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Mixtures of environmentally relevant endocrine disrupting chemicals affect mammary gland development in female and male rats

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ABSTRACT

Estrogenic chemicals are able to alter mammary gland development in female rodents, but little is known on the effects of anti-androgens and mixtures of endocrine disrupting chemicals (EDCs) with dissimilar modes of action.

Pregnant rat dams were exposed during gestation and lactation to mixtures of environmentally relevant EDCs with estrogenic, anti-androgenic or dissimilar modes of action (TotalMix) of 100-, 200- or 450-fold high end human intake estimates. Mammary glands of prepubertal and adult female and male offspring were examined.

Oestrogens increased mammary outgrowth in prepubertal females and the mRNA level of matrix metalloproteinase-3, which may be a potential biomarker for increased outgrowth. Mixtures of EDCs gave rise to ductal hyperplasia in adult males. Adult female mammary glands of the TotalMix group showed morphological changes possibly reflecting increased prolactin levels. In conclusion both estrogenic and anti-androgenic chemicals given during foetal life and lactation affected mammary glands in the offspring.

1. Introduction

Sexual differentiation during foetal development and infancy is highly dependent on hormonal influence. Disturbance of the hormonal balance by endocrine disrupting chemicals (EDCs) may have important impact on the development of a wide range of hormone sensitive organs in the young animal or child. In rodents, perinatal exposure to EDCs has been shown to affect offspring and some changes are persistent in adulthood [1–3]. The mammary glands are formed during sexual differentiation in utero. Androgens are responsible for the male-like differentiation of mammary glands, blocking the nipple formation [4,5] and oestrogens are important for the female development [4,6]. Several environmental chemicals have been demonstrated to have estrogenic or anti-androgenic properties and may thus interfere with early mammary gland development.

Estrogenic chemicals have been shown to affect early mammary gland development in rodent females and to accelerate mammary gland growth. In rat studies, an increase in the area and the number of terminal end buds (TEBs) have been shown in prepubertal female mammary glands when exposed to estrogenic compounds such as genistein and estradiol benzoate [7,8]. Moreover, studies have shown increased lobular or alveolar hyperplasia [9–12] and secretory dilation of alveoli of adult female mammary glands exposed to oestrogens [9,13]. TEBs are undifferentiated proliferative structures in rat mammary glands where neoplastic transformation primarily occurs, and an increase in the number of TEBs may be a sign of increased risk of mammary cancer later in life [14]. Lobules are differentiated structures and lobule development with alveolar or lobular hyperplasia, on the other hand, may be a sign of accelerated growth or increased differentiation of the mammary gland. Oestrogens such as genistein and ethinyl estradiol have also been shown to affect male mammary glands, increasing the area, density and branching of the gland [15–17], suggesting an enhanced growth of mammary glands in males.

Anti-androgenic chemicals are well-known to affect male offspring in experimental studies. For example, phthalates are known to induce malformations of the reproductive organs, reduce the

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ano-genital distance and increase nipple retention in rats [18–20]. However, potential effects of anti-androgens on mammary gland development have only been described in a few studies. Saad et al. [21] observed increased branching in whole mounts of pubertal female mammary glands following perinatal exposure to vinclozolin. Histology mammary glands from adult males exposed perinatally to di-*n*-butyl phthalate (DBP) showed increased incidence of vacuolar degeneration in alveolar cells and alveolar atrophy [22]. Moreover, developmental exposure to a mixture of anti-androgenic pesticides in a study by Jacobsen et al. [1] showed effects indicating an increased feminization of adult male mammary glands. Thus, anti-androgens appear to affect both female and male mammary glands, though the data are scarce.

Humans are exposed to a wide range of chemicals with estrogenic and anti-androgenic properties. To mimic human exposure, an environmentally relevant mixture of EDCs with dissimilar modes of action was studied for effects on male and female mammary development in perinatally exposed rats. This paper describes the effects of a mixture of chemicals known to be mainly estrogenic (Emix), a mixture of chemicals known to be mainly anti-androgenic (Amix) and a total mixture of both the estrogenic and the anti-androgenic compounds. Additionally, paracetamol has been shown to have anti-androgenic capacities [23,24] and was tested alone and included in the total mixture (TotalMix). The TotalMix included the following 13 chemicals: the phthalates DBP and di-(2-ethylhexyl) phthalate (DEHP), the pesticides vinclozolin, prochloraz, procymidone, linuron and epoxiconazole, the pesticide metabolite p,p′-DDE, the UV-filter substances octyl methoxycinnamate (OMC) and 4-methyl-benzylidene camphor (4-MBC), the phenolic compound bisphenol A, the preservative butyl paraben and the analgesic compound paracetamol. As previously reported, this mixture affected a range of anti-androgenic endpoints during early development [18].

