Male reproductive development is intricately dependent on fetal androgen action. Consequently, disrupted androgen action during fetal life can interfere with the development of the reproductive system resulting in adverse effects on reproductive function later in life. One biomarker used to evaluate fetal androgen action is the anogenital distance (AGD), the distance between the anus and the external genitalia. A short male AGD is strongly associated with genital malformations at birth and reproductive disorders in adulthood. AGD is therefore used as an effect readout in rodent toxicity studies aimed at testing compounds for endocrine activity and anti-androgenic properties, and in human epidemiological studies to correlate fetal exposure to endocrine disrupting chemicals to feminization of new-born boys. In this review, we have synthesized current data related to intrauterine exposure to xenobiotics and AGD measurements. We discuss the utility of AGD as a retrospective marker of in utero anti-androgenicity and as a predictive marker for male reproductive disorders, both with respect to human health and rodent toxicity studies. Finally, we highlight four areas that need addressing to fully evaluate AGD as a biomarker in both a regulatory and clinical setting.

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A pragmatic approach for human risk assessment of chemical mixtures
Humans are continuously exposed to complex chemical mixtures from foods and the environment. Due to our inadequate understanding of mixture effects, tools to assess the combined risk of mixed chemical exposures have been difficult to develop. In recent years, regulatory authorities across the world have made considerable progress towards developing pragmatic frameworks to deal with combined exposure to multiple chemicals for risk assessment purposes. These approaches require a high level of information about chemical exposures and toxicities, information that often is lacking. We see this data gap as delaying urgently needed improvements in chemical safety. Herein, we present a pragmatic step-by-step procedure for mixture risk assessment and propose tools for grouping of chemicals. Until we have a better understanding of adverse outcome pathways, we suggest that grouping of chemicals for mixture risk assessment be based on integrated in vivo and in vitro data, read-across as well as computational methods such as QSAR models or integrative systems biology. These latter methods can be used to predict inherent hazards or modes/mechanisms of action and to group the chemicals in cases where no experimental data exist.

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Contributors: Boberg, J., Dybdahl, M., Petersen, A., Hass, U., Svingen, T., Vinggaard, A. M.
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Publication information
Distinct transcriptional profiles of the female, male and finasteride-induced feminized male anogenital region in rat fetuses

A short anogenital distance (AGD) in males is a marker for incomplete masculinization and a predictor of adverse effects on male reproductive health. For this reason, AGD is used to assess the endocrine disrupting potential of chemicals for risk assessment purposes. The molecular mechanisms underpinning this chemically induced shortening of the AGD, however, remains unclear. Although it is clear that AR-mediated signaling is essential, evidence also suggest the involvement of other signaling pathways. This study presents the first global transcriptional profile of the anogenital tissue in male rat fetuses with chemically induced short AGD, also including comparison to normal male and female control animals. The anti-androgenic drug finasteride (10mg/kg bw/day) was used to induce short AGD by exposing time-mated Sprague Dawley rats at gestation days (GD) 7-21. The AGD was 37% shorter in exposed male fetuses compared to control males at GD21. Transcriptomics analysis on anogenital tissues revealed a sexually dimorphic transcriptional profile. More than 350 genes were found to be differentially expressed between the three groups. The expression pattern of four genes of particular interest (Esr1, Padi2, Wnt2 and Sfrp4) was also tested by RT-qPCR analyses, indicating that estrogen and Wnt2 signaling play a role in the sexually dimorphic development of the anogenital region. Our transcriptomics profiles provide a stepping-stone for future studies aimed at characterizing the molecular events governing development of the anogenital tissues, as well as describing the detailed Adverse Outcome Pathways for short AGD; an accepted biomarker of endocrine effects for chemical risk assessment.

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Contributors: Lindgren Schwartz, C. V., Vinggaard, A. M., Christiansen, S., Darde, T. A., Chalmel, F., Svingen, T.
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**Workshop on the validation and regulatory acceptance of Innovative 3R approaches in regulatory toxicology - Evolution versus revolution**
At a joint workshop organized by RIVM and BfR, international experts from governmental institutes, regulatory agencies, industry, academia and animal welfare organizations discussed and provided recommendations for the development, validation and implementation of innovative 3R approaches in regulatory toxicology. In particular, an evolutionary improvement of our current approach of test method validation in the context of defined approaches or integrated testing strategies was discussed together with a revolutionary approach based on a comprehensive description of the physiological responses of the human body to chemical exposure and the subsequent definition of relevant and predictive in vitro, in chemico or in silico methods. A more comprehensive evaluation of biological relevance, scientific validity and regulatory purpose of new test methods and assessment strategies together with case studies that provide practical experience with new approaches were discussed as essential steps to build up the necessary confidence to facilitate regulatory acceptance.
Workshop on acceleration of the validation and regulatory acceptance of alternative methods and implementation of testing strategies
This report describes the proceedings of the BfR-RIVM workshop on validation of alternative methods which was held 23 and 24 March 2017 in Berlin, Germany. Stakeholders from governmental agencies, regulatory authorities, universities, industry and the OECD were invited to discuss current problems concerning the regulatory acceptance and implementation of alternative test methods and testing strategies, with the aim to develop feasible solutions. Classical validation of alternative methods usually involves one to one comparison with the gold standard animal study. This approach suffers from the reductionist nature of an alternative test as compared to the animal study as well as from the animal study being considered as the gold standard. Modern approaches combine individual alternatives into testing strategies, for which integrated and defined approaches are emerging at OECD. Furthermore, progress in mechanistic toxicology, e.g. through the adverse outcome pathway approach, and in computational systems toxicology allows integration of alternative test battery results into toxicity predictions that are more fine-tuned to the human situation. The road towards transition to a mechanistically-based human-focused hazard and risk assessment of chemicals requires an open mind towards stepping away from the animal study as the gold standard and defining human biologically based regulatory requirements for human hazard and risk assessment.
Characterizing novel molecular mechanisms for short AGD – a biomarker of fetal testicular dysfunction

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Research output: Contribution to journal › Journal article – Annual report year: 2018 › Research › peer-review

Combination effects of pesticides on birth weight and metabolic programming in rat offspring
Risk assessment of pesticides is generally based on the no observed adverse effect levels (NOAELs) for single compounds. However, humans are typically exposed to a mixture of several pesticides. The objectives of the project were to investigate whether a mixture of environmentally relevant pesticides cause decreased birth weights at dose levels below NOAELs for the individual pesticides in rats, to evaluate whether the mixture effect is best predicted by the independent action or the dose-addition model, to investigate the influences of developmental pesticide exposure on metabolic programming of the offspring using biomarkers for obesity and type 2 diabetes, and to give input for regulatory considerations.

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Source-ID: 162458889
Research output: Book/Report › Report – Annual report year: 2018 › Commissioned

Effects on metabolic parameters in young rats born with low birth weight after exposure to a mixture of pesticides
Pesticide exposure during fetal life can lead to low birth weight and is commonly observed in reproductive toxicology studies. Associations have also been found in low birth weight babies born from pesticide-exposed gardeners. Since low birth weight is also linked to metabolic disorders, it can be speculated that early life exposure to pesticides could increase the risk of becoming obese or developing diabetes later in life. We have analyzed potential long-term effects of gestational and lactational exposure to a low dose mixture of six pesticides that individually can cause low birth weight: Cyromazine, MCPB, Pirimicarb, Quinoclamine, Thiram, and Ziram. Exposed male offspring, who were smaller than controls, displayed
some degree of catch-up growth. Insulin and glucagon regulation was not significantly affected, and analyses of liver and pancreas did not reveal obvious histopathological effects. Efforts towards identifying potential biomarkers of metabolic disease-risk did not result in any strong candidates, albeit leptin levels were altered in exposed animals. In fat tissues, the key genes Lep, Nmb and Nmbr were altered in high dosed offspring, and were differentially expressed between sexes. Our results suggest that early-life exposure to pesticides may contribute to the development of metabolic disorders later in life.

**Environmental Toxicology: Pesticides**
Pesticides are toxic substances that are deliberately released into our environment to kill or control living organisms. They have many beneficial qualities with regards to their intended use, but also carry with them potential harmful side effects for other living organisms that are inadvertently exposed, including humans. In this article, we will discuss some of the possible toxic effects they can have on male reproductive health. Of particular concern are pesticides with endocrine disrupting properties, which have been shown to interfere with male reproductive development. Early life exposure can prevent proper masculinization, having permanent consequences for the offspring.
organic pollutants (POPs). For humans, foods represent the main source of exposure, but common house dust can also be a significant source of exposure in small children.

Phthalates and bisphenol A can interfere with male reproductive development by inducing reproductive organ malformations and impaired sperm production. Also persistent halogenated chemicals may be intentionally or unintentionally present in plastics and migrate/leak to foods or the environment causing concern for male reproductive function.

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Erratum to: The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study
https://doi-org.proxy.findit.dtu.dk/10.1007/s11356-010-0396-x

In the original article wrong unites were quoted in Table 3 (page 508) and Table 4 (page 510) as well as in the paragraph 3.2 Core chemical exposure experiments on page 509. Also in paragraph 2.3 Selection and testing of chemicals the link to the Supplemental Materials (ESM) was missing. The correct versions of the tables and the paragraph as well as the ESM link are provided below.

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Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis

Glyphosate has been suggested to be an endocrine disrupting chemical capable of disrupting male reproduction. There are conflicting data, however, with studies reporting on effects from exposure to either glyphosate alone or to herbicide formulations, making comparisons difficult. We assessed rat testis histopathology and androgen function following two weeks exposure to either glyphosate at 2.5 and 25 mg/kg bw/day (5x and 50x Acceptable Daily Intake, ADI, respectively), or equivalent high dose of glyphosate in a herbicide formulation, Glyfonova. We observed no significant effects on testes or testosterone synthesis in rats exposed to glyphosate. Limited effects were observed in rats exposed to Glyfonova, with a small upregulation of the steroidogenic genes Cyp11a1 and Cyp17a1. We conclude that glyphosate alone has no effects on adult rat testis at exposure levels up to 25 mg/kg bw/day. Glyfonova induced only minor effects on steroidogenic gene expression, likely caused by additives other than glyphosate.

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Glyphosate alone does not adversely affect testicular androgen function in mature rats

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Perineal gene expression profiling in rats with chemically induced short anogenital distance

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Organisations: National Food Institute, Research group for Molecular and Reproductive Toxicology, Copenhagen Center for Health Technology, French Institute of Health and Medical Research
Contributors: Schwartz, C. V. L., Vinggaard, A. M., Christiansen, S., Chalmel, F., Svingen, T.
PFHxS Causes Developmental Hypothyroxinemia Without Affecting Behavioral Tests in Rat Offspring

Introduction
Thyroid hormones (TH) are critical for mammalian brain development. In humans, low maternal serum thyroxine (T4) levels are associated with neurological deficiencies and cognitive impairment. Perfluorohexane sulfonate (PFHxS) is a widespread environmental contaminant found in human serum, tissues and milk. We have shown that PFHxS decrease serum thyroxine in pregnant rat dams and their offspring. Here, we further investigate effects on the thyroid system, neurodevelopment, and combination effects of PFHxS and a mixture of environmentally relevant endocrine disruptors.

Methods
Perfluorohexane sulfonate (PFHxS, 0, 0.05, 5 or 25 mg/kg/day with and without EDmix, a mixture of 12 endocrine disruptors e.g. phthalates, pesticides, UV-filters, Bisphenol A and butyl paraben) was administered (p.o.) to Wistar rat dams (n = 16-20/dose group) from gestation day (GD) 7 through postnatal day (PD) 22. Offspring were assessed in activity boxes and the radial arm maze.

Results/discussion
PFHxS not only decreased serum T4 levels in dams and offspring but in the high dose also reduced T3 to 84% of controls in both dams (PD 22) and pups (PD 16). The hypothalamic-pituitary-thyroid (HPT) axis was not activated based on lack of effect on serum TSH, thyroid gland histology, weight and thyroid gene expression levels. Developmental hypothyroxinemia did not appear to increase physical activity levels in young and adult offspring. However, the expected sex difference was absent on PD 115 in low dose PFHxS (0.05 mg/kg) and at high doses in combination with EDmix (5 mg/kg +EDmix and 25 mg/kg + EDmix). Slight effects on offspring learning and memory did not appear correlated to decreased TH levels during development.

Conclusions
PFHxS decreased circulating levels of T3 and T4 in pregnant rat dams and their offspring without apparent compensation by the HPT axis. The thyroid hormone disruption was not associated with detectable learning and memory deficits. Rather findings suggest that PFHxS may disrupt sexual differentiation of the brain. Standard behavioral assays appear insensitive to adverse effects on brain development caused by thyroid hormone disruption. Hence, there is a need for development of sensitive assays to protect human thyroid function. Does not reflect EPA policy.
proof of principle showing that adverse effects on anogenital distance in male offspring, which is a unique and non-invasive marker for male reproductive health effects in animals and humans, can be predicted for selected pesticides.

We investigated this strategy on 9 pesticides and selected 3 compounds – fludioxonil, cyprodinil and dimethomorph – for in vivo ‘validation’ of the alternative approach in rats. Predicted fetal levels were within a factor of 2 from measured concentrations, and all three compounds showed a shortened AGD in vivo.

In conclusion, our approach can be used to avoid unnecessary animal testing and focus on compounds that most likely will produce in vivo activity.

