALTROMIN GmbH, Lage, Germany) and acidified tap water (to prevent microbial growth) were provided ad libitum.

On the day after arrival (GD4), the dams were pseudorandomly distributed into groups of eight animals with similar body weight (bw) distributions. Mixtures were administered by gavage from GD7 to the day before expected birth (GD21) and from pup day (PD) 1 until PD13. However, as most of the exposed dams in study 1 were unable to give birth, only dams from study 2 were dosed from PD1 to PD13.

The substances used were corn oil (vehicle) (Sigma-Aldrich, Brøndby, Denmark), and procymidone, epoxyconazole, tebuconazole, mancozeb and prochloraz. All chemicals were purchased in a technical quality from VWR- Bie & Berntsen (Herlev, Denmark).

The composition of the pesticide mixture (Pmix) was chosen according to the doses of each individual pesticide that caused no major effects on pregnancy length and pup survival in our earlier studies (Vinggaard et al., 2005a; Taxvig et al., 2007). The animals were dosed with vehicle (control) or 25, 50, 75, 100 or 125% of Pmix (see Table 1). The doses used in study 1 were 75, 100 and 125%, whereas the doses studied in study 2 were 25 and 50% of Pmix. However, the dams dosed with 125% of Pmix exhibited signs of acute neurotoxicity after 2 days of dosing. Consequently, the dose was decreased to 100% of Pmix and the dams were included in the 100% group. The dams were inspected twice a day for general toxicity.

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Pmix-25%</th>
<th>Pmix-50%</th>
<th>Pmix-75%</th>
<th>Pmix-100%</th>
<th>Pmix-125%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epoxiconazole</td>
<td>3.75</td>
<td>7.50</td>
<td>11.25</td>
<td>15.00</td>
<td>18.75</td>
</tr>
<tr>
<td>Mancozeb</td>
<td>6.25</td>
<td>12.50</td>
<td>18.75</td>
<td>25.00</td>
<td>31.25</td>
</tr>
<tr>
<td>Prochloraz</td>
<td>8.75</td>
<td>17.50</td>
<td>26.25</td>
<td>35.00</td>
<td>43.75</td>
</tr>
<tr>
<td>Tebuconazole</td>
<td>12.50</td>
<td>25.00</td>
<td>37.50</td>
<td>50.00</td>
<td>62.50</td>
</tr>
<tr>
<td>Procymidone</td>
<td>12.50</td>
<td>25.00</td>
<td>37.50</td>
<td>50.00</td>
<td>62.50</td>
</tr>
<tr>
<td>Pestimix, total</td>
<td>43.75</td>
<td>87.50</td>
<td>131.25</td>
<td>175.00</td>
<td>218.75</td>
</tr>
</tbody>
</table>

The mixture ratio was based on doses causing no effects on gestation lengths for the individual pesticides, i.e. Pmix-100%.
including changes in clinical appearance (e.g. sedation and tremor). Body weights were recorded on GD4 and daily during the dosing period to monitor a decrease or increase in weight gain and the number of pregnant dams is presented in Table 2.

The animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee.

Delivery and post-natal development

In study 1, all control animals gave birth as expected on GD22–23, whereas most of the exposed dams exhibited severe problems with parturition. It was therefore decided to end the study on GD25 and perform caesarean section on animals that had not yet given birth. The dams that had already given birth and their pups were sacrificed the same day. The dams were weighed and decapitated after CO₂/O₂ anaesthesia, uteri were taken out, and the number of live or dead fetuses, resorptions and implantations were registered.

The weights of dams and individual pups were recorded after delivery both in the animals in study 1 which were able to give birth and in all the pregnant animals in study 2. The pups were counted, sexed and checked for anomalies. Pups found dead were macroscopically investigated for changes when possible. The expected day of delivery, GD23, was designated pup day (PD) 1 for the pups. Thereby, the age of the pups was not related to the time of conception, but was rather similar to post-natal age as the animals gave birth on GD22–24. Body weight of offspring in study 2 was recorded on PD6 and 13.

Anogenital distance and nipple retention

Anogenital distance (AGD) was measured in the offspring at birth (PD1) using a stereomicroscope. On PD13, all male and female pups were examined for the presence of areolas/nipples (NR), described as a dark focal area (with or without a nipple bud) located where nipples are normally present in female offspring. Female rats normally have 12–13 nipples.

