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Persistent developmental toxicity in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides

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ABSTRACT

There is growing concern of permanent damage to the endocrine and nervous systems after developmental exposure to endocrine disrupting chemicals. In this study the permanent reproductive and neurobehavioral effects of combined exposure to five endocrine disrupting pesticides, epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone, were examined. Pregnant and lactating rat dams were dosed with a mixture of the five pesticides at three different doses, or with the individual pesticides at one of two doses.

Adverse effects were observed in young and adult male offspring from the group exposed to the highest dose of the mixture. These included reduced prostate and epididymis weights, increased testes weights, altered prostate histopathology, increased density of mammary glands, reduced sperm counts, and decreased spatial learning. As no significant effects were seen following single compound exposure at the doses included in the highest mixture dose, these results indicate cumulative adverse effects of the pesticide mixture.

1. Introduction

Exposure to endocrine disrupting chemicals (EDCs) during early life may cause long-term health effects, and can influence both the sexual and neurological development of the offspring, even until it reaches maturity or middle age [1–3]. In the Western world, findings of declining human semen quality and a high prevalence of congenital malformations of reproductive organs and hormone-dependent cancers [4–6], as well as a high prevalence of children being diagnosed with ADHD and other neurological disorders [7,8] are causing concern.

Previous research indicates that a wide range of pesticides may act as endocrine disrupters. The azole fungicides prochloraz, tebuconazole and epoxiconazole have been shown to react through several endocrine disrupting mechanisms, and to induce various endocrine disrupting effects [9–15]. Common features for the azole fungicides are that they increase gestational length and affect steroid hormone levels in fetuses and/or dams. In addition, studies indicate that prochloraz may also affect thyroid hormone levels and cause effects on the sexually dimorphic development of the brain [11]. Furthermore, it has been shown that procymidone competitively antagonizes binding to the androgen receptor (AR), and consequently affects the reproductive development in male offspring [16,17]. Mancozeb, a fungicide from the dithiocarbamate group, mainly acts via disruption of the thyroid hormone system and is therefore suspected of affecting brain development [18,19].

Although animal studies have shown that some pesticides can disrupt male sexual differentiation during development, the individual pesticides alone have so far not been shown to contribute to adverse human effects at relevant exposure levels. However, initial observations in epidemiological studies [20–22] point in the same direction as what has been seen in laboratory experiments

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with endocrine disrupting chemicals, namely that substantial mixture effects occur even though the individual chemicals are present at low, ineffective doses [23–25]. Cumulative effects can be seen when small and statistically insignificant effects of each compound are added to induce statistically significant effects when these compounds are mixed. These findings have stimulated interest in exploring the consequences of combined exposures to environmentally relevant mixtures of endocrine disrupting pesticides.

Currently, there are no data on the effects of combined developmental exposure to endocrine disrupting pesticides, which have the potential for affecting both reproductive and brain development. It is important to keep in mind that some pesticides may act through both sex- and thyroid hormone related mechanisms. Furthermore thyroid hormone disrupting pesticides may also affect testicular development [26] while anti-androgenic pesticides may disturb the sexually dimorphic development of the brain [11,27]. Consequently, it is relevant to study combined effects of pesticides with such dissimilar modes of action. Therefore, this study aimed at exploring whether combined developmental exposure to endocrine disrupting pesticides at low doses, i.e. doses below NOAEL for the single pesticides, would lead to adverse developmental toxicity effects. In the present paper we report results on thyroid and reproductive organs and behavioral endpoints from pre-pubertal and adult animals that have been exposed pre- and postnatally to a mixture of the five endocrine disrupting pesticides; procymidone, prochloraz, tebuconazole, epoxiconazole and mancozeb. Data on maternal endpoints, postnatal development and genital malformation frequencies from this study as well as mathematical modeling of the mixture results and in vitro studies with the same mixture of pesticides are presented in Hass et al. [28].

2. Materials and methods

2.1. Chemicals

Before initiating the study, the mixture ratio and dose levels of the individual pesticides were chosen as presented in Hass et al. [28]. In summary, the mixture ratio for the five pesticides was chosen based on the NOAEL for effects on increased gestation length in dams and perinatal mortality in the offspring. Upon choosing the mixture ratio, two range-finding studies were performed in order to test for toxicity and endocrine disrupting effects of various mixture doses. The first was in non-pregnant animals while the second was in pregnant animals [29].

The 5 pesticides used were procymidone (CAS no. 32809-16-8, purity 99.5), epoxiconazole (CAS no. 106325-08-8, purity 99.0), tebuconazole (CAS no. 107534-96-3, purity 98.5), mancozeb (CAS no. 8018-01-7, purity 76.0) and prochloraz (CAS no. 67747-09-5, purity 98.5). All chemicals were purchased in a technical quality from VWR – Bie & Berntsen, Herlev, Denmark. Corn oil (Sigma–Aldrich, Brøndby, Denmark) was used as vehicle.

2.2. Animals and exposure

The mixture study was performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee. Animals received a complete rodent diet and acidified tap water ad libitum, and were housed under standard conditions with 12 h reverse light–dark cycle with light starting at 9 p.m. and continuing throughout the night until 9 a.m. In this way behavioral testing could be performed during the animals' active period. For further information on housing conditions please consult Hass et al. [28].