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Transcriptional profiling of the chemically induced feminized anogenital region in male rats

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Transcriptional profiling of the chemically induced feminized anogenital region in male rats

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Transcriptional profiling of the chemically-induced feminized male rat anogenital region

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Research output: Contribution to conference › Conference abstract for conference – Annual report year: 2018 › Research › peer-review

An effect-directed strategy for characterizing emerging chemicals in food contact materials made from paper and board

Food contact materials (FCM) are any type of item intended to come into contact with foods and thus represent a potential source for human exposure to chemicals. Regarding FCMs made of paper and board, information pertaining to their chemical constituents and the potential impacts on human health remains scarce, which hampers safety evaluation. We describe an effect-directed strategy to identify and characterize emerging chemicals in paper and board FCMs. Twenty FCMs were tested in eight reporter gene assays, including assays for the AR, ER, AhR, PPARγ, Nrf2 and p53, as well as mutagenicity. All FCMs exhibited activities in at least one assay. As proof-of-principle, FCM samples obtained from a sandwich wrapper and a pizza box were carried through a complete step-by-step multi-tiered approach. The pizza box exhibited ER activity, likely caused by the presence of bisphenol A, dibutyl phthalate, and benzylbutyl phthalate. The sandwich wrapper exhibited AR antagonism, likely caused by abietic acid and dehydroabietic acid. Migration studies confirmed that the active chemicals can transfer from FCMs to food simulants. In conclusion, we report an effect-directed strategy that can identify hazards posed by FCMs made from paper and board, including the identification of the chemical(s) responsible for the observed activity.

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DOIs:
10.1016/j.fct.2017.05.061
Enniatin B and beauvericin are common in Danish cereals and show high hepatotoxicity on a high-content imaging platform

Mycotoxins are fungi-born metabolites that can contaminate foods through mould-infected crops. They are a significant food/feed-safety issue across the globe and represent a substantial financial burden for the world economy. Moreover, with a changing climate and fungal biota, there is now much discussion about emerging mycotoxins that are measurable at significant levels in crops world-wide. Unfortunately, we still know very little about the bioavailability and toxic potentials of many of these less characterized mycotoxins, including the large family of enniatins. In this study, we present new occurrence data for enniatin A, A1, B, B1 and beauvericin in four Danish crops: oat, wheat, and barley from the 2010 harvest, and rye from 2011 harvest. The occurrence of the four enniatins were B>B1>A1>A. Enniatin B was detected in 100% of tested samples regardless of crop type. In addition to occurrence data, we report a proof-of-concept study using a human-relevant high-content hepatotoxicity, or "quadroprobe," assay to screen mycotoxins for their cytotoxic potential. The assay was sensitive for most cytotoxic compounds in the 0.009–100 µM range. Among eight tested mycotoxins (enniatin B, beauvericin, altenuariol, deoxynivalenol, aflatoxin B1, andrastin A, citrinin, and penicillic acid), enniatin B and beauvericin showed significant cytotoxicity at a concentration lower than that for aflatoxin B1, which is the archetypal acute hepatotoxic and liver-carcinogenic mycotoxin. Hence, the quadroprobe hepatotoxicity assay may become a valuable assessment tool for toxicity assessment of mycotoxins in the future.
Environmental influences on ovarian dysgenesis - developmental windows sensitive to chemical exposures

A woman's reproductive health and ability to have children directly affect numerous aspects of her life, from personal well-being and socioeconomic standing, to morbidity and lifespan. In turn, reproductive health depends on the development of correctly functioning ovaries, a process that starts early during fetal life. Early disruption to ovarian programming can have long-lasting consequences, potentially manifesting as disease much later in adulthood. A growing body of evidence suggests that exposure to chemicals early in life, including endocrine-disrupting chemicals, can cause a range of disorders later in life, such as those described in the ovarian dysgenesis syndrome hypothesis. In this Review, we discuss four specific time windows during which the ovary is particularly sensitive to disruption by exogenous insults: gonadal sex determination, meiotic division, follicle assembly and the first wave of follicle recruitment. To date, most evidence points towards the germ cell lineage being the most vulnerable to chemical exposure, particularly meiotic division and follicle assembly. Environmental chemicals and pharmaceuticals, such as bisphenols or mild analgesics (including paracetamol), can also affect the somatic cell lineages. This Review summarizes our current knowledge pertaining to environmental chemicals and pharmaceuticals, and their potential contributions to the development of ovarian dysgenesis syndrome. We also highlight knowledge gaps that need addressing to safeguard female reproductive health.

Human biomonitoring in risk assessment: analysis of the current practice and 1st examples on the use of HBM in risk assessments of HBM4EU priority chemicals

Human biomonitoring in risk assessment: analysis of the current practice and 1st examples on the use of HBM in risk assessments of HBM4EU priority chemicals

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Mechanisms of action involved in chemically induced effects on male reproductive health

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Mellemkødet sladrer om skadelige kemikalier

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Corrigendum to "Reproductive and behavioral effects of diisononyl phthalate (DINP) in perinatally exposed rats" [Reprod. Toxicol. 31 (2) (2011) 200–209]

General information
Publication status: Published
Organisations: National Food Institute, Research Group for Reproductive Toxicology, Research Group for Molecular Toxicology, Technical University of Denmark
Fluorinated alkyl substances and technical mixtures used in food paper-packaging exhibit endocrine-related activity in vitro

Migration of chemicals from packaging materials to foods may lead to human exposure. Polyfluoroalkyl substances (PFAS) can be used in technical mixtures (TMs) for use in food packaging of paper and board, and PFAS have been detected in human serum and umbilical cord blood. The specific structures of the PFAS in TMs are often unknown, but polyfluorinated alkyl phosphate esters (PAPs) have been characterized in TMs, food packaging, and in food. PAPs can be metabolized into fluorotelomer alcohols (FTOHs) and perfluoroalkyl carboxylic acids (PFCAs). Some PFAS have endocrine activities, highlighting the need to investigate these effects. Herein, we studied the endocrine activity of less characterized PFAS, including short-chain PFCAs and FTOHs, PAPs, and TMs of unknown chemical composition. Long-chain PFCAs were also included. We applied seven assays covering effects on estrogen, glucocorticoid, androgen, and peroxisome proliferator-activated receptor (PPAR) activity, as well as steroidogenesis in vitro and ex vivo. In general, PAPs, FTOHs, TMs, and long-chain PFCAs showed estrogenic activity through receptor activation and/or increasing 17β-estradiol levels. Furthermore, short- and long-chain PFCAs activated PPARα and PPARγ. Collectively, this means that (i) PAPs, FTOHs, and PFCAs exhibit endocrine activity through distinct and sometimes different mechanisms, (ii) two out of three tested TMs exhibited estrogenic activity, and (iii) short-chain FTOHs showed estrogenic activity and short-chain PFCAs generally activate both PPARα and PPARγ with similar potency and efficacy as long-chain PFCAs. In conclusion, several new and divergent toxicological targets were identified for different groups of PFAS.
**Intrauterine Exposure to Paracetamol and Aniline Impairs Female Reproductive Development by Reducing Follicle Reserves and Fertility**

Studies report that fetal exposure to paracetamol/acetaminophen by maternal consumption can interfere with male reproductive development. Moreover, recent biomonitoring data report widespread presence of paracetamol in German and Danish populations, suggesting exposure via secondary (nonpharmaceutical) sources, such as metabolic conversion from the ubiquitous industrial compound aniline. In this study, we investigated the extent to which paracetamol and aniline can interfere with female reproductive development. Intrauterine exposure to paracetamol by gavage of pregnant dams resulted in shortening of the anogenital distance in adult offspring, suggesting that fetal hormone signaling had been disturbed. Female offspring of paracetamol-exposed mothers had ovaries with diminished follicle reserve and reduced fertility. Fetal gonads of exposed animals had also reduced gonocyte numbers, suggesting that the reduced follicle count in adults could be due to early disruption of germ cell development. However, ex vivo cultures of ovaries from 12.5 days post coitum fetuses showed no decrease in proliferation or expression following exposure to paracetamol. This suggests that the effect of paracetamol occurs prior to this developmental stage. Accordingly, using embryonic stem cells as a proxy for primordial germ cells we show that paracetamol is an inhibitor of cellular proliferation, but without cytotoxic effects. Collectively, our data show that intrauterine exposure to paracetamol at levels commonly observed in pregnant women, as well as its precursor aniline, may block primordial germ cell proliferation, ultimately leading to reduced follicle reserves and compromised reproductive capacity later in life.

**General information**

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**Juvenile Male Rats Exposed to a Low-Dose Mixture of Twenty-Seven Environmental Chemicals Display Adverse Health Effects**

Humans are exposed to a large number of environmental chemicals in their daily life, many of which are readily detectable in blood or urine. It remains uncertain if these chemicals can cause adverse health effects when present together at low doses. In this study we have tested whether a mixture of 27 chemicals administered orally to juvenile male rats for three months could leave a pathophysiological footprint. The mixture contained metals, perfluorinated compounds, PCB, dioxins, pesticides, heterocyclic amines, phthalate, PAHs and others, with a combined dose of 0.16 (Low dose), 0.47 (Mid dose) or 1.6 (High dose) mg/kg bw/day. The lowest dose was designed with the aim of obtaining plasma or urine concentrations in rats at levels approaching those observed in humans. Some single congeners were administered at doses representative of combined doses for chemical groups. With this baseline, we found effects on weight, histology and gene expression in the liver, as well as changes to the blood plasma metabolome in all exposure groups, including low-dose. Additional adverse effects were observed in the higher dosed groups, including enlarged kidneys and
alterations to the metabolome. No significant effects on reproductive parameters were observed.

**Late-life effects on testosterone production following in utero exposure to the pesticide fludioxonil**

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**Multiple endocrine disrupting effects in rats perinatally exposed to butylparaben**
Parabens comprise a group of preservatives commonly added to cosmetics, lotions and other consumer products. Butylparaben has estrogenic and anti-androgenic properties and is known to reduce sperm counts in rats following perinatal exposure. Whether butylparaben exposure can affect other endocrine sensitive endpoints, however, remains largely unknown. In this study, time-mated Wistar rats (n=18) were orally exposed to 0, 10, 100 or 500 mg/kg bw/day of butylparaben from gestation day 7 to pup day 22. Several endocrine-sensitive endpoints were adversely affected. In the two highest dose groups, the anogenital distance of newborn male and female offspring was significantly reduced, and in prepubertal females, ovary weights were reduced and mammary gland outgrowth was increased. In male offspring, sperm count was significantly reduced at all doses from 10 mg/kg bw/day. Testicular CYP19a1 (aromatase) expression was reduced in prepubertal, but not adult animals exposed to butylparaben. In adult testes, Nr5a1 expression was reduced at all doses, indicating persistent disruption of steroidogenesis. Prostate histology was altered at prepuberty and adult prostate weights were reduced in the high dose group. Thus, butylparaben exerted endocrine disrupting effects on both male and female offspring. The observed adverse developmental effect on sperm count at the lowest dose is highly relevant to risk assessment, as this is the lowest observed adverse effect level in a study on perinatal exposure to butylparaben.

**General information**
Non-targeted screening for contaminants in paper and board food-contact materials using effect-directed analysis and accurate mass spectrometry

Due to large knowledge gaps in chemical composition and toxicological data for substances involved, paper and board food-contact materials (P&B FCM) have been emerging as a FCM type of particular concern for consumer safety. This study describes the development of a step-by-step strategy, including extraction, high-performance liquid chromatography (HPLC) fractionation, tentative identification of relevant substances and in vitro testing of selected tentatively identified substances. As a case study, we used two fractions from a recycled pizza box sample which exhibited aryl hydrocarbon receptor (AhR) activity. These fractions were analysed by gas chromatography (GC) and ultra-HPLC (UHPLC) coupled to quadrupole time-of-flight mass spectrometers (QTOF MS) in order tentatively to identify substances. The elemental composition was determined for peaks above a threshold, and compared with entries in a commercial mass spectral library for GC-MS (GC-EI-QTOF MS) analysis and an in-house built library of accurate masses for substances known to be used in P&B packaging for UHPLC-QTOF analysis. Of 75 tentatively identified substances, 15 were initially selected for further testing in vitro; however, only seven were commercially available and subsequently tested in vitro and quantified. Of these seven, the identities of three pigments found in printing inks were confirmed by UHPLC tandem mass spectrometry (QqQ MS/MS). Two pigments had entries in the database, meaning that a material relevant accurate mass database can provide a fast tentative identification. Pure standards of the seven tentatively identified substances were tested in vitro but could not explain a significant proportion of the AhR-response in the extract. Targeted analyses of dioxins and PCBs, both well-known AhR agonists, was performed. However, the dioxins could explain approximately 3% of the activity observed in the pizza box extract indicating that some very AhR active substance(s) still remain to be identified in recycled low quality P&B.
Perfluorononanoic acid in combination with 14 chemicals exerts low-dose mixture effects in rats

Humans are simultaneously exposed to several chemicals that act jointly to induce mixture effects. At doses close to or higher than no-observed adverse effect levels, chemicals usually act additively in experimental studies. However, we are lacking knowledge on the importance of exposure to complex real-world mixtures at more relevant human exposure levels. We hypothesised that adverse mixture effects occur at doses approaching high-end human exposure levels. A mixture (Mix) of 14 chemicals at a combined dose of 2.5 mg/kg bw/day was tested in combination with perfluorononanoic acid (PFNA) at doses of 0.0125 (Low PFNA), 0.25 (Mid PFNA) and 5 (High PFNA) mg/kg bw/day by oral administration for 14 days in juvenile male rats. Indication of a toxicokinetic interaction was found, as simultaneous exposure to PFNA and the Mix caused a 2.8-fold increase in plasma PFNA concentrations at Low PFNA. An increase in testosterone and dihydrotestosterone plasma concentrations was observed for Low PFNA + Mix. This effect was considered non-monotonic, as higher doses did not cause this effect. Reduced LH plasma concentrations together with increased androgen concentrations indicate a disturbed pituitary-testis axis caused by the 15-chemical mixture. Low PFNA by itself increased the corticosterone plasma concentration, an effect which was normalised after simultaneous exposure to Mix. This combined with affected ACTH plasma concentrations and down-regulation of 11β HSD mRNA in livers indicates a disturbed pituitary-adrenal axis. In conclusion, our data suggest that mixtures of environmental chemicals at doses approaching high-end human exposure levels can cause a hormonal imbalance and disturb steroid hormones and their regulation. These effects may be non-monotonic and were observed at low doses. Whether this reflects a more general phenomenon that should be taken into consideration when predicting human mixture effects or represents a rarer phenomenon remains to be shown.