Section PD13, organ weights and assessment of malformations in male external genitalia

The animals were weighed and decapitated after CO₂/O₂ anaesthesia. Testis, epididymis, ventral prostate, seminal vesicles, and liver were excised and weighed from one male per litter. From one female per litter, the uterus and ovary were excised and weighed. The external genitalia of all male offspring were inspected for genital dysgenesis and scored on a scale from 0 to 3, with the observer being blinded with respect to dose group. The scores were:

- Score 0 (no effect): Normal genital tubercle, with the urethral opening found at the tip of the genital tubercle and the preputial skin intact.
- Score 1 (mild dysgenesis of the external genitals): A small cavity on the inferior side of the genital tubercle or a minor cleft in the preputial opening was observed, estimated 0.5–1.4 on an arbitrary scale. The size of the genital tubercle was decreased.

### Table 2 Pregnancy and weight data

<table>
<thead>
<tr>
<th></th>
<th>Controla+b</th>
<th>Pmix-25%b</th>
<th>Pmix-50%b</th>
<th>Pmix-75%a</th>
<th>Pmix-100%a</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. pregnant dams</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Maternal bw gain GD7–21 (g)</td>
<td>83.4 ± 3.8</td>
<td>82.6 ± 3.4</td>
<td>74.4 ± 4.7</td>
<td>75.0 ± 10.0</td>
<td>53.7 ± 6.9**</td>
</tr>
<tr>
<td>Maternal bw gain GD7–PD1 (g)</td>
<td>9.3 ± 1.8</td>
<td>4.8 ± 3.5</td>
<td>4.9 ± 3.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gestational length (d)</td>
<td>23 ± 0.0</td>
<td>23.5 ± 0.1*</td>
<td>24.1 ± 0.2**</td>
<td>24.8 ± 0.1**</td>
<td>24.6 ± 0.2**</td>
</tr>
<tr>
<td>Pup prenatal mortality (%)</td>
<td>7.7 ± 3.6</td>
<td>15.7 ± 12.9</td>
<td>72.8 ± 10.7**</td>
<td>93.9 ± 2.8**</td>
<td>92.8 ± 4.4***</td>
</tr>
<tr>
<td>Birth weight, male pups (g)</td>
<td>6.3 ± 0.2</td>
<td>6.2 ± 0.3</td>
<td>6.0 ± 0.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Birth weight, female pups (g)</td>
<td>6.1 ± 0.1</td>
<td>6.0 ± 0.2</td>
<td>5.5 ± 0.03**</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Prostate weight (mg)</td>
<td>5.8 ± 0.4</td>
<td>3.6 ± 0.4*</td>
<td>2.1 ± 0.3*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Left testis weight (mg)</td>
<td>36.9 ± 1.6</td>
<td>33.3 ± 2.5</td>
<td>33.7 ± 1.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Right testis weight (mg)</td>
<td>36.0 ± 2.0</td>
<td>33.2 ± 3.0</td>
<td>33.0 ± 1.6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Epididymis weight (mg)</td>
<td>23.6 ± 0.5</td>
<td>17.4 ± 1.6*</td>
<td>14.9 ± 1.2*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Uterus weight (mg)</td>
<td>17.1 ± 2.4</td>
<td>14.0 ± 1.3</td>
<td>12.1 ± 2.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Liver weight – male pups (g)</td>
<td>0.64 ± 0.03</td>
<td>0.69 ± 0.09</td>
<td>0.85 ± 0.1*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Liver weight – female pups (g)</td>
<td>0.61 ± 0.02</td>
<td>0.7 ± 0.06</td>
<td>0.72 ± 0.01**</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Liver weight dams (g)</td>
<td>10.4 ± 0.3</td>
<td>12 ± 0.4</td>
<td>11.2 ± 0.2</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Data represent group means, based on litter mean ± SEM.
*p < 0.05, **p < 0.01 and ***p < 0.0001.
Birth weight was analysed using the number of offspring as a covariate; organ weights were analysed using body weight as a covariate.
–, no data because of caesarean section in the groups Pmix-75% and Pmix-100%.

aStudy number 1; bStudy number 2; a+b The control group is representing both study 1 and 2.
Score 2 (moderate dysgenesis of the external genitals): The preputial cleft was larger, estimated 1.5–2.4 on an arbitrary scale. The urethral opening was situated halfway down towards the base of the genital tubercle (hypospadias).