As noted, mammary effects of EDCs have been described in both prepubertal and adult rats [8,10,15,16]. The aim of the present study was to investigate the mammary effects of perinatal exposure to environmentally EDCs relevant for humans and to investigate how early exposure to anti-androgens and oestrogens in combination may affect mammary gland development throughout life. Moreover, a molecular biomarker for prediction of prepubertal mammary changes was investigated. In rodent studies, examination of the entire mammary gland during development is possible by evaluation of mammary whole mounts, i.e. mammary tissue fixed on a glass slide and stained for microscopic investigation. To determine the optimal age for evaluation of the mammary gland whole mounts, we performed an initial study (study A) comparing results of neonatal, prepubertal and adult mammary whole mount evaluation following exposure to the TotalMix. Female and male mammary gland whole mounts of offspring were evaluated for outgrowth, density and number of TEBs. In the following study (study B), whole mounts of prepubertal animals were prepared and the effect of each sub-component of the TotalMix (Emix, Amix and paracetamol) was compared to the effect of the TotalMix. To enable identification of a molecular biomarker for endocrine disruption affecting mammary gland development, microarray analysis was performed in dose groups showing significant changes in mammary outgrowth prepubertally. Based on literature and a microarray, relevant genes were selected for gene expression analysis with quantitative real time PCR (qPCR). Histological sections of adult mammary glands were evaluated for adult offspring. Data on reproductive parameters and effects in ageing rats from study A are published in Christiansen et al. [18] and Isling et al. [3], and reproductive parameters and data on preweaning rats from study B are published in Axelstad et al. [25].

2. Materials and methods

2.1. Chemicals

In these two studies, dose levels were selected based on human intake data. A detailed description of the TotalMix composition and choice of dose-levels can be found in Christiansen et al. [18]. The highest dose of the TotalMix (TotalMix-450) represented 450-fold "high human intake levels" of each compound. Paracetamol was present at a dose level of 360 mg/kg bw/day in the TotalMix-450, a dose level of paracetamol that corresponds to approximately 7-fold human intake levels in individuals consuming 6 paracetamol tablets of 500 mg per day. Study B also included the same dose of paracetamol in the dose-group receiving paracetamol only. Study B also included 100- and 200-fold "high human intake levels" for the total mixture (TotalMix-100 and TotalMix-200), and 200-fold and 450-fold "high human intake levels" for the anti-androgen (Amix-200 and Amix-450) as well as the oestrogen (Emix-200 and Emix-450) sub-mixtures. The ratios and amounts of the single compounds in the mixtures are shown in Table 1 and are described in details in Axelstad et al. [25]. Corn oil (VWR-Bie&Berntsen A/S, Søborg, Denmark) was used as vehicle and a control compound.

2.2. Animals and experimental design

Two studies were performed using comparable study designs. Time-mated nulliparous female Wistar rats were supplied on gestation day (GD) 3 and on GD 4 dams were assigned to dose groups with similar weight distributions in all groups. Dams were dosed by gavage during morning hours from GD 7 to GD 21. The day after expected delivery was defined as pup day (PD) 1. Dosing of dams was resumed during lactation PD 1–22. However, when animals were exposed to paracetamol, dosing of paracetamol during pregnancy was restricted to GD 13–19 to avoid potential effects on embryonic implantation [26]. Paracetamol was excluded from the mixture on GD 20–21 to avoid potential effects on birth, and on PD 14–22 paracetamol was added to the mixture for blood measurements of chemicals (to be reported elsewhere). Hence, in the case of TotalMix, dams were dosed with TotalMix without paracetamol during the dosing periods GD 7–12 and PD 1–13. Animals were administered acidified tap water (in glass bottles) and soy- and alfalfa free diet (Altromin 1314, GmBH, Lage Germany) ad libitum. Animals were housed in semi-transparent polycarbonate cages ($15 \times 27 \times 43$ cm) with Aspen bedding (Tapvei, Brogaarden, Gentofte, Denmark) in controlled environmental conditions with $22 \pm 1\,^{\circ}$C, 10 air changes per hour, air humidity $55\% \pm 5$ and 12 h light–dark cycles with lights on from 9PM to 9AM. Until GD 17, pregnant rat dams were housed in pairs and thereafter alone. The animal studies were performed under conditions approved by the Danish Animal Experiments Inspectorate and by the in-house Animal Welfare Committee of the National Food Institute at the Technical University of Denmark.

The first series of studies (study A) aimed to determine the optimal timing of mammary examination in whole mounts, and mammary effects were examined in neonatal (PD 6), prepubertal (PD 22) and post-pubertal (PD 49 or 55) offspring exposed to the mixture. In the second study (study B), the same mixture was subdivided into anti-androgens, oestrogens and paracetamol, and mammary effects at PD 22 of each sub-mixture were compared with effects of the total-mixture.

Study A was performed in two blocks. In each block 28 dams were assigned to 2 groups ($n = 14$): control (0 mg/kg) and TotalMix-450. In study B, 152 dams were assigned to 9 groups ($n = 20$ in control and TotalMix-100 groups and $n = 16$ in each of the other groups): control (0 mg), 3 TotalMix groups (TotalMix-100, TotalMix-200 and Totalix-450), 2 Amix groups (Amix-200 and