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Source-ID: 104650189
Research output: Contribution to journal › Journal article – Annual report year: 2015 › Research › peer-review
Perinatal exposure to mixtures of endocrine disrupting chemicals reduces female rat follicle reserves and accelerates reproductive aging

Exposure to endocrine disrupting chemicals (EDCs) during development can have negative consequences later in life. In this study we investigated the effect of perinatal exposure to mixtures of human relevant EDCs on the female reproductive system. Rat dams were exposed to a mixture of phthalates, pesticides, UV-filters, bisphenol A, butyl-paraben, as well as paracetamol. The compounds were tested together (Totalmix) or in subgroups with anti-androgenic (AAmix) or estrogenic (Emix) potentials. Paracetamol was tested separately. In pre-pubertal rats, a significant reduction in primordial follicle numbers was seen in AAmix and PM groups, and reduced plasma levels of prolactin was seen in AAmix. In one-year-old animals, the incidence of irregular estrous cycles was higher after Totalmix-exposure and reduced ovary weights were seen in Totalmix, AAmix, and PM groups. These findings resemble premature ovarian insufficiency in humans, and raises concern regarding potential effects of mixtures of EDCs on female reproductive function.

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The risk of chemical cocktail effects and how to deal with the issue

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Transferring in vivo exposure into in vitro assays using silicone to assess the endocrine activity of POPs accumulated in human breast implants

General information
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Organisations: Department of Environmental Engineering, Environmental Chemistry, National Food Institute, Research group for Analytical Food Chemistry, Copenhagen Center for Health Technology
Contributors: Gilbert, D., Mayer, P., Pedersen, M., Vinggaard, A. M.
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Chemical cocktail effects

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Cocktail: Danmarks største forskningsprojekt om cocktaileffekter i fødevarer

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Publication information
Place of publication: Søborg
Endocrine activity of persistent organic pollutants accumulated in human silicone implants — Dosing in vitro assays by partitioning from silicone

Persistent organic pollutants (POPs) accumulated in human tissues may pose a risk for human health by interfering with the endocrine system. This study establishes a new link between actual human internal POP levels and the endocrine active dose in vitro, applying partitioning-controlled dosing from silicone to the H295R steroidogenesis assay: (1) Measured concentrations of POPs in silicone breast implants were taken from a recent study and silicone disks were loaded according to these measurements. (2) Silicone disks were transferred into H295R cell culture plates in order to control exposure of the adrenal cells by equilibrium partitioning. (3) Hormone production of the adrenal cells was measured as toxicity endpoint. 4-Nonylphenol was used for method development, and the new dosing method was compared to conventional solvent-dosing. The two dosing modes yielded similar dose-dependent hormonal responses of H295R cells. However, with the partitioning-controlled freely dissolved concentrations (Cfree) as dose metrics, dose–response curves were left-shifted by two orders of magnitude relative to spiked concentrations. Partitioning-controlled dosing of POPs resulted in up to 2-fold increases in progestagen and corticosteroid levels at Cfree of individual POPs in or below the femtomolar range. Silicone acted not only as source of the POPs but also as a sorption sink for lipophilic hormones, stimulating the cellular hormone production. Methodologically, the study showed that silicone can be used as reference partitioning phase to transfer in vivo exposure in humans (silicone implants) to in vitro assays (partition-controlled dosing). The main finding was that POPs at the levels at which they are found in humans can interfere with steroidogenesis in a human adrenocortical cell line.

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Exposure to perfluorononanoic acid combined with a low-dose mixture of 14 human-relevant compounds disturbs energy/lipid homeostasis in rats

Humans are constantly exposed to a significant number of compounds and many are readily detected in human body fluids. Worryingly, several of these compounds are either suspected to be, or have already been shown to be harmful to humans either individually or in combination. However, the potential consequences of low-dose exposure to complex mixtures remain poorly understood. We have profiled the effects on rat blood plasma and liver homeostasis using metabolomics and transcriptomics following 2-week exposure to either a mixture of 14 common chemicals (Mix), perfluorononanoic acid (PFNA) at low (0.0125 mg/kg/day) or mid (0.25 mg/kg/day) doses, or a combination of Mix and PFNA. In blood plasma, 63 and 64 metabolites were significantly changed upon exposure to Mix alone or PFNA + Mix, respectively. Twelve of the metabolites were identified and comprised mainly lipids, with various lipid classes differentially affected across study groups. In the liver, expression of 182 and 203 genes—mainly related to energy homeostasis and lipid metabolism—were differentially expressed upon exposure to PFNA alone or PFNA + Mix, respectively. In general, Mix alone affected lipid metabolism evident in blood plasma, whereas effects on lipid metabolism in the liver were mainly driven by PFNA. This study verifies that a chemical mixture given at high-end human exposure levels can affect lipid homeostasis and that the combined use of metabolomics and transcriptomics can provide complimentary information allowing for a detailed analysis of affected signaling pathways.
Mixture effects of twenty-seven environmental contaminants given to rats at doses comparable to human exposure

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Research output: Contribution to conference › Poster – Annual report year: 2015 › Research › peer-review

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

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.

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Perinatal exposure to mixtures of anti-androgenic chemicals causes proliferative lesions in rat prostate

BACKGROUND:
Elevated levels of endogenous or exogenous estrogens during fetal life can induce permanent disturbances in prostate growth and predispose to precancerous lesions. Recent studies have indicated that also early anti-androgen exposure may affect prostate cancer risk.

METHODS:
We examined the influence of perinatal exposure to mixtures of anti-androgenic and estrogenic chemicals on prostate development. Wistar rats were exposed from gestation day 7 to postnatal day 22 to a mixture of 8 anti-androgenic compounds (AAMix), a mixture of four estrogenic compounds (EMix), or paracetamol or a mixture of all 13 compounds (TotalMix) in mixture ratios reflecting human exposure levels.

RESULTS:
Ventral prostate weights were reduced by the TotalMix and AAMix in pre-pubertal rats. Histological changes in prostate appeared with increasing age and indicated a shift from the normal age-dependent epithelial atrophy towards hyperplasia. These lesions showed similarities to pre-cancerous lesions in humans. Increased proliferation was observed already in pre-puberty and it was hypothesized that this could be associated with reduced ERβ signaling, but no clear conclusions could be made from gene expression studies on ERβ-related pathways. The influences of the estrogenic chemicals and paracetamol on prostate morphology were minor, but in young adulthood the estrogen mixture reduced ventral prostate mRNA levels of Igf1 and paracetamol reduced the mRNA level of Ppdc3.

CONCLUSIONS:
Mixtures of endocrine disruptors relevant for human exposure was found to elicit persistent effects on the rat prostate following perinatal exposure, suggesting that human perinatal exposure to environmental chemicals may increase the risk of prostate cancer later in life. Prostate.
OBJECTIVES:
In the same birth cohort, we investigated whether prenatal POP exposure was associated with serum concentrations of insulin and leptin among 5-year-old children, thus possibly mediating the association with overweight and obesity at 7 years of age.

METHODS:
The analyses were based on a prospective Faroese Birth Cohort (n=656), recruited between 1997 and 2000. Major POPs, polychlorinated biphenyls (PCBs), p,p'-dichlorodiphenyldichloroethylene (DDE) and hexachlorobenzene (HCB), were measured in maternal pregnancy serum and breast milk. Children were followed-up at the age of 5 years where a non-fasting blood sample was drawn; 520 children (273 boys and 247 girls) had adequate serum amounts available for biomarker analyses by Luminex® technology. Insulin and leptin concentrations were transformed from continuous to binary variables, using the 75th percentile as a cut-off point. Multiple logistic regression was used to investigate associations between prenatal POP exposures and non-fasting serum concentrations of insulin and leptin at age 5 while taking into account confounders.

RESULTS:
Girls with highest prenatal POP exposure were more likely to have high non-fasting insulin levels (PCBs 4th quartile: OR=3.71; 95% CI: 1.36, 10.01. DDE 4th quartile: OR=2.75; 95% CI: 1.09, 6.90. HCB 4th quartile: OR=1.98; 95% CI: 1.06, 3.69) compared to girls in the lowest quartile. No significant associations were observed with leptin, or among boys. A mediating effect of insulin or leptin on later obesity was not observed.

CONCLUSION:
These findings suggest, that for girls, prenatal exposure to POPs may play a role for later development of metabolic diseases by affecting the level of insulin.
A computational approach to mechanistic and predictive toxicology of pesticides

Emerging challenges of managing and interpreting large amounts of complex biological data have given rise to the growing field of computational biology. We investigated the applicability of an integrated systems toxicology approach on five selected pesticides to get an overview of their modes of action in humans, to group them according to their modes of action, and to hypothesize on their potential effects on human health. We extracted human proteins associated to prochloraz, tebuconazole, epoxiconazole, procymidone, and mancozeb and enriched each protein set by using a high confidence human protein interactome. Then, we explored modes of action of the chemicals, by integrating protein-disease information to the resulting protein networks. The dominating human adverse effects affected were reproductive disorders followed by adrenal diseases. Our results indicated that prochloraz, tebuconazole, and procymidone exerted their effects mainly via interference with steroidogenesis and nuclear receptors. Prochloraz was associated to a large number of human diseases, and together with tebuconazole showed several significant associations to Testicular Dysgenesis Syndrome. Mancozeb showed a differential mode of action, involving inflammatory processes. This method provides an efficient way of overviewing data and grouping chemicals according to their mode of action and potential human adverse effects. Such information is valuable when dealing with predictions of mixture effects of chemicals and may contribute to the development of adverse outcome pathways.
Applicability of Computational Systems Biology in Toxicology

Systems biology as a research field has emerged within the last few decades. Systems biology, often defined as the antithesis of the reductionist approach, integrates information about individual components of a biological system. In integrative systems biology, large data sets from various sources and databases are used to model and predict effects of chemicals on, for instance, human health. In toxicology, computational systems biology enables identification of important pathways and molecules from large data sets; tasks that can be extremely laborious when performed by a classical literature search. However, computational systems biology offers more advantages than providing a high-throughput literature search; it may form the basis for establishment of hypotheses on potential links between environmental chemicals and human diseases, which would be very difficult to establish experimentally. This is possible due to the existence of comprehensive databases containing information on networks of human protein–protein interactions and protein–disease associations. Experimentally determined targets of the specific chemical of interest can be fed into these networks to obtain additional information that can be used to establish hypotheses on links between the chemical and human diseases. Such information can also be applied for designing more intelligent animal/cell experiments that can test the established hypotheses. Here, we describe how and why to apply an integrative systems biology method in the hypothesis-generating phase of toxicological research.

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Source-ID: u::10984
Research output: Contribution to journal › Journal article – Annual report year: 2013 › Research › peer-review

Are Structural Analogues to Bisphenol A Safe Alternatives?
Background: Bisphenol A (BPA) is a chemical with widespread human exposure suspected of causing low-dose effects. Thus, a need for developing alternatives to BPA exists. Structural analogues of BPA have already been detected in foods and humans. Due to the structural analogy of the alternatives, there is a risk of effects similar to BPA.Objectives: The aim was to elucidate and compare the hazards of bisphenol B (BPB), bisphenol E (BPE), bisphenol F (BPF), bisphenol S (BPS) and 4-cumylphenol (HPP) to BPA.Methods: In vitro studies on steroidogenesis, receptor activity, and biomarkers of effect, as well as Quantitative Structure-Activity Relationship (QSAR) modeling Results: All test compounds caused the same qualitative effects on estrogen receptor and androgen receptor activities, and most of the alternatives exhibited potencies within the same range as BPA. Hormone profiles for the compounds indicated a specific mechanism of action on steroidogenesis which generally lead to decreased androgen, and increased estrogen and progesteragen levels. Differential effects on corticosteroid synthesis were observed suggesting a compound-specific mechanism. Overall, BPS was less estrogenic and antiandrogenic than BPA, but BPS showed the largest efficacy on 17α-hydroxyprogesterone
(17α-OH progesterone). Finally, there were indications of DNA damage, carcinogenicity, oxidative stress, effects on metabolism, and skin sensitization of one or more of the test compounds. Conclusions: Interference with the endocrine system was the predominant effect of the test compounds. A substitution of BPA with these structural analogues should be carried out with caution.

