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Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides

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ABSTRACT

The present study investigated whether a mixture of low doses of five environmentally relevant endocrine disrupting pesticides, epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone, would cause adverse developmental toxicity effects in rats. In rat dams, a significant increase in gestation length was seen, while in male offspring increased nipple retention and increased incidence and severity of genital malformations were observed. Severe mixture effects on gestation length, nipple retention and genital malformations were seen at dose levels where the individual pesticides caused no or smaller effects when given alone. Generally, the mixture effect predictions based on dose-additivity were in good agreement with the observed effects. The results indicate that there is a need for modification of risk assessment procedures for pesticides, in order to take account of the mixture effects and cumulative intake, because of the potentially serious impact of mixed exposure on development and reproduction in humans.

1. Introduction

The high prevalence of disorders related to the endocrine system, *e.g.* hormone-dependent cancers, fertility problems and congenital malformations of reproductive organs, is a point of increasing concern in the western world [1,2]. Many genetic, environmental and lifestyle factors may be involved in these adverse effects, one of them possibly being exposures to endocrine disrupting chemicals (EDCs).

Currently, risk assessment of chemicals including pesticides is based on the no observed adverse effect levels (NOAELs) for effects of single compounds. Based on results from animal studies, exposure to single endocrine disrupting chemicals (EDCs) generally does not cause concern for adverse reproductive effects in humans. Humans are, however, exposed to a mixture of several EDCs [3,4], and during the last decade, scientific and regulatory focus has gradually begun shifting towards examining the effects of mixtures. Since 2005 the European Union member states have for example been obliged to evaluate and if possible refine existing methodologies in order to take combined actions of pesticides into account during risk assessment and especially when establishing maximum residue levels (MRLs) [5].

In studies where experimental animals have been exposed to several endocrine disrupters simultaneously, substantial mixture effects on reproductive development have been seen even though each of the individual EDCs were present at low, ineffective doses [6–8]. In addition, there are indications that cumulative exposure to EDCs including pesticides may play a role in relation to effects on human development, as epidemiological studies have reported increased prevalence of cryptorchidism in sons of women working as gardeners [9] or living on farms where pesticides have been used [10,11]. Furthermore, epidemiological studies have found associations between cryptorchidism and hypospadia and total estrogenic load measured in mother's placenta extracts [12], and association between congenital cryptorchidism and levels of certain organochlorine pesticides in breast milk [13]. Swan et al. [4] found that decreases in anogenital distance among male infants are associated with prenatal phthalate exposure, and Pierik et al. [14]

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identified paternal exposures to pesticides and smoking as factors associated with these congenital malformations. Thus, it is important to bear in mind that exposures to endocrine disrupters may contribute to a combined adverse effect, even though the effects of the single compounds are undetectable [6,8,15]. The findings from animal mixture studies should have major implications for the human risk assessment of EDCs, as they imply that the current use of NOAELs for single chemicals may lead to an underestimation of the potential risk for humans exposed to mixtures of chemicals.

A wide range of pesticides is suspected to act as endocrine disrupters, and there can be many different mechanisms causing the observed effects in animal studies. The five presently investigated pesticides, epoxiconazole, mancozeb, prochloraz, tebuconazole and procymidone, were selected based on information on pesticide use in Denmark and within the EU, and on their endocrine disrupting activity and effects. Procymidone competitively antagonizes the binding of androgens to the androgen receptor, and thereby mainly affects the reproductive development in male offspring [16,17]. Common features of the azole fungicides epoxiconazole, prochloraz and tebuconazole are that they increase gestational length in dams, virilize female pups and affect steroid hormone levels in fetuses and/or dams [18–20]. Mancozeb acts mainly *via* disruption of the thyroid hormone system and is therefore suspected of disrupting brain development [21,22].

The applied extended developmental toxicity model in rats has previously been employed by our group to characterize the effects of developmental exposure to EDCs on male offspring, and detailed mixture experiments have revealed that statistically significant mixture effects can be observed when anti-androgenic chemicals are combined at levels below their individual NOAELs [6,7]. Before initiating the experimental study presented here, the mixture ratio and dose levels of the individual pesticides were chosen based on earlier obtained dose–response relationships for each of the five pesticides. Hereafter, range-finding studies were performed to find a combined dose that represented the pesticides at doses close to their individual NOAELs, without causing major effects on the pregnant animals or on pup survival. Data from these range-finding studies are presented in Jacobsen et al. [23] and were used to set the dose levels of the mixture and of the individual compounds. Three different doses of the pesticide mixture and two doses of the individual pesticides were investigated in pregnant rat dams, and gestation length as well as anogenital distance, nipple retention and incidence of congenital malformations in the offspring were registered. Based on single chemical dose–response curves obtained in both previous and the present study, the effects of the pesticide mixture were predicted using the mathematical models dose-addition and independent action. Other endpoints sensitive to endocrine disruption, such as reproductive organ weights and histopathology, semen quality and a wide range of neurobehavioral endpoints were also measured in the study, and are presented in Jacobsen et al. [24]. Furthermore, chemical analysis of the pesticides in blood samples from dams and offspring were used for evaluating if mixture exposure and exposure to the single pesticides would lead to comparable blood levels. These results are presented in Herrmann et al. [25].