Score 3 (severe dysgenesis of the external genitals): The preputial cleft was large, estimated 2.5–3.5 on an arbitrary scale. The urethral opening was situated further than halfway down the inferior side of the genital tubercle to the base of the genital tubercle (hypospadias). At the base of the genital tubercle, a groove extending laterally was observed (similar to control females at PD13).

Hormone analysis

Progesterone were analysed in serum from 1–5 male and 1–3 female pups in 4–5 litters per dose group at PD13. Testosterone and estradiol were analysed in serum from 1 to 3 male or 1 to 3 female pups in 3–5 litters respectively. Serum from the pups in each litter were pooled by sex. Testosterone, estradiol and progesterone were extracted from the serum as previously described (Vinggaard, 2005b) and the hormones were measured by time-resolved fluorescence using commercially available fluoroimmuno-assay kits (PerkinElmer Life Sciences, Turku, Finland).

Statistics

For all analyses, the alpha level was set at 0.05 and the litter was the statistical unit. Data were examined for normal distribution and homogeneity of variance, and if relevant, transformed. In cases where normal distribution and homogeneity of variance could not be obtained by data transformation, a non-parametric Kruskall–Wallis test was used, followed by Wilcoxon’s test for pair wise comparisons. Data with normal distribution and homogeneity of variance were analysed using analysis of variance (ANOVA). When more than one pup from each litter was examined, statistical analyses were adjusted using litter as an independent, random and nested factor in ANOVA. Birth weights were analysed using the number of offspring per litter as covariate and organ weights were analysed using body weight as a covariate.

Anogenital distance data were analysed by the calculated AGD-index, namely, AGD divided by the cube root of body weight. The cube root was used because this converts a three-dimensional end point (weight) into a one-dimensional such as the AGD (Gallavan et al., 1999; Gray et al., 1999).

Analysing the level of demasculinization of male pups, the scores were categorized into a binary variable with scores 0 and 1 (no hypospadias) and scores 2 and 3 (mild and severe hypospadias). Statistical analyses of the effects on level of demasculinization were performed using Fisher’s exact test.

The number of nipple/areolas was assumed to follow a binomial-distribution with a response range between 0 and \( \theta_{max} \) with \( \theta_{max} \) being equal to the biologically possible maximal number of nipples in rats, either 12 or 13. The choice of \( \theta_{max} \) was decided on considering the global fit (information criterion of Schwarz). To account for litter effects on NR, correlation structures between number of nipple/areolas and litter were modelled by the Generalized Estimating Equations method as in Hass et al. (2007). All statistical analyses were performed using the SAS procedure PROC GENMOD (SAS Institute Inc, Cary, NC, USA).

Results

Pregnancy data and post-natal survival

There were no significant effects on maternal body weight gain from GD7–21 and GD7–PD1 in dams exposed to Pmix-75% or lower (Table 2). However, maternal body weight gain from GD7–21 in dams exposed to the highest dose of the mixture (Pmix-100%) was significantly decreased (Table 2).

Gestation length was significantly increased in all dosed groups (Table 2) and 5 of 7 dams in Pmix-75% and 9 of 14 dams in Pmix-100% were unable to give birth and had to be sacrificed on GD25 (Fig. 1a).

The number of liveborn pups significantly decreased and the perinatal pup loss was significantly increased at Pmix-50% and higher when compared with controls (Table 2, Fig. 1b). No effects on birth weight were observed in male pups compared with controls, whereas the female pups exposed to mix-50% had a significantly decreased birth weight (Table 2).

No data are shown on birth weight in pups exposed to mix-75% and mix-100%, as there were too few live pups to assess this end point properly.

AGD and NR

It was only possible to record AGD in a few litters in study 1 as most of the dams were unable to give birth (data not shown). In study 2, the mixture produced dose-dependent changes in AGD index with a significant increase seen in females and a decrease in males (Fig. 2a). Nipple retention was significantly and dose-dependently increased in male pups in both groups exposed to the mixture i.e. Pmix-25% and Pmix-50% (Fig. 2b).

Autopsy, organ weight and dysgenesis PD13

No effects were observed on weight of the testes or the uterus in male and female offspring respectively. Weights...
of prostate and epididymis in male pups were decreased in Pmix-25% and Pmix-50% exposed animals (Table 2). The liver weights of both male and female pups were elevated in the Pmix-50%-treated animals, but no effects were observed on liver weights of the dams (Table 2).