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Compounds in food packaging materials - toxicological profiling of knowns and unknowns
Food contact materials (FCMs) are sources of food contamination and human chemical exposure. Some chemicals in these materials are known to cause adverse effects, but many are poorly characterized for their potential toxicological hazards making risk assessment a challenge. The aim of the project was to obtain knowledge on the potential hazards posed by chemicals present in FCMs through examining compounds with known usages or suspected of being used in these materials, namely bisphenol A (BPA), BPA analogues and fluorinated substances. Furthermore, we developed a strategy to identify problematic compounds present in these materials. Specific focus was placed on in vitro endpoints assessing endocrine activity. BPA, five BPA analogues, and 19 fluorinated substances including fluorochemical containing technical mixtures (TMs) were investigated. The in vitro assays included the androgen receptor (AR), estrogen receptor (ER), aryl hydrocarbon receptor, retinoic acid receptor, glucocorticoid receptor, p53, and nuclear factor (erythroid-derived 2)-like 2 reporter gene assays, and the H295R steroidogenesis assay. The FCM strategy was a step-by-step procedure in which extracts from FCMs of paper and board were tested in vitro, active extracts were fractionated and tested in vitro, tentative identification was performed in active fractions, and tentatively identified compounds were tested in vitro and quantified in the extract. BPA analogues generally led to similar estrogenic and antiandrogenic effects in vitro compared to BPA. However, the BPA analogue BPS caused less marked effects on most of these endpoints, but led to a more pronounced effects on progestagen levels compared to BPA. Likewise, the effects on corticoid levels in the H295R steroidogenesis assay differed between the six compounds. These data suggest that the tested analogues overall have similar effects on the parameters investigated, though some differed. Therefore based on the presented data, the BPA analogues may not be suitable BPA alternatives. The fluorinated chemicals exhibited endocrine activities distinct from one another, but subgroups of fluorinated chemicals had similar profiles. Polyfluoroalkyl phosphate ester surfactants (PAPs) generally decreased progestagen and androgen levels, fluorotelomer alcohols (FTOHs) generally increased ER activity, and long-chained perfluorinated alkyl carboxylic acids (PFCAs) generally increased 17β-estradiol levels. Two TMs caused estrogenicity in vitro, whereas none of the short-chained PFCAs caused effects. It is recommended to conduct further studies on polyfluorinated chemicals as well as TMs to obtain more information on the implications of these differences in effect. The developed FCM strategy proved useful for identifying potential problematic compounds in FCMs of paper and board. All extracts from FCMs led to effects in at least one of the applied in vitro assays and we successfully identified five causative agents in two FCMs when applying the full strategy. It is recommended to test more FCMs of paper and board with the strategy to obtain information on other potentially problematic compounds present in these materials. The presented data overall suggest that some compounds present in FCMs or suspected of being used can exert endocrine activities in vitro, though the implications of these findings with respect to effects in humans and exposure to humans remain largely unknown. Nevertheless, it is of concern that so many of the materials and compounds led to effects and
calls for further studies to be conducted. The data obtained in this PhD can be used as a prioritization tool for these purposes.

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**Discovery and Early Clinical Development of 2-{6-[2-(3,5-Dichloro-4-pyridyl)acetyl]-2,3-dimethoxyphenoxy}-N-propylacetamide (LEO 29102), a Soft-Drug Inhibitor of Phosphodiesterase 4 for Topical Treatment of Atopic Dermatitis**

Development of orally available phosphodiesterase 4 (PDE4) inhibitors as anti-inflammatory drugs has been going on for decades. However, only roflumilast has received FDA approval. One key challenge has been the low therapeutic window observed in the clinic for PDE4 inhibitors, primarily due to PDE4 mediated side effects. Here we describe our approach to circumvent this issue by applying a soft-drug concept in the design of a topically acting PDE4 inhibitor for treatment of dermatological diseases. We used a fast follower approach, starting from piclamilast. In particular, simultaneous introduction of 2'-alkoxy substituents and changing an amide to a keto linker proved to be beneficial when designing potential soft-drug candidates. This effort culminated in identification of LEO 29102 (20), a potent, selective, and soft-drug PDE4 inhibitor with properties suitable for patient-friendly formulations giving efficient drug delivery to the skin. Compound 20 has reached phase 2 and demonstrated clinically relevant efficacy in the treatment of atopic dermatitis.

**Green Toxicology – Application of predictive toxicology**
Humans are constantly challenged by exposure to a cocktail of chemicals that can have negative health effects, and fetuses and young children are particularly vulnerable. Therefore, we need safer chemicals in order to reduce any potential environmental and human hazards. A solid framework to design safer chemicals and to identify problematic compounds already in use such as industrial compounds, drugs, pesticides and cosmetics, is required. Green toxicology is the application of predictive toxicology to the production of chemicals with the specific intent of improving their design for hazard reduction. This objective is partly achieved through core principles of green chemistry. However, better utilization
of existing predictive toxicological tools alongside new inventions is still required. For this, input from toxicologists early in the chemical enterprise is necessary to make informed choices on molecular design. Current tools, including (Quantitative) Structure-Activity Relationships ((Q)SARs) for predicting toxicity, Physiologically Based Kinetic (PBK) dynamic modeling for predicting absorption, distribution, metabolism and excretion, as well as human cell-based methods, deserve to be applied in chemical risk assessment to a greater extent than is currently the case. Greater focus on these tools, their strengths and weaknesses, should be part of chemistry training at the university level, thus ensuring constant focus on the issue and fostering new inventions into the future.

Late-life effects on rat reproductive system after developmental exposure to mixtures of endocrine disrupters.

This study examined late-life effects of perinatal exposure of rats to a mixture of endocrine-disrupting contaminants. Four groups of 14 time-mated Wistar rats were exposed by gavage from gestation day 7 to pup day 22 to a mixture of 13 anti-androgenic and estrogenic chemicals including phthalates, pesticides, u.v.-filters, bisphenol A, parabens, and the drug paracetamol. The groups received vehicle (control), a mixture of all 13 chemicals at 150-times (TotalMix150) or 450-times (TotalMix450) high-end human exposure, or 450-times a mixture of nine predominantly anti-androgenic chemicals (AAMix450). Onset of puberty and estrous cyclicity at 9 and 12 months of age were assessed. Few female offspring showed significantly regular estrus cyclicity at 12 months of age in the TotalMix450 and AAMix450 groups compared with controls. In 19-month-old male offspring, epididymal sperm counts were lower than controls, and in ventral prostate an overrepresentation of findings related to hyperplasia was observed in exposed groups compared with controls, particularly in the group dosed with anti-androgens. A higher incidence of pituitary adenoma at 19 months of age was found in males and females in the AAMix450 group. Developmental exposure of rats to the highest dose of a human-relevant mixture of endocrine disrupters induced adverse effects late in life, manifested as earlier female reproductive senescence, reduced sperm counts, higher score for prostate atypical hyperplasia, and higher incidence of pituitary tumors. These delayed effects highlight the need for further studies on the role of endocrine disrupters in hormone-related disorders in aging humans.
Low-dose effects of bisphenol A on early sexual development in male and female rats.
Bisphenol A (BPA) is widely detected in human urine and blood. BPA has been reported to impair many endpoints for reproductive and neurological development; however, it is controversial whether BPA has effects in the microgram per kilogram dose range. The aim of the current study was to examine the influence of BPA on early sexual development in male and female rats at dose levels covering both regulatory no observed adverse effect levels (NOAELs) (5 and 50 mg/kg bw per day) as well as doses in the microgram per kilogram dose range (0.025 and 0.25 mg/kg bw per day). Time-mated Wistar rats (n=22) were gavaged during pregnancy and lactation from gestation day 7 to pup day 22 with 0, 0.025, 0.25, 5 or 50 mg/kg bw per day BPA. From 0.250 mg/kg and above, male anogenital distance (AGD) was significantly decreased, whereas decreased female AGD was seen from 0.025 mg/kg bw per day and above. Moreover, the incidence of nipple retention in males appeared to increase dose relatedly and the increase was statistically significant at 50 mg/kg per day. No significant changes in reproductive organ weights in the 16-day-old males and females and no signs of maternal toxicity were seen. The decreased AGD at birth in both sexes indicates effects on prenatal sexual development and provides new evidence of low-dose adverse effects of BPA in rats in the microgram per kilogram dose range. The NOAEL in this study is clearly below 5 mg/kg for BPA, which is used as the basis for establishment of the current tolerable daily intake (TDI) by EFSA; thus a reconsideration of the current TDI of BPA appears warranted.

Polyfluorinated alkyl phosphate ester surfactants - current knowledge and knowledge gaps
Fluorochemicals are a diverse group of synthetically produced compounds with the unique ability to repel water as well as oil. This property makes them ideal for multiple purposes in a variety of consumer and industrial products. Fluorochemicals have been detected in the environment, as well as in human blood, urine and milk. Due to their long half-life in human beings, there is an increased risk that exposure to these compounds can cause adverse effects. However, except for perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), there is a large data gap regarding toxicological information on fluorochemicals. Polyfluorinated alkyl phosphate ester surfactants (PAPs) belong to the group of polyfluorinated alkyl surfactants. They have been detected in indoor dust and are widely used in food-contact materials, from which they have the ability to migrate into food. Toxicological data on PAPs are very limited, but current studies indicate that some PAPs have the potential to interfere with sex hormone synthesis in vitro. Disturbance of the sex hormone balance in foetal life has been suggested to be an important mechanism involved in adverse effects on, for example, male reproductive health and development. The current lack of toxicological data on PAPs impairs the risk assessment of this group of compounds. However, until more toxicological data on PAPs are available, the limited data currently accessible give reason to believe that these compounds might have the ability to cause potentially adverse effects, as seen for other perfluorinated chemicals, including some metabolic products of PAPs.

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A low dose chemical mixture modulates the effect of PFNA in male rats

Purpose: When mathematical models are applied for assessment of chemical mixture effects, assumptions of lack of synergy or potentiation have to be made; Thus joint effects of chemicals are anticipated not to be unexpectedly high. The present investigation was designed to test whether a chemical mixture at human relevant doses interacts with perfluorononanoic acid (PFNA) by synergy or potentiation.

Methods: Male rats were given PFNA at 0.0125, 0.25 or 5 mg/kg bw/day for 14 days with or without a mixture containing 12 endocrine disrupters (total dose 2.5 mg/kg bw/day), bergamottin from grapefruit juice (0.2 mg/kg bw/day), and glabridin from liquorice (0.3 mg/kg bw/day). Rats were examined pathologically, plasma hormones and mRNA levels of several enzymes were measured, and metabolomic profiling of plasma was performed.

Results and conclusion: High dose PFNA exhibited liver steatosis and effects on testes enzyme mRNA levels. These effects were similar both with and without mixture. In contrast, co-treatment with mixture increased both relative and absolute liver weights of the 5 mg/kg/day PFNA, suggesting that liver toxicity was exacerbated by the mixture.

These data suggest that a chemical mixture at a human relevant dose potentiates the response of the rat to PFNA, but not at a human relevant dose of PFNA. Thus, with the parameters measured to date, this investigation supports the hypothesis that synergism or potentiation due to ADME interactions are absent at human relevant doses.
Are structural analogues to bisphenol A a safe alternative?

Bisphenol A (BPA) is often used in polycarbonate plastics, coatings of food and drink cans, and in thermal papers. Foods are thought to be a major human exposure route and human biomonitoring data suggest widespread exposure. BPA is suspected of contributing to effects such as increased birth weight, behavioral changes in children, cardiovascular disease, and diabetes. Regulatory initiatives and increased public awareness, related to the potential adverse effects of BPA, has led to an incitement to find alternative compounds. Structural analogues of BPA are available on the market, some of which are found in foods and have been measured in humans. Due to the structural analogy there is an inherent risk that these compounds may lead to similar effects as BPA.

The aim of this study was to characterize the toxicological profile of BPA and the five analogues using in vitro assays assessing effects on ER, AR, aryl hydrocarbon receptor (AhR), retinoic acid receptor (RAR) and glucocorticoid receptor (GR) activation, effects on steroidogenesis, potential to cause oxidative stress (Nrf2 assay) and genotoxic potential (P53 assay).

Overall the qualitative effects were similar for the BPs tested; however differences in quantitative effects were observed in some cases. All BPs showed antiandrogenic and estrogenic potential and potential to affect steroidogenesis. The site of interference in steroidogenesis appears specific. Results obtained from Nrf2, p53, and AhR reporter gene assays showed that some or all BPs have the potential to activate these assays at high concentrations (LOEC > 50 μM).
Concentration addition, independent action and generalized concentration addition models for mixture effect prediction of sex hormone synthesis in vitro.

Humans are concomitantly exposed to numerous chemicals. An infinite number of combinations and doses thereof can be imagined. For toxicological risk assessment the mathematical prediction of mixture effects, using knowledge on single chemicals, is therefore desirable. We investigated pros and cons of the concentration addition (CA), independent action (IA) and generalized concentration addition (GCA) models. First we measured effects of single chemicals and mixtures thereof on steroid synthesis in H295R cells. Then single chemical data were applied to the models; predictions of mixture effects were calculated and compared to the experimental mixture data. Mixture 1 contained environmental chemicals adjusted in ratio according to human exposure levels. Mixture 2 was a potency adjusted mixture containing five pesticides. Prediction of testosterone effects coincided with the experimental Mixture 1 data. In contrast, antagonism was observed for effects of Mixture 2 on this hormone. The mixtures contained chemicals exerting only limited maximal effects. This hampered prediction by the CA and IA models, whereas the GCA model could be used to predict a full dose response curve. Regarding effects on progesterone and estradiol, some chemicals were having stimulatory effects whereas others had inhibitory effects. The three models were not applicable in this situation and no predictions could be performed. Finally, the expected contributions of single chemicals to the mixture effects were calculated. Prochloraz was the predominant but not sole driver of the mixtures, suggesting that one chemical alone was not responsible for the mixture effects. In conclusion, the GCA model seemed to be superior to the CA and IA models for the prediction of testosterone effects. A situation with chemicals exerting opposing effects, for which the models could not be applied, was identified. In addition, the data indicate that in non-potency adjusted mixtures the effects cannot always be accounted for by single chemicals.
Dietary relevant mixtures of phytoestrogens inhibit adipocyte differentiation in vitro

Phytoestrogens (PEs) are naturally occurring plant components, with the ability to induce biological responses in vertebrates by mimicking or modulating the action of endogenous hormones. Single isoflavones have been shown to affect adipocyte differentiation, but knowledge on the effect of dietary relevant mixtures of PEs, including for instance lignans, is lacking. In the current study dietary relevant mixtures of isoflavones and their metabolites, lignans and their metabolites, coumestrol, and a mixture containing all of them, were examined for effects on adipogenesis in 3T3-L1 adipocytes, as well as tested for their PPARγ activating abilities. The results showed that mixtures of isoflavonoid parent compounds and metabolites, respectively, a mixture of lignan metabolites, as well as coumestrol concentration-dependently inhibited adipocyte differentiation. Furthermore, a mixture of isoflavonoid parent compounds, and a mixture of isoflavonoid metabolites were found to have PPARγ activating abilities. These results suggest that PEs can affect pathways known to play a role in obesity development, and indicate that the inhibitory effect on adipocyte differentiation does not appear to be strictly associated with PPARγ activation/inhibition. The current study support the hypothesis that compounds with endocrine activity can affect pathways playing a role in the development obesity and obesity related diseases.