The study included 14 groups of animals, and was performed in 4 blocks with a week between each block. The 14 groups were as equally as possible distributed among the 4 blocks, and the animals used were 198 time-mated nulliparous, young adult female Wistar rats (HanTac:WH, Taconic Europe, Ejby, Denmark). The animals were observed twice daily for signs of toxicity and body weights were recorded daily during the entire dosing period.

On the day after arrival at gestation day (GD) 4, the dams were distributed into groups with similar body weight (bw) distributions. They were given 4 days after arrival to adapt to the reversed light–dark cycle before beginning the exposure. Dams were dosed daily by gavage, from GD 7 to pup day (PD) 16. For more detailed information on dosing scheme please consult Hass et al. [28].

In Table 1 the composition of the pesticide mixture, the doses of the pesticides administered individually and in mixture and the number of litters in each group are shown. Four groups of 22 rat dams were given daily oral doses of 0, 14.6 (8.3% of NOAEL), 29.2 (17% of NOAEL) or 43.8 (25% of NOAEL) mg/kg/day of the mixture of the 5 pesticides, whereas ten groups of 10 or 12 time-mated rats were similarly dosed with two doses of the individual pesticides. The lowest dose of each pesticide was similar to the dose included in the highest mixture dose and the highest dose of the single pesticides was 4 times higher, corresponding to 25% of NOAEL and to NOAEL, respectively, for effects on gestation length and perinatal mortality. Due to low pregnancy rate the number of litters in each dose group was unfortunately somewhat lower (Table 1).

In Fig. 1 an overview of the study design is given. Results from offspring sacrificed on PD 16 and after weaning are presented in the present paper, whereas results from dams and the younger pups are presented in Hass et al. [28].

2.3. Sacrifice on PD 16

On PD 16, 1–3 male and 1–3 female pups per litter were randomly selected for autopsy. Pups were weighed, decapitated and trunk blood was collected for hormone analysis. Uterus, ovaries, thyroids and liver were dissected from one female pup per litter. Uterus, ovaries and livers were weighed, whereas the thyroid was excised on the thyroid cartilage in order to obtain optimal histological preservation. Uterus, one ovary, alternately left and right, a section of the liver and the thyroid were fixed in formalin and processed for paraffin embedding.

Testes, epididymides, ventral prostate, seminal vesicle, levator ani/bulbocavernosus muscle (LABC), bulbourethral glands, liver and thyroids were dissected from one male pup per litter and weighed. One testis per male (alternately left and right) was fixed in Bouin's fixative and processed for paraffin embedding. Epididymides, seminal vesicles and thyroids (cleared from the thyroid cartilage) were fixed in formalin and processed for paraffin embedding.

Histological evaluation was made of testes, thyroids and of those organs in which statistically significant changes in organ weights were seen. One section per organ was stained with hematoxylin and eosin for histological evaluation, and for thyroids only the mixture groups were evaluated.

2.4. Sacrifice on PD 22 and 50

On PD 22, 1–3 male and 1–3 female pups per litter were weaned. Dams were decapitated in CO_2/O_2 anesthesia and the numbers of uterine implantation sites was counted. Trunk blood was collected and used for hormone analysis. The male and female pups which were not to be kept after weaning were decapitated on PD 22 in CO_2/O_2 anesthesia and blood samples were collected for hormone analyses. On PD 50 1–2 males and females per litter from control and mixture groups were decapitated in CO_2/O_2 anesthesia and blood samples were collected for hormonal analyses.

2.5. Mammary glands

From controls and the three mixture groups (groups 1–4), one male and one female per litter at PD 22 and 1–2 males and 1–2 females per litter at PND 50 were used for investigation of effects on mammary gland development. At both ages, the 4th abdominal mammary gland was excised for whole mount preparation, and on PD 50 the contralateral 4th abdominal mammary gland from males and females was excised for histological analysis. Alternately left and right glands were used for each purpose. For histologic examination, mammary glands were fixed in formalin and stained with hematoxylin and eosin. Female mammary glands were evaluated for tubuloalveolar and lobuloalveolar morphology. Male mammary glands were evaluated for secretory material in the ducts and vacuolization of the epithelium in controls and the highest mixture group. The mammary gland whole mounts were fixed in formalin, stained with alum carmine, dehydrated and mounted. The mammary glands were scanned on a flatbed scanner and outline area, longitudinal growth, and transverse growth were measured using Image Pro Express (Media Cybernetics). The density was scored on a scale from 1 to 5 (with 5 representing most dense mammary glands) with appropriate scoring criteria according to age and gender. The number of terminal end buds (TEBs) was counted in the mammary glands at PD 22. Whole mounts of females PD 50 were only evaluated for density due to the large gland size and overlapping branches hampering outgrowth measurements and TEB number assessment.

2.6. Onset of puberty

Onset of puberty was registered in all weaned male and female offspring. In female offspring sexual maturity was assessed by determining day of vaginal opening (VO) as described by Goldman et al. [30]. All weaned females were examined daily from PD 30 to PD 42. In male offspring the onset of puberty was assessed as time of preputial separation (PPS) [31,32]. Males were examined daily from PD 34 to PD 50. On the day of VO or PPS the age and weight of the animals were recorded.

2.7. Behavioral testing

The investigations were performed during the animals' dark cycle, i.e. their active period, from 9 a.m. to 4 p.m., in dimly lit rooms. The experimenter was kept unaware as to which group an individual rat belonged, and exposed and control