The incidence of hypospadias was increased with increasing dose (Fig. 2c). In the Pmix-25%, the males had either no, mild or moderate dysgenesis (score 0–2), whereas all of the males in the Pmix-50% group showed severe dysgenesis of the genitalia (score 3). No animals in the control group showed any malformations.

Hormone levels
No statistically significant effects of exposure of Pmix (25, 50%) on progesterone, testosterone or estradiol serum levels were revealed in dams, male or female pups

Discussion
The aim of these range-finding studies was to assess whether there would be joint effects on pregnancy length and pup survival when the five pesticides were present at doses that individually did not produce observable effects on these endpoints in our earlier studies. Moreover, an aim was also to find the dose that should be the highest
The observed anti-androgenic effects on the sexual differentiation of the male offspring seen as decreased AGD, nipple retention, decreased prostate and epididymis weight and hypospadias are likely because of the combined exposure to the threeazole fungicides and procymidone as similar effects to some extent have been seen for the individual pesticides in our earlier studies and by others (Ostby et al., 1999; Wolf et al., 1999; Noriega et al., 2005; Vinggaard et al., 2005a; Laier et al., 2006; Hass et al., 2007; Taxvig et al., 2007; Christiansen et al., 2009).

Prochloraz caused increased AGD at 50 mg/kg and nipple retention at 30 mg/kg in male offspring (Vinggaard et al., 2005a; Laier et al., 2006). Genital malformations were observed at 150 mg/kg prochloraz, but no effects on epididymis or prostate were found at the same dose (Laier et al., 2006; Christiansen et al., 2009). Prochloraz is also able to induce increased testicular progesterone concentrations in male rat foetuses (Vinggaard et al., 2005a; Laier et al., 2006; Blystone et al., 2007). Recent studies suggest a previously unidentified role for the progesterone receptor, possibly interacting with the androgen receptor, in disturbed genital tubercle development (Willingham et al., 2006).

Tebuconazole caused nipple retention at 50 and 100 mg/kg/day, whereas epoxyconazole did not induce observable nipple retention at 15 and 50 mg/kg/day (Taxvig et al., 2007). Decreased weight of prostate, but not epididymis, has been observed at 50 mg/kg epoxyconazole, whereas tebuconazole did not affect prostate or epididymis weights up to 100 mg/kg in studies performed in our laboratory (Taxvig et al., 2007).

Procymidone has in our laboratory induced decreased AGD and nipple retention at 25 mg/kg/day, but not at 10 mg/kg/day, while a decreased prostate weight was observed at 10 mg/kg (Hass et al., 2007; Metzdorff et al., 2007). No hypospadias was observed at 25 mg/kg in adult male rats or at 14.1 mg/kg in immature male rats (Metzdorff et al., 2007; Christiansen et al., 2008).

In a similarly designed study in our laboratory, Mancozeb has not shown effects on NR and AGD at doses below 100 mg/kg (Axelstad, Christiansen & Hass, unpublished data from our laboratory).

Thus, the individual doses (Table 1) of each of the threeazole fungicides and mancozeb in the pesticide mixture Pmix-25%-50% were clearly lower than those causing no effects on male sexual differentiation, whereas the dose of procymidone was close to this dose level (12.5 mg/kg).

The increased AGD observed in the female offspring is likely to be caused by the combined exposure to the threeazole fungicides as our earlier studies have documented similar effects of prochloraz, epoxyconazole and tebuconazole (Laier et al., 2006; Taxvig et al., 2007). This effect may be caused by increased progesterone levels in

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Table 3 Hormone levels in study 2

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Control</th>
<th>Pmix-25%</th>
<th>Pmix-50%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone levels,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>males (nM)</td>
<td>0.7 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>females (nM)</td>
<td>1.2 ± 0.5</td>
<td>0.9 ± 0.8</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Progesterone levels,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dams (nM)</td>
<td>129.5 ± 44.5</td>
<td>95.0 ± 25.4</td>
<td>82.5 ± 65.6</td>
</tr>
<tr>
<td>Testosterone levels,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>males (nM)</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>0.7 ± 0.4</td>
</tr>
<tr>
<td>Estradiol levels,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>females (nM)</td>
<td>0.03 ± 0.01</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

Data represent group mean, based on pooled serum ± SD, N = 3–5 litters in each group.
the dams (Willingham et al., 2006). The progesterone as well as testosterone and estradiol levels in the current study were not significantly changed in the dams or in the pups, but this may be as a result of the low number of samples taken in this range finding study with a limited number of litters per group.