Effects of perinatal ethinyl estradiol exposure in male and female Wistar rats

Perinatal exposure to endocrine disrupting chemicals with estrogenic activity can adversely affect reproductive development, but few studies evaluating estrogen-sensitive endpoints have been performed in Wistar rats. Therefore, time-mated Wistar rats (n=10) were gavaged during gestation and lactation with 0, 5, 15 or 50μg/kg bw/day of ethinyl estradiol. This potent estrogen was found to induce an increased number of nipples and reduced ovary weight in female offspring. Malformations of female genitalia were found in young as well as adult offspring, as an increased AGD was seen at birth and a deeper urethral slit length was seen in adulthood. In prepubertal male offspring, estrogen-regulated gene expression in ventral prostate was increased dose-dependently and a decreased ventral prostate weight was seen at 15μg/kg. Female external sexual characteristics and prostate development were found to be targets for exposure to estrogenic compounds and may be of interest in studies on estrogenic environmental compounds.
Endocrine disrupting effects in rats perinatally exposed to a dietary relevant mixture of phytoestrogens

Dietary phytoestrogens may prevent certain human diseases, but endocrine activity has been reported in animal studies. Sprague-Dawley rats were exposed perinatally to a 1-, 10- or 100-fold “high human dietary intake” mixture of 12 phytoestrogens consisting of mainly the lignan secoisolarici resinol and the isoflavones genistein and daidzein. This mixture induced persistent adverse effects, as adult male mammary glands showed hypertrophic growth. A reduced anogenital distance in newborn males indicated an anti-androgenic mode of action. Testosterone levels, testis and prostate weights, and expression of selected genes in testis and prostate were unaffected. Decreased serum estradiol was seen in genistein-exposed dams. This study indicated adverse effects at high intake levels in rats, but does not provide evidence for risk of phytoestrogen-mediated endocrine disruption at normal human dietary consumption levels. Further studies are warranted to increase the knowledge upon which risk assessment on dietary phytoestrogen exposure during pregnancy and infancy is based.
Fluorochemicals used in food packaging inhibit male sex hormone synthesis
Polyfluoroalkyl phosphate surfactants (PAPS) are widely used in food contact materials (FCMs) of paper and board and have recently been detected in 57% of investigated materials. Human exposure occurs as PAPS have been measured in blood; however knowledge is lacking on the toxicology of PAPS. The aim of this study was to elucidate the effects of six fluorochemicals on sex hormone synthesis and androgen receptor (AR) activation in vitro. Four PAPS and two metabolites, perfluorooctanoic acid (PFOA) and 8:2 fluorotelomer alcohol (8:2 FTOH) were tested. Hormone profiles, including eight steroid hormones, generally showed that 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH led to decreases in androgens (testosterone, dehydroepiandrosterone, and androstenedione) in the H295R steroidogenesis assay. Decreases were observed for progesterone and 17-OH-progesterone as well. These observations indicated that a step prior to progestagen and androgen synthesis had been affected. Gene expression analysis of StAR, Bzrp, CYP11A, CYP17, CYP21 and CYP19 mRNA showed a decrease in Bzrp mRNA levels for 8:2 monoPAPS and 8:2 FTOH indicating interference with cholesterol transport to the inner mitochondria. Cortisol, estrone and 17β-estradiol levels were in several cases increased with exposure. In accordance with these data CYP19 gene expression increased with 8:2 diPAPS, 8:2 monoPAPS and 8:2 FTOH exposures indicating that this is a contributing factor to the decreased androgen and the increased estrogen levels. Overall, these results demonstrate that fluorochemicals present in food packaging materials and their metabolites can affect steroidogenesis through decreased Bzrp and increased CYP19 gene expression leading to lower androgen and higher estrogen levels.

Identification of a Novel Androgen Receptor Mutation in a Family With Multiple Components Compatible With the Testicular Dysgenesis Syndrome
Context: Androgen signaling via the androgen receptor (AR) is essential for normal testis development and male reproductive functions. We describe a rare family with 3 males affected by a mild disorder of sex determination compatible with testicular dysgenesis syndrome (TDS), including subfertility, cryptorchidism, hypospadias, and testicular cancer, caused by a novel AR mutation.Objective: The aim of this study was to describe the phenotype of the affected males, characterize functionally the novel AR mutation, and discuss the significance of partial androgen insufficiency in the pathogenesis of TDS.Participants: The proband, his first cousin, and a nephew underwent a detailed clinical investigation including genetic tests, whereas four female members of the family were tested for the specific AR mutation.Results: A novel AR mutation, c.2214T>G;p.Ile738Met, was identified in the affected family members. Functional analysis of the mutation in a gene-reporter assay showed a 50% reduction in AR-induced transcriptional activity. The affected males had elevated LH and T in accordance with decreased AR signaling. The histology and immunohistochemical profile of the testis tissue from the 2 patients with testicular cancer showed features consistent with insufficient testis development and TDS.Conclusion: The presence of all hallmarks of TDS, including germ cell cancer, in a family with a novel AR mutation causing a partial decrease in AR function is in line with the concept that reduced androgen signaling may contribute to the
development of TDS. It also seems consistent with the hypothesis that environmental factors interfering with this pathway can play a role in the pathogenesis of TDS.

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In vitro–in vivo correlations for endocrine activity of a mixture of 5 currently used pesticides
Two pesticide mixtures consisting of three and five currently used pesticides, respectively, were investigated for potential endocrine disrupting effects. Mix 3 consisted of bitertanol, propiconazole, and cypermethrin and Mix 5 contained the same 3 pesticides plus malathion and terbuthylazine. All five single pesticides and the two mixtures were tested in vitro in H295R cells for effects on steroidogenesis. All five pesticides individually and both mixtures affected steroidogenesis. The mixtures caused an increase in progesterone and a decrease in testosterone, and Mix 5 also increased estradiol, indicating increased aromatase activity.

The pesticide-mixtures were also investigated in vivo in pregnant rats dosed from gestational day 7 to 21, followed by examination of dams and fetuses. All 5 pesticides could be detected in the amniotic fluid, demonstrating exposure of the fetuses. Decreased estradiol and increased T4 levels were seen in dams exposed to Mix 5. Neither of the two mixtures showed any effects on fetal hormone levels, but Mix 5 did cause a significant increase in aromatase mRNA levels in adrenal glands from female fetuses. Overall, aromatase induction in adrenals was found for Mix 5 both in vitro and in vivo. However, the hormonal responses in vitro were only partly reflected in vivo, probably due to ADME interactions, as the amniotic pesticide levels were negatively affected by the number of compounds present in the mixtures. Nonetheless, the H295R assay gives hints on interference with steroidogenesis, and it is suggested that one underlying mechanism for these pesticides is disturbance of steroidogenic enzymes.

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In vitro - in vivo correlations for endocrine activity of a mixture of currently used pesticides

Two pesticide mixtures were investigated for potential endocrine activity. Mix 3 consisted of bitertanol, propiconazole, and cypermethrin, and Mix 5 included malathion and terbuthylazine in addition to the three pesticides in Mix 3. All five single pesticides and the two mixtures were investigated for their ability to affect steroidogenesis in vitro in H295R cells. The pesticides alone and both mixtures affected steroidogenesis with both mixtures causing increase in progesterone and decrease in testosterone. For Mix 5 an increase in estradiol was seen as well, indicating increased aromatase activity. The two mixtures were also investigated in pregnant rats dosed from gestational day 7 to 21, followed by examination of dams and fetuses. Decreased estradiol and reduced placental testosterone were seen in dams exposed to Mix 5. Also a significant increase in aromatase mRNA-levels in female adrenal glands was found for Mix5. However, either of the two mixtures showed any effects on fetal hormone levels in plasma or testis, or on anogenital distance. Overall, potential aromatase induction was found for Mix 5 both in vitro and in vivo, but not for Mix 3, an effect likely owed to terbuthylazine in Mix 5. However, the hormonal responses in vitro were only partly reflected in vivo, probably due to some toxicokinetic issues, as the pesticide levels in the amniotic fluid also were found to be negatively affected by the number of compounds present in the mixtures. Nonetheless, the H295R assay gives hints on conceivable interference with steroidogenesis, thus generating hypotheses on in vivo effects.
Non-monotonous dose–response curves observed for some endpoints, after pre- and postnatal exposure of rats to the fungicide epoxiconazole
Predictive value of cell assays for developmental toxicity and embryotoxicity of conazole fungicides.
This paper evaluates in vivo predictability of a battery of in vitro tests covering developmental toxicity and embryotoxicity of five widely used conazole fungicides. The conazoles were investigated in the embryonic stem cell test, and data were compared to in vivo embryotoxicity data. The same conazoles were evaluated on the basis of data from a battery of cell assays for endocrine activity, including assays for AR, ER, AhR, and sex hormone synthesis, and data were compared to in vivo developmental toxicity data. Overall, the ranking of the five conazole fungicides based on in vitro data were in reasonably good agreement with available in vivo effects. Ketoconazole and epoxiconazole are the most potent embryotoxic compounds, whereas prochloraz belongs to the most potent developmental toxicants. In conclusion, a rough prediction of the ranking of these conazole fungicides for in vivo toxicity data was possible by a holistic evaluation of data from a panel of cell-based assays.

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Triclosan exposure reduces thyroxine levels in pregnant and lactating rat dams and in directly exposed offspring
Thyroid-disrupting chemicals can potentially disrupt brain development. Two studies investigating the effect of the antibacterial compound triclosan on thyroxine (T4) levels in rats are reported. In the first, Wistar rat dams were gavaged with 75, 150 or 300 mg triclosan/kg bw/day throughout gestation and lactation. Total T4 serum levels were measured in dams and offspring, and all doses of triclosan significantly lowered T4 in dams, but no significant effects on T4 levels were seen in the offspring at the end of the lactation period. Since this lack of effect could be due to minimal exposure through maternal milk, a second study using direct per oral pup exposure from postnatal day 3–16 to 50 or 150 mg triclosan/kg bw/day was performed. This exposure pointed to significant T4 reductions in 16 day old offspring in both dose groups. These results corroborate previous studies showing that in rats lactational transfer of triclosan seems limited. Since an optimal study design for testing potential developmental neurotoxicants in rats, should include exposure during both the pre- and postnatal periods of brain development, we suggest that in the case of triclosan, direct dosing of pups may be the best way to obtain that goal. © 2013 Elsevier Ltd. All rights reserved.

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

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.

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Applicability of the GCA model for effect prediction of endocrine disrupters in mixture

General information
Developmental toxicity effects in experimental animals after mixed exposure to endocrine disrupting pesticides

Eleven environmental relevant chemicals were investigated for their ability to affect adipogenesis in vitro, biomarker release from adipocytes and PPARα and γ activation. We found that butylparaben stimulated adipogenesis in 3T3-L1 adipocytes and increased release of leptin, adiponectin and resistin from the cells. Butylparaben activated PPARγ as well, which may be a mediator of the adipogenic effect. Polychlorinated biphenyl (PCB)153 also stimulate adipogenesis and biomarker release, but did not affect PPARs. The data indicates that PPARγ activating chemicals often stimulate adipocyte differentiation although PPARy activation is neither a requirement nor a guarantee for stimulation. Four out of the eleven chemicals (bisphenol A, mono-ethylhexyl phthalate, butylparaben, PCB 153) caused increased adipogenesis. The release of adipocyte-secreted hormones was sometimes but not always correlated with the effect on adipocyte differentiation. Eight chemicals were able to cause increased leptin release. These findings strengthen the hypothesis that chemicals can interfere with pathways related to obesity development.
Effect of environmental pollutants on obesity parameters in vitro

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Fluorochemicals used in food packaging inhibit male sex hormone synthesis

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

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.
Differential effects of environmental chemicals and food contaminants on adipocyte differentiation, biomarker release, and PPARα/γ activation

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Reproductive and behavioral effects of diisononyl phthalate (DINP) in perinatally exposed rats

Disisononyl phthalate (DINP) is a plasticizer abundantly used in consumer products as a substitute for other plasticizers prohibited in certain products due to reproductive toxicity. As anti-androgenic effects of DINP are suspected, DINP effects on reproduction and sexually dimorphic behavior were studied. Pregnant Wistar rats were gavaged from gestation day 7 to postnatal day (PND) 17 with vehicle, 300, 600, 750 or 900mg DINP/kg bw/day. In fetal testes histopathological effects typical of phthalates were observed. In male offspring, DINP caused increased nipple retention, reduced anogenital distance, reduced sperm motility and increased sperm count. DINP affected spatial learning as female offspring performed better than controls and similarly to control males in the Morris Water Maze, indicating masculinization of behavior in DINP exposed females. These results show that DINP causes anti-androgenic effects on reproductive development, though less potent than DEHP, DBP and BBP, and further safety evaluation of DINP appears warranted.