One goal of mixture toxicology is to estimate the toxicities of untested mixtures of chemicals on basis of information on individual components. For predicting combined effects of the five pesticides, we assumed that each pesticide in the mixture did not exacerbate or diminish the effects of the other pesticides. The choice of an appropriate model for calculation of additivity expectations is essential for assessments of mixture effects, because it is in relation to these additivity expectations that combination effects are judged in terms of synergisms or antagonisms [26]. Mixture effects according to "additivity" assumptions can be calculated by using two alternative concepts, dose addition and independent action. Dose addition looks at mixture effects in terms of a "dilution principle". It assumes that one chemical can be replaced totally or in part by an equal fraction of an equi-effective dose of another, without diminishing the overall combined effect [27]. Dose addition is often used for mixtures composed of chemicals that act through a similar or common mode of action [28–32]. Its application to the present mixture of five pesticides appeared justified because all pesticides are endocrine disruptors and – apart from mancozeb – produce a common effect outcome, *i.e.* anti-androgenic effects in male offspring. However, it can be argued with equal justification that the similarity assumption for dose addition is not applicable to the chosen mixture because the pesticides produce their effects by a diversity of molecular mechanisms. For this reason, we also employed the alternative concept of independent action to estimate additivity expectations. Independent action assumes that the joint effects of a combination of agents can be calculated by adopting the statistical concept of independent events [33]. It is viewed as appropriate for mixtures of chemicals with diverse modes of action [28], however, dose addition and independent action often yield additive mixture effect predictions within the same range.

The overall aim was to explore whether a mixture of environmentally relevant endocrine disrupting pesticides with dissimilar modes of action would cause adverse developmental toxicity effects in rats at dose levels below NOAELs for the individual pesticides. Furthermore, the aim was to investigate whether dose-additivity or independent action predictions of the expected mixture effects resulted in useful estimates compared to the observed mixture effects for relevant endpoints. The mixture and the single pesticides were also investigated using *in vitro* assays, in order to compare these results with those from the rat study, and evaluate the usability of alternative *in vitro* methods for estimating potential mixture effects.

2. Materials and methods

2.1. Chemicals

The five pesticides used were epoxiconazole (CAS no. 106325-08-8, purity 99.0), mancozeb (CAS no. 8018-01-7, purity 76.0), prochloraz (CAS no. 67747-09-5, purity 98.5), tebuconazole (CAS no. 107534-96-3, purity 98.5) and procymidone (CAS no. 32809-16-8, purity 99.5). 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 animal studies were performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee.

The study included 198 time-mated nulliparous, young adult animals (Han-Tac:WH, Taconic Europe, Ejby, Denmark) distributed to 14 groups of animals. It was performed in 4 blocks with a week between each block and the 14 groups were as equally as possible distributed among the 4 blocks. The animals were housed in pairs until GD (Gestation Day) 18 and alone thereafter under standard conditions in semi-transparent polycarbonate cages (15 cm × 27 cm × 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 9 p.m., light intensity 500 lx, temperature 21 ± 2 °C, humidity 50% ± 5%, ventilation 8 air changes per h). 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) was provided *ad libitum*. The animals were observed twice daily for signs of toxicity and body weights were recorded daily during the dosing period.

On the day after arrival, at gestation day (GD) 4, the time-mated animals were pseudorandomly distributed into groups with similar body weight (bw) distributions. They were given four days after arrival to adapt to the reversed light-dark cycle before beginning the exposure. Independently of actual day of delivery, the expected day of delivery, GD 23, was designated pup day (PD) 1 for the pups. Thereby, the age of the pups related to the time of conception, but was rather similar to postnatal age. Dams were dosed daily by gavage, from GD 7 to 21 and from the day after birth to pup (PD) 16, and were treated at a constant volume of 2 ml/kg/day, with individual doses based on the body weight of the animal on the day of dosing.