Prostate and liver weights were reduced in the present study and it would be relevant to measure changes in gene expression in these organs. In the prostate, androgen-regulated genes such as ornithine decarboxylase and Prostate binding protein subunit C3 (PPB C3) are known to be altered by anti-androgenic compounds (Nellemann et al., 2005). Hepatic expression of growth hormone as well as drug metabolizing enzymes is sexually dimorphic and may be altered by xenobiotics (Waxman & Holloway, 2009). As mentioned above, detailed molecular and endocrine endpoints will be addressed in a later study with more litters per group.

In conclusion, the findings from these range-finding studies showed that combined exposure to the five pesticides induced marked adverse effects on parturition and pup survival at doses where the individual pesticides appear to induce no such effects. The significance of these findings for human risk assessment must be emphasized because they clearly indicate that risk assessment based on single endocrine disrupters alone underestimates the risk for adverse effects when exposure is to several pesticides with common effect outcomes regardless of mechanism.

The sexual differentiation of the offspring was also significantly affected. However, based on a range-finding study with a limited number of litters, only cautious conclusions can be drawn. Consequently, sexual differentiation of the offspring, as well as the predictability of the combination effects based on dose-additivity modelling, is currently studied more thoroughly in a large mixture dose-response study in our laboratory.

Acknowledgements

Lillian Sztuk, Heidi Letting, Birgitte Møller Plesning, Ulla El-Baroudy, Vibeke Kjær, Dorthe Hansen and Sarah Grundt Simonsen are thanked for their excellent technical assistance. This work is part of the research project ‘Developmental toxicity effects in experimental animals after mixed exposure to endocrine disrupting pesticides’ and financial support from the Danish Environmental Protection Agency’s Pesticide Research Programme is gratefully acknowledged.

References


Pesticide mixture: endocrine disruption


Panel discussion

Ana Soto (Boston, USA)

In your 5 chemical mixture experiment with antiandrogens on fetal rats you observed decreased anogenital distance (AGD) in males and increased AGD in females. What is the mechanism causing this effect in females?

Ulla Hass (Søborg, Denmark)

Not many such studies have been performed in females and the mechanisms are unknown. It is hypothesised that these chemicals in addition to their antiandrogenic effect also have very weak androgenic activity. They bind to the androgen receptor (AR) but have a very poor stimulatory effect. In males, the binding to AR competes with the much more strongly acting testosterone and therefore the receptor is blocked causing reduced AGD. In the females where there is no testosterone, the weak androgenic activity causes increased AGD. This is one possibility but more research is required and the role of progesterone must also be considered.

Fred vom Saal (Columbia, USA)

Literature from the 1970s described elongated AGD in female rodents developing in utero between two males. The testosterone in the females comes from the rodent placenta which, unlike the human placenta, has no aromatase activity and has high levels of C17-20 lyase and 17β-hydroxysteroidalase: the major sex steroids from the rodent placenta are androstenedione and testosterone, and not progesterone. Pregnant rodent females have serum levels of >1ng/ml testosterone, and 10ng/ml androstenedione. The AGD can be reduced in these masculinised rodent fetuses by antiandrogens such as flutamide or cyproterone acetate. Papers on this finding have been published by Clemens and myself.

Ulla Hass

Thank you for that valuable information which we shall now be assessing.

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Vasantha Padmanabhan (Ann Arbor, USA)
John Marshall has studied the interaction of progesterone and androgen in the human polycystic ovary syndrome. Progesterone is a major negative feedback regulator of LH. High androgen levels such as seen in PCOS can reduce sensitivity to progesterone. A similar scenario may occur in your studies. You are looking at several antiandrogens in unison. Have you plans to assess the response to antiandrogenic with androgenic, or antiandrogenic with oestrogenic EDCs in combination as occurs in real life situations?

Ulla Hass
We are about to test the Contamed mix, which is a mixture of antiandrogen and oestrogen; but not antiandrogen with androgen because I am unaware of environmental pollutants which are androgenic and therefore this might not be of relevance.

Niels E Skakkebæk (Copenhagen, Denmark)
Your hypothesis concerning increased AGD in females is supported by older studies performed in the 1970s using cyproterone acetate (CA) which showed weak virilisation effects on female fetuses when the pregnant dams were exposed to CA.