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The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study

In response to increasing concerns regarding the potential of chemicals to interact with the endocrine system of humans and wildlife, various national and international programs have been initiated with the aim to develop new guidelines for the screening and testing of these chemicals in vertebrates. Here, we report on the validation of an in vitro assay, the H295R steroidogenesis assay, to detect chemicals with the potential to inhibit or induce the production of the sex steroid hormones testosterone (T) and 17β-estradiol (E2) in preparation for the development of an Organization for Economic Cooperation and Development (OECD) test guideline. A previously optimized and pre-validated protocol was used to assess the potential of 28 chemicals of diverse structures and properties to validate the H295R steroidogenesis assay. These chemicals are comprised of known endocrine-active chemicals and “negative” chemicals that were not expected to have effects on the targeted endpoints, as well as a number of test chemicals with unknown modes of action at the level of the steroidogenic pathway. A total of seven laboratories from seven countries participated in this effort. In addition to effects on hormone production, confounding factors, such as cell viability and possible direct interference of test substances with antibody-based hormone detection assays, were assessed. Prior to and during the conduct of exposure experiments, each laboratory had to demonstrate that they were able to conduct the assay within the margin of predefined performance criteria. With a few exceptions, all laboratories met the key quality performance parameters, and only 2% and 7% of all experiments for T and E2, respectively, were excluded due to exceedance of these parameters. Of the 28 chemicals analyzed, 13 and 14 tested affected production of T and E2, respectively, while 11 and 8 did not result in significant effects on T and E2 production, respectively. Four and six chemicals produced ambiguous results for effects on T and E2 production, respectively. However, four of these cases each for T and E2 were associated with only one laboratory after a personnel change occurred. Significant interference of test chemicals with some of the antibody-based hormone detection systems occurred for four chemicals. Only one of these chemicals, however, significantly affected the ability of the detection system to categorize the chemical as affecting E2 or T production. With one exception, the H295R steroidogenesis assay protocol successfully identified the majority of chemicals with known and unknown modes of interaction as inducers or inhibitors of T and E2 production. Thus it can be considered a reliable screen for chemicals that can alter the production of sex steroid hormones. One of the remaining limitations associated with the H295R steroidogenesis assay protocol is the relatively small basal production of E2 and its effect on quantifying the decreased production of this hormone with regard to the identification of weak inhibitors. An initial comparison of the data produced in this study with those from in vivo studies from the literature demonstrated the potential of the H295R steroidogenesis assay to identify chemicals affecting hormone homeostasis in whole organisms. Particularly promising was the lack of any false negatives during the validation and the very low number of false positives (1 out of 28 chemicals for each T and E2). Based on the results obtained during this validation study and the accordingly revised test protocols, an OECD draft test guideline was developed and submitted to the OECD working group of the national coordinators of the test guidelines program (WNT) for comments in December 2009.

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Effects of Nutrition Relevant Mixtures of Phytoestrogens on Steroidogenesis, Aromatase, Estrogen, and Androgen Activity

Phytoestrogens (PEs) are naturally occurring plant components produced in a large range of plants. They can induce biologic responses in vertebrates by mimicking or modulating the action or production of endogenous hormones. This study examined mixtures of 12 food relevant PEs for effects on steroid hormone production, aromatase activity, estrogenic activity, and for interaction with the androgen receptor. The results show that a mixture of all tested PEs increased estradiol production and decreased testosterone production in H295R human adrenal corticocarcinoma cells, indicating an induced aromatase activity. Furthermore, exposure of the H295R cells to isoflavonoids caused a decrease in testosterone production, and various mixtures of PEs significantly stimulated MCF-7 human breast adenocarcinoma cell growth and induced aromatase activity in JEG-3 choriocarcinoma cells. The estrogenic effect in the MCF7 cells of the isoflavonoid mixture and coumestrol was supported by an observed increase in progesterone receptor protein expression as well as a decreased ER expression. Overall, the results support that nutrition-relevant concentrations of PEs both alone and in mixtures possess various endocrine disrupting effects, all of which need to be considered when assessing the effects on human health.
Synergistic Disruption of External Male Sex Organ Development by a Mixture of Four Antiandrogens

By disrupting the action of androgens during gestation, certain chemicals present in food, consumer products and the environment can induce irreversible demasculinisation and malformations of sex organs among male offspring. However, the consequences of simultaneous exposure to such chemicals are not well described, especially when they exert their actions by differing molecular mechanisms. Objectives: To fill this gap, we investigated the effects of mixtures of a widely used plasticizer, di(2-ethylhexyl) phthalate (DEHP), two fungicides present in food, vinclozolin and prochloraz, and a pharmaceutical, finasteride, on landmarks of male sexual development in the rat, including changes in anogenital distance, retained nipples, sex organ weights and malformations of genitalia. These chemicals were chosen because they disrupt androgen action according to differing mechanisms of action. Results: Strikingly, the effect of combined exposure to the selected chemicals on malformations of external sex organs was synergistic, and the observed responses were
greater than would be predicted from the toxicities of the individual chemicals. In relation to other hallmarks of disrupted male sexual development, including changes in anogenital distance, retained nipples, and sex organ weights, the combined effects were dose additive. When the four chemicals were combined at doses equal to no-observed-adverse-effect levels estimated for nipple retention, significant reductions in anogenital distance were observed in male offspring. Conclusions: Since unhindered androgen action is essential for human male development in foetal life, these findings are highly relevant to human risk assessment. Evaluations that ignore the possibility of combination effects may lead to considerable underestimations of risks associated with exposures to chemicals that disrupt male sexual differentiation.

Do parabens have the ability to interfere with steroidogenesis?

The effects of ethyl and butyl paraben on steroidogenesis were evaluated in rats exposed in utero. Pregnant Wistar rats were dosed from gestational day (GD) 7 to GD 21, followed by examination of the dams, and the fetuses. Additionally, both parabens were tested in vitro in the H295R steroidogenesis assay and in the T-screen assay, the later to test for their ability to act as thyroid hormone receptor agonist or antagonist. In the in utero exposure toxicity study, neither ethyl nor butyl paraben showed any treatment-related effects on testosterone production, anogenital distance, or testicular histopathology. However, butyl paraben caused a significant decrease in the mRNA expression level of estradiol receptor-beta in fetal ovaries, and also significantly decreased the mRNA expression of steroidogenic acute regulatory protein and peripheral benzodiazepine receptor in the adrenal glands. In vitro butyl paraben increased the proliferation of the GH3 cells in the T-Screen assay, thereby acting as a weak thyroid hormone receptor agonist. In the adrenal H295R steroidogenesis assay both ethyl and butyl paraben caused a significant increase in the progesterone formation. Overall, the results indicate that butyl paraben might have the ability to act as endocrine disruptor by interfering with the transport of cholesterol to the mitochondrion, thereby interfering with steroidogenesis, but also that the two tested parabens do not show clear endocrine disrupting capabilities in our short-term in vivo experiment.
Do azole fungicides possess an endocrine disrupting hazard?

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Endocrine disrupting properties in vivo of widely used azole fungicides
The endocrine-disrupting potential of four commonly used azole fungicides, propiconazole, tebuconazole, epoxiconazole and ketoconazole, were tested in two short-term in vivo studies. Initially, the antiandrogenic effects of propiconazole and tebuconazole (50, 100 and 150 mg/kg body weight/day each) were examined in the Hershberger assay. In the second study, pregnant Wistar rats were dosed with propiconazole, tebuconazole, epoxiconazole or ketoconazole (50 mg/kg/day each) from gestational day (GD) 7 to GD 21. Caesarian sections were performed on dams at GD 21. Tebuconazole and propiconazole demonstrated no antiandrogenic effects at doses between 50 and 150 mg/kg body weight/day in the Hershberger assay. In the in utero exposure toxicity study, ketoconazole, a pharmaceutical to treat human fungal infections, decreased anogenital distance and reduced testicular testosterone levels, demonstrating a demasculinizing effect on male fetuses. Tebuconazole, epoxiconazole and ketoconazole induced a high-frequency of post-implantation loss, and both ketoconazole and epoxiconazole caused a marked increase in late and very late resorptions. Overall the results show that many of the commonly used azole fungicides act as endocrine disruptors in vivo, although the profile of action in vivo varies. As ketoconazole is known to implicate numerous endocrine-disrupting effects in humans, the concern for the effects of the other tested azole fungicides in humans is growing.

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Higher levels of ethyl paraben and butyl paraben in rat amniotic fluid than in maternal plasma after subcutaneous administration.

Parabens are a group of antimicrobial preservatives widely used in cosmetics, pharmaceuticals, and in foods. Previous in vitro and in vivo studies have shown weak estrogenic effects of some parabens. Thus, especially, exposure of fetus and infants via the mother is a matter of concern. In order to obtain more knowledge about the distribution of ethyl paraben and butyl paraben in pregnant rats and pups after perinatal exposure, the presented study was designed. The data show response and distribution of ethyl paraben and butyl paraben in maternal rat plasma, pools of amniotic fluids, placenta, whole-body fetuses, and in fetal liver after dosing of dams with 100, 200, and 400 mg/kg body weight (bw)/day from gestational day 7 to 21. After cesarean section of dams, the fluids and tissues were collected, deconjugated, and purified by solid-phase extraction, and ethyl paraben and butyl paraben were analyzed by liquid chromatography-tandem mass spectrometry. Markedly higher levels of ethyl paraben compared to butyl paraben were found in all fluids and tissues. Both ethyl paraben and butyl paraben in maternal plasma, livers, and whole-body tissues from fetus seemed to be saturated after dosing with \( \geq 100 \) mg/kg bw/day, while both compounds were excreted into amniotic fluid in a dose-dependent manner. Significant difference was found between the level of ethyl paraben in maternal plasma and amniotic fluid after dosing with 200 mg/kg bw/day as well as between the levels of butyl paraben in maternal plasma and amniotic fluid after dosing with 100, 200, and 400 mg/kg bw/day.

Dysgenesis and histological changes of genitals and perturbations of gene expression in male rats after in utero exposure to antiandrogen mixtures

We investigated the ability of a mixture of three androgen receptor antagonists to induce disruption of male sexual differentiation after perinatal exposure. The aim was to assess whether the joint effects of vinclozolin, flutamide, and procyomidone can be predicted based on dose-response data of the individual chemicals. Chemicals were administered orally to pregnant Wistar rats from gestational day 7 to postnatal day 16. Changes in reproductive organ weights and of androgen-regulated gene expression in prostates from male rat pups were chosen as end points for extensive dose-
response studies. With all end points, the joint effects of the three antiandrogens were dose additive. Histological evaluations showed that dysgenesis and hypoplasia of prostates, seminal vesicles, and epididymis were seen with the highest mixture doses. No changes were observed in any single-compound low-dose group for these lesions, nor were there histopathological changes in the testes. Pronounced dysgenesis of external genitals was observed with all doses of the mixture, and severe dysgenesis was seen with a mixture for which the individual compounds caused no effects. A combination of doses of each chemical that on its own did not produce significant reductions in the weights of seminal vesicles and PBP C3 expression induced a marked mixture effect. Thus, antiandrogens cause additive effects on end points of various molecular complexities such as alterations at the morphological and the molecular level. Exposure to antiandrogens, which appears to exert only small effects when judged on a chemical-by-chemical basis, may induce marked responses in concert with, possibly unrecognized, similarly acting chemicals.

Effects of azole fungicides on the function of sex and thyroid hormones

Azole-fungicides are frequently used in Denmark. Epoxiconazole, prociconazole, and tebuconazole had endocrine disrupting properties in cell based assays. In rats, epoxiconazole and tebuconazole increased gestational length, maternal progesterone level, and masculinized female-offspring. Besides, tebuconazole caused feminization of male-offspring. Similar effects were previously demonstrated for prochloraz. The results indicate that azole-fungicides in general have endocrine disrupting properties.

Endocrine-disrupting activities in vivo of the fungicides tebuconazole and epoxiconazole

The triazole fungicides tebuconazole and epoxiconazole were investigated for reproductive toxic effects after exposure during gestation and lactation. Rats were dosed with epoxiconazole (15 or 50 mg/kg bw/day) or tebuconazole (50 or 100
mg/kg bw/day) during pregnancy from gestational day (GD) 7 and continued during lactation until postnatal day (PND) 16. Some dams were randomly chosen for cesarean section at GD 21 to evaluate effects on sexual differentiation in the fetuses. Other dams delivered normally, and the pups were examined (e.g., anogenital distance [AGD] and hormone levels) at birth, at PND 13 or PND 16, and semen quality was assessed in adults. Both tebuconazole and epoxiconazole affected reproductive development in the offspring after exposure in utero. Both compounds virilized the female offspring as shown by an increased AGD PND 0. Furthermore, tebuconazole had a feminizing effect on male offspring as shown by increased nipple retention. This effect was likely caused by the reduced testosterone levels seen in male fetuses. Tebuconazole increased the testicular concentrations of progesterone and 17 alpha-hydroxyprogesterone in male fetuses, indicating a direct impact on the steroid synthesis pathway in the Leydig cells. The high dose of epoxiconazole had marked fetotoxic effects, while the lower dose caused increased birth weights. The increased birth weights may be explained by a marked increase in testosterone levels in dams during gestation. Common features for azole fungicides are that they increase gestational length, virilize female pups, and affect steroid hormone levels in fetuses and/or dams. These effects strongly indicate that one major underlying mechanism for the endocrine-disrupting effects of azole fungicides is disturbance of key enzymes like CYP17 involved in the synthesis of steroid hormones.

In vitro and in vivo screening of azole fungicides for antiandrogenic effects

In general, azole fungicides have a low acute toxicity, but we have only little knowledge about their potential health risks at low chronic exposures. Previously we have shown that prochloraz has multiple potential mechanisms of action in cell-based assays, and prochloraz possessed antiestrogenic and antiandrogenic effects both in vitro and in vivo. Two other azole fungicides, tebuconazole and epoxiconazole, have now been investigated for antiandrogenic effects in vitro and in vivo as well. The fungicides were screened in two well-established cell assays, including testing for agonistic and antagonistic effects on AR in transfected CHO cells, using an AR reporter gene assay. The compounds were also analyzed for effects on steroidogenesis in H295R cells, a human adrenocorticocarcinoma cell line, used to detect effects on steroid production. In vitro tebuconazole and epoxiconazole proved to be antagonists of the AR, and in the H295R cell assay, they were able to inhibit testosterone and estradiol levels, and increase progesterone levels. In an in vivo study, designed to test for developmental effects on rat offspring after prenatal exposure, the effects on hormone levels in male fetuses and morphological signs of feminization of the male offspring were investigated. Tebuconazole caused an increase in testicular 17alpha-hydroxyprogesterone and progesterone levels, and a decrease in testosterone levels in male fetuses. Epoxiconazole had no effect on any of the measured hormone levels. Furthermore, tebuconazole increased the AGD in female pups and resulted in an increased number of nipples in male pups, a tendency that was also seen for epoxiconazole, though it was not statistically significant. In conclusion, the results obtained in vitro are in good agreement with the effects observed in vivo. Tebuconazole showed antiandrogenic effects both in vitro and in vivo. Antiandrogenic effects were also seen for epoxiconazole in vitro, however the dominating effect observed in vivo was a high frequency of stillbirths at the highest dose.
The OECD validation program of the H295R steroidogenesis assay for the identification of in vitro inhibitors and inducers of testosterone and estradiol production. Phase 2: Inter-laboratory pre-validation studies

Background, Goals and Scope. In response to concerns that have been raised about chemical substances that may alter the function of endocrine systems and result in adverse effects on human health, an OECD initiative was undertaken to develop and validate in vitro and in vivo assays to identify chemicals that may interfere with endocrine systems of vertebrates. Here we report on studies that were conducted to develop and standardize a cell-based screening assay using the H295R cell line to prioritize chemicals that may act on steroidogenic processes in humans and wildlife. These studies are currently ongoing as part of the 'Special Activity on the Testing and Assessment of Endocrine Disruptors' within the OECD Test Guidelines Program to review, develop, standardize, and validate a number of in vitro and in vivo toxicological assays for testing and assessment of chemicals concerning their potential to interact with the endocrine system of vertebrates. Study Design. Six laboratories from five countries participated in the pre-validation studies. Each laboratory tested the effects of three model chemicals on the production of testosterone (T) and estradiol (E2) using the H295R Steroidogenesis Assay. Chemicals tested were well described inducers or inhibitors of steroidogenic pathways (forskolin, prochloraz and fadrozole). All experiments were conducted in 24 well plates following standard protocols. Six different doses per compound were analyzed in triplicate per plate. A quality control (QQ plate was run in conjunction with the chemical exposure plate to account for inter-assay variation. Each chemical exposure was conducted two or three times. Results. All laboratories successfully detected increases and/or decreases in hormone production by H295R cells after exposure to the different model compounds and there was good agreement in the pattern of response for all groups. Forskolin increased both T and E2 while fadrozole and prochloraz decreased production of both hormones. All chemicals affected hormone production in a close-dependent manner with the exception of fadrozole which caused maximum inhibition of E2 at the two least concentrations tested. Some inter-laboratory differences were noted in the alteration of hormone production measured in chemically exposed cells. However, with the exception of the production of T measured at one laboratory in cells exposed to forskolin, the EC(50)s calculated were comparable (coefficients of variation 34-49%) for all hormones. Discussion and Perspectives. The results indicated that the H295R Steroidogenesis Assay protocol was robust, transferable and reproducible among all laboratories. However, in several instances that were primarily related to one laboratory there were unexplained minor uncertainties related to the inter-laboratory hormone production variation. Based on the findings from this Phase 2 prevalidation study, the H295R Steroidogenesis Assay protocol is currently being refined. The next phase of the OECD validation program will test the refined protocol among the same group of laboratories using an extended set of chemicals (similar to 30) that will include positive and negative chemical controls as well as a broad spectrum of different potential inducers and inhibitors of steroidogenic pathways.
Diisobutyl phthalate has comparable anti-androgenic effects to di-n-butyl phthalate in fetal rat testis

Phthalates are widely used as plasticizers in various consumer products and building materials. Some of the phthalates are known to interfere with male reproductive development in rats, and di-n-butyl phthalate (DBP), diethylhexyl phthalate (DEHP) and butyl benzyl phthalate (BBP) were recently banned for use in toys in the EU mainly due to their reproductive toxicity. Diisobutyl phthalate (DiBP) has similar structural and application properties as DBP, and is being used as a substitute for DBP. However, knowledge on male reproductive effects of DiBP in experimental animals is lacking. Methods: In the current study, four groups of pregnant Wistar rats were exposed to either 0 mg/kg bw/day or 600 mg/kg bw/day of DiBP from gestation day (GD) 7 to either GD 19 or GD 20/21. Male offspring was examined at GD 19 or GD 20/21 for effects on testicular testosterone production and testicular histopathology. Changes in anogenital distance (AGD) were evaluated as an indication of feminisation of males. Results: Anogenital distance was statistically significantly reduced at GD 20/21 to-ether with reductions in testicular testosterone production and testicular testosterone content. Histopathological effects (Leydig cell hyperplasia, Sertoli cell vacuolisation, central location of gonocytes and presence of multinuclear gonocytes) known for DBP and DEHP were observed in testes of DiBP-exposed animals at GD 20/21. Additionally, immunohistochemical expression of P450scc and STAR proteins in Leydig cells was reduced by DiBP. At GD 19, these effects on anogenital distance, testosterone levels and histopathology were less prominent. Conclusion: In this study, GD 20/21 rather than GD 19 appears to be the optimal time for investigating changes in anogenital distance, testosterone levels, and testicular histopathology. DiBP has similar testicular and developmental effects as DBP and DEHP, and although more developmental and especially postnatal studies are needed to clearly identify the reproductive effects of DiBP, this study indicates a reason for concern about the use of DiBP as a substitute for DBP. (c) 2005 Elsevier Ireland Ltd. All rights reserved.

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Estrogenic effects in vitro and in vivo of the fungicide fenarimol

The fungicide fenarimol has the potential to induce endocrine disrupting effects via several mechanisms since it possesses both estrogenic and antiandrogenic activity and inhibits aromatase activity in cell culture studies. Hence, the integrated response of fenarimol in vivo is not easy to predict. In this study, we demonstrate that fenarimol is also estrogenic in vivo. causing significantly increased uterine weight in ovariectomized female rats. In addition, mRNA levels of the estrogen responsive gene lactoferrin (LF) were decreased in uteri, serum FSH levels were increased, and T3 levels decreased in fenarimol-treated animals. To our knowledge, only two other pesticides (o,p-DDT and methoxychlor) have previously been reported to induce an estrogenic response in the rodent uterotrophic bioassay. A pronounced xenoestrogenicity in serum samples from rats treated with fenarimol and estradiol benzoate (E2B) separately or in combination was observed, demonstrating the usefulness of this approach for estimating the integrated internal exposure to xenoestrogens. The MCF-7 cell proliferation assay was used to investigate further the dose-response curves for the estrogenic, antiestrogenic, and aromatase inhibiting properties of fenarimol in vitro. The results indicates that fenarimol exhibits a dual effect being aromatase inhibitor at low concentrations and estrogenic at higher concentrations.

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In vitro screening of azole fungicides for antiandrogenic effects – comparison with in vivo effects

In general, azole fungicides have a low acute toxicity but little is known about their potential health risks at low chronic exposures. We have recently shown that prochloraz has multiple potential mechanisms of action, including interaction with the estrogen and androgen receptors, in cell-based assays. In vitro prochloraz proved to be an activator of the aryl hydrocarbon receptor (AhR), to inhibit aromatase activity and to possess antiestrogenic and antiandrogenic effects both in vitro and in vivo. Another two azole fungicides, tebuconazole and epoxiconazole, have now been investigated for antiandrogenic effects in vitro and in vivo as well. The fungicides were screened in two well-established cell assays, including testing for agonistic and antagonistic effects on AR in transfected CHO cells, using the AR reporter gene assay. Secondly the compounds were analyzed for effects on steroidogenesis in H295R cells, a cell line, which produces a wide range of steroid hormones in measurable quantities, including testosterone, progesterone and estradiol, a property that makes it suitable as a screening assay to detect effects on steroidogenesis. In the in vitro tebuconazole and epoxiconazole showed antiandrogenic effects, and in the H295R cell assay, tebuconazole and epoxiconazole were like prochloraz able to inhibit testosterone and estradiol levels and increase progesterone levels. For the in vivo testing, a study was conducted testing the developmental effects on offspring after prenatal exposure, by looking at endpoints like hormone levels in male fetuses and sign of feminization of the male offspring. In vivo tebuconazole had an increasing effect on the testicular level of 17alpha-hydroxyprogesterone and progesterone, a decreasing effect on the level of testosterone, but no effect on testicular testosterone and progesterone production. Epoxiconazole showed now effect. Furthermore tebuconazole increased the AGD in female pups and resulted in an increased number of nipples in male pups at PND 13, a tendency that was also seen for epoxiconazole, though it was not significant. In conclusion the results obtained in vitro is in good agreement with the effects observed in vivo. Tebuconazole showed antiandrogenic effects both in vitro and in vivo. Antiandrogenic effects were also seen for epoxiconazole in vitro, however the observed effects in vivo was not quite what might be predicted from the in vitro experiments, which can be due to the fact, that in vitro generally is more sensitive than in vivo.

Kombinationseffekter af pesticider

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Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz

The fungicide prochloraz has got multiple mechanisms of action that may influence the demasculinizing and reproductive toxic effects of the compound. In the present study, Wistar rats were dosed perinatally with prochloraz (50 and 150 mg/kg/day) from gestational day (GD) 7 to postnatal day (PND) 16. Caesarian sections were performed on selected dams at GD 21, while others were allowed to give birth to pups that were followed until PND 16. Prochloraz caused mild dysgenesis of the male external genitalia as well as reduced anogenital distance and retention of nipples in male pups. An increased anogenital distance indicated virilization of female pups. Effects on steroidogenesis in male fetuses became evident as decreased testicular and plasma levels of testosterone and increased levels of progesterone. Ex vivo synthesis of both steroid hormones was qualitatively similarly affected by prochloraz. Immunohistochemistry of fetal testes showed increased expression of 17 alpha-hydroxylase/17,20-lyase (P450c17) and a reduction in 17 beta-hydroxysteroid dehydrogenase (type 10) expression, whereas no changes in expression of genes involved in testicular steroidogenesis were observed. Increased expression of P450c17 mRNA was observed in fetal male adrenals, and the androgen-regulated genes ornithine decarboxylase, prostatic binding protein C3 as well as insulin-like growth factor I mRNA were reduced in ventral prostates PND 16. These results indicate that reduced activity of P450c17 may be a primary cause of the disrupted fetal steroidogenesis and that an altered androgen metabolism may play a role as well. In vitro studies on human adrenocortical carcinoma cells supported the findings in vivo as reduced testosterone and increased progesterone levels were observed. Overall, these results together indicate that prochloraz acts directly on the fetal testes to inhibit steroidogenesis and that this effect is exhibited at protein, and not at genomic, level. (c) 2005 Elsevier Inc. All rights reserved.

Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis

Diethylhexyl phthalate (DEHP) is widely used as a plasticizer in consumer products and is known to disturb the development of the male reproductive system in rats. The mechanisms by which DEHP exerts these effects are not yet fully elucidated, though some of the effects are related to reduced fetal testosterone production. The present study investigated the effects of four different doses of DEHP on fetal testicular histopathology, testosterone production and expression of proteins and genes involved in steroid synthesis in fetal testes. Pregnant Wistar rats were gavaged from GD 7 to 21 with vehicle, 10, 30, 100 or 300 mg/kg bw/day of DEHP. In male fetuses examined at GD 21, testicular testosterone production ex vivo and testicular testosterone levels were reduced significantly at the highest dose. Histopathological effects on gonocytes were observed at 100 and 300 mg/kg bw/day, whereas Leydig cell effects were mainly seen at 300 mg/kg bw/day. Quantitative RT-PCR revealed reduced testicular mRNA expression of the steroidogenesis related factors SR-B1, STAR, PBR and P450scc. Additionally, we observed reduced mRNA expression of the nuclear receptor SF-1, which regulates certain steps in steroid synthesis, and reduced expression of the cryptorchidism-associated Insl-3. Immunohistochemistry showed clear reductions of STAR, PBR, P450scc and PPAR gamma protein levels in fetal Leydig cells, indicating that DEHP affects regulation of certain steps in cholesterol transport and steroid synthesis. The suppression of testosterone levels observed in phthalate-exposed fetal rats was likely caused by the low expression of these receptors and enzymes involved in steroidogenesis. It is conceivable that the observed effects of DEHP on the expression of nuclear receptors SF-1 and PPAR gamma are involved in the downregulation of
steroidogenic factors and testosterone levels and thereby underlie the disturbed development of the male reproductive system. (c) 2006 Elsevier Ireland Ltd. All rights reserved.

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**Prochloraz: an imidazole fungicide with multiple mechanisms of action**

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**Antiandrogenic effects in short-term in vivo studies of the fungicide fenarimol**
The fungicide fenarimol has estrogenic and antiandrogenic activity and inhibits aromatase activity in vitro. We tested, whether fenarimol had antiandrogenic effects in vivo. In a Hershberger assay, fenarimol given orally to castrated testosterone-treated male rats caused markedly reduced weights of ventral prostate, seminal vesicles, musc. levator anitbulbocavernosus, and bulbourethral glands. Qualitatively similar, but weaker, effects were also evident in intact fenarimol-exposed young adult males. except that prostates were not significantly affected. Changes in androgen-regulated gene expression were determined by real-time RTPCR in ventral prostates and fenarimol caused a pronounced decrease of prostate binding protein C3 (PBP C3), ornithin decarboxylase (ODC), and insulin-like-growth factor 1 (IGF-1) mRNA levels. The antiandrogenic drug flutamide, included as a positive control, caused down-regulation of PBP C3 mRNA and up-regulation of TRPM-2 mRNA levels. Serum T4 levels were reduced after fenarimol treatment and a tendency towards increased LH levels was seen. However, no effects on testosterone levels or testosterone production ex vivo could be revealed. Taken together these results indicate that fenarimol acts as an antiandrogen in vivo having effects
qualitatively comparable to those of flutamide on organ level, whereas differential effects on gene expression were observed. In an additional Hershberger test, the effects of fenarimol were compared to those of estradiol benzoate, prochloraz and the aromatase inhibitor fadrozole. The data indicate a similar mode of action of fenarimol and prochloraz in the males, whereas no indications were found that the estrogenic or aromatase inhibitory properties had important impact on the effects observed in the males. Thus, it is suggested that fenarimol mediates its antiandrogenic effects at least partly via antagonism of androgen receptors.

**Gene expression changes in rat prostate after activation or blocking of the androgen and estrogen receptor**
Several endpoints of different molecular complexity were studied in the Hershberger assay in order to evaluate the specificity and suitability of this test as a broad screening model. Androgen and estrogen receptors were activated or blocked, and expression of typical estrogen- or androgen responsive genes (complement C3, ER alpha, ER beta, AR, TRPM-2, PBPC3, ODC, and IGF-1 mRNA) was analyzed in rat ventral prostate by real time RT-PCR. Administration of estradiol benzoate (EB) to castrated testosterone-treated rats had no effect on reproductive organ weights or gene expression levels and the anti-estrogen, ICI 182780, only affected ODC expression. Therefore, estrogenic or anti-estrogenic compounds would not be expected to seriously affect the outcome of a Hershberger test. However, EB given alone to castrated rats resulted in various effects. EB increased seminal vesicle weight, an effect reversed by ICI 182780, and affected TRPM-2, PBPC3, ODC, IGF-1, AR, and ERα mRNA levels. AR expression in the prostate seemed to be under regulation of both estrogens and androgens, as ICI 182780 inhibited the testosterone-induced AR expression, and flutamide inhibited the EB-induced AR expression. These data indicate that estrogens have various effects in castrated male rats and that expression of several genes is under multi-hormonal control in the ventral prostate. However, interactions between estrogens and androgens do not play a major role in the Hershberger assay, as simultaneous TP administration abolished the effects of EB. First choice of gene expression profiles in the Hershberger assay to study androgenic or anti-androgenic effects would be the traditional, TRPNI-2 and PBPC3, supplemented with the new complement C3.
Gene expression profiling of rat liver after in utero exposure to the fungicide prochloraz using GeneChip (R) Rat Genome Array

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Low-dose effects of anti-androgens in male rat offspring after perinatal exposure

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Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring

Prochloraz is a commonly used fungicide that has shown multiple mechanisms of action in vitro. It antagonizes the androgen and the estrogen receptors, agonizes the Ah receptor, and inhibits aromatase activity. In vivo prochloraz acts antiandrogenically in the Hershberger assay by reducing weights of reproductive organs, affecting androgen-regulated gene expressions, and increasing luteinizing hormone (LH) levels. The purpose of this study was to investigate reproductive toxic effects after exposure during gestation and lactation to prochloraz alone and a mixture of five pesticides (deltamethrin, methiocarb, prochloraz, simazine, and tribenuron-methyl). Prochloraz (30 mg/kg/day) or the mixture (20 mg/kg/day) was dosed to pregnant Wistar dams from gestational day (GD) 7 until postnatal day (PND) 16. Some dams were taken for cesarean section at GD 21, and others were allowed to give birth. Results showed that prochloraz and the mixture significantly reduced plasma and testicular testosterone levels in GD 21 male fetuses, whereas testicular progesterone was increased. Gestational length was increased by prochloraz. Chemical analysis of the rat breast milk showed that prochloraz was transferred to the milk. In males a significant increase of nipple retention was found, and the bulbourethral gland weight was decreased, whereas other reproductive organs were unaffected. In addition cytochrome P450 (CYP)1A activities in livers were induced by prochloraz, possibly as a result of Ah receptor activation. Behavioral studies showed that the activity level and sweet preference of adult males were significantly increased. Overall these results strongly indicate that prochloraz feminizes the male offspring after perinatal exposure, and that these effects are
due, at least in part, to diminished fetal steroidogenesis.

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**Hormonforstyrrende effekter af kombinationer af pesticider**

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**Interlaboratory comparison of four in vitro assays for assessing androgenic and antiandrogenic activity of environmental chemicals**

We evaluated and compared four in vitro assays to detect androgen agonists and antagonists in an international interlaboratory study. Laboratory 1 used a cell proliferation assay (assay 1) with human mammary carcinoma cells stably transfected with human androgen receptor. The other laboratories used reporter gene assays, two based on stably transfected human prostate carcinoma cells (assay 2) or human mammary carcinoma cells (assay 4), and the third based on transient transfection of Chinese hamster ovary cells (assay 3). Four laboratories received four coded compounds and two controls: two steroidal androgens, two antiandrogens, an androgenic control, 5alpha-dihydrotestosterone (DHT), and an antiandrogenic control, bicalutamide (ICI 176,334). All laboratories correctly detected the androgenic activity of 4-androsten-3,17-dione and 17alpha-methyl-testosterone. For both compounds, the calculated androgenic potencies relative to the positive control (RAPs) remained within one order of magnitude. However, laboratory 3 calculated a 50-fold higher RAP for 4-androsten-3,17-dione. All assays detected and quantified the antiandrogenic effect of vinclozolin [median inhibitory concentration (IC50) values ranging from 1.1 x 10(-7) M to 4.7 x 10(-7) M]. In assays 2 and 3, vinclozolin showed partial androgenic activity at the highest concentrations tested. For vinclozolin, calculated antiandrogenic potencies relative to bicalutamide (RAAPs) differed no more than a factor of 10, and IC50 values matched those of bicalutamide. Similarly, we found antiandrogenic activity for tris-(4-chlorophenyl) methanol. RAAP values were between 0.086 and 0.37. Three assays showed cytotoxicity for this compound at or above 1 x 10(-5) M. In summary, all assays proved sensitive screening tools to detect and quantify androgen receptor-mediated androgenic and antiandrogenic effects of these chemicals accurately, with coefficients of variation between 8 and 90%.

**General information**

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Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats

The plasticizer di(2-ethylhexyl)phthalate (DEHP) exhibits antiandrogenic effects in perinatally exposed male rats. Di(2-ethylhexyl) adipate (DEHA) and diisononyl phthalate (DINP) are currently being evaluated as potential substitutes for DEHP, but similarities in structure and metabolism of DEHP with DEHA and DINP have led to the hypothesis that similarities in action may also exist. Pregnant Wistar rats were gavaged during gestation and lactation with vehicle, DEHP (300 or 750 mg/kg bodyweight per day), DINP (750 mg/kg bodyweight per day), DEHP (750 mg/kg bodyweight per day) in combination with DEHA (400 mg/kg bodyweight per day), or DEHP (300 mg/kg bodyweight per day) in combination with DINP (750 mg/kg bodyweight per day). DINP and DEHP were both shown to reduce testicular testosterone production ex vivo and testosterone levels in testes and plasma of male fetuses at gestation day 21, indicating a similar mechanism of action for DINP and DEHP. Additionally, plasma LH levels in male fetuses were elevated. Neonatal anogenital distance was reduced and the number of nipples at postnatal day 13 increased in DEHP-exposed male offspring. Serum inhibin B levels were significantly reduced in DEHP-exposed prepubertal male offspring, and in a few adult males. No modulating effects of DEHA on the endocrine effects of DEHP were detected, but a tendency towards an accumulating effect of DEHP and DINP in combination on suppression of testosterone synthesis was seen. (C) 2003 Elsevier Inc. All rights reserved.
The combined antiandrogenic effects of five commonly used pesticides

In this study, mixture effects of five dissimilarly acting pesticides were analyzed for antiandrogenic effects in vitro and in vivo. Deltamethrin, methiocarb, prochloraz, simazine, and tribenuron-methyl are all commonly used for agricultural and horticultural purposes. Concentration-response curves for the inhibition of R1881-induced transcriptional activity of the androgen receptor (AR) in vitro of each pesticide alone and in an equimolar mixture were obtained. The IC25 values for deltamethrin, methiocarb, prochloraz, and the mixture were 5.8, 5.8, 3.5, and 7.5 μM, respectively. Simazine and tribenuron-methyl were ineffective. Applying the isobole method resulted in an isobole coefficient of 0.94 at IC25 for the effect of the mixture, indicating additive effects of the compounds. Comparison of observed effects and effects calculated by assuming additivity also strongly indicated additive effects of the pesticides in vitro. In vivo, each of the five pesticides and a mixture of the pesticides were tested for antiandrogenic effects in castrated testosterone-treated Wistar rats. The mixture induced a significant change of weights of the levator ani/bulbocavernous muscle and adrenal glands. Changes in gene expression in ventral prostates were observed as distinct effects on levels of ornithin decarboxylase (ODC) mRNA and effects on levels of prostate binding protein subunit C3 (PBP C3) mRNA. No pesticide-induced effect on the level of testosterone-repressed prostatic message 2 (TRPM-2) mRNA was observed, whereas flutamide increased TRPM-2 levels. In conclusion, the pesticides were found to act additively in vitro. In vivo, the organ weight changes indicated that the pesticides had an accumulating effect that was not observed for the individual pesticides. Several pesticide-induced gene expression changes were observed, indicating that these are either very sensitive antiandrogenic end-points or that these changes are induced by a pathway not related to AR.

Di(2-ethylhexyl) adipate (DEHA) induced developmental toxicity but not antiandrogenic effects in pre- and postnatally exposed Wistar rats

Di(2-ethylhexyl) adipate (DEHA) induced developmental toxicity but not antiandrogenic effects in pre- and postnatally exposed Wistar rats.
Effect of in utero-administered coumestrol, equol, and organic selenium on biomarkers for phase 2 enzyme capacity and redox status

The aim of the present study was to investigate the effect of in utero administration of coumestrol, equol, and selenium-enriched yeast on selected hepatic phase 2 enzymes, plasma hormone levels, and markers for redox status in plasma and red blood cells (RBCs). The test compounds were administered via the diet to pregnant Sprague-Dawley rats throughout gestation. Within 24 h following delivery dams and offspring were sacrificed, and blood, liver and reproductive organs were sampled. Coumestrol, equol, and selenium-enriched yeast did not significantly affect hepatic glutathione S-transferase (GST), quinone reductase (QR), or RBC glutathione peroxidase (GP(x)) in the offspring, whereas significant increases in GST QR, and GP(x) activities in dams were observed following administration of selenium-enriched yeast. The level of 17beta-estradiol in offspring from coumestrol-exposed dams was significantly increased compared with the control. The present results indicate that selenium-enriched yeast, coumestrol, and equol affect selected hepatic phase 2 enzymes and GP(x) in RBC in dams, whereas the offspring in general were refractive to the employed treatments. Further studies are warranted to investigate whether the observed in utero effects imposed by the selected plant compounds confer permanent alterations on the health status of the animal resulting in an altered resistance to cancer.

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Effects of currently used pesticides in the AhR-CALUX assay: comparison between the human TV101L and the rat H4IIE cell line

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates many of the biologic and toxicological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. The in vitro chemically activated luciferase expression (CALUX) assay has been proven to be a rapid and sensitive assay for assessing the potency of AhR-activating compounds. We have used the AhR-CALUX assay to investigate the AhR-mediated activity of the persistent organochlorine insecticide dieldrin and twenty-two pesticides currently used in Denmark by employing the rat H4IIE and the human TV101L hepatoma cell lines. In comparison the results indicated that the rat H4IIE cell line is more sensitive than the human TV101L for detection of TCDD inducing AhR-CALUX activity. The pesticides iprodione, chlorpyrifos and prochlororaz showed dose-dependent AhR agonistic effects in both cell lines at concentrations above 10, 1 and 1 μM, respectively. However, some pesticides (methiocarb, chlorothalonil, tribenuron-methyl, paclobutrazol and tolchlofos-methyl) elicited differential responses in the two cell lines.

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Effects of dietary antioxidants and 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ) on preneoplastic lesions and on oxidative damage, hormonal status, and detoxification capacity in the rat

The potential beneficial or adverse affect of prolonged dietary administration of moderate to high doses (1-100 mg/kg diet) of the antioxidants, lycopene, quercetin and resveratrol or a mixture of lycopene and quercetin was investigated in male F344 rats. Selected markers for toxicity and defense mechanisms were assayed in blood, liver and colon and the impact of the antioxidant administrations on putative preneoplastic changes in liver and colon was assessed. The dietary carcinogen, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) (200 mg/kg diet) served as a pro-oxidant, genotoxicity and general toxicity control. IQ increased the levels of protein and DNA oxidation products in plasma, the area of glutathione S-transferase-placental form positive (GST-P) foci in the liver as well as the number of colonic aberrant crypt foci (ACF). All antioxidants and the antioxidant combination significantly increased the level of lymphocytic DNA damage, to an extent comparable with the effect induced by IQ. In contrast to the control group where no GST-P foci were detected, GST-P foci were detected in animals exposed to quercetin, lycopene and the combination of the two. However, the increase in the volume of GST-P foci did not reach statistical significance. The present results indicate that moderate to high doses of common dietary antioxidants can damage lymphocyte DNA and induce low levels of preneoplastic liver lesions in experimental animals. Long-term exposure to moderate to high doses of antioxidants may thus via pro-oxidative mechanisms and non-oxidative mechanisms modulate carcinogenesis.

Male reproductive effects of octylphenol and estradiol in Fischer and Wistar rats

4-tert-Octylphenol is a non-ionic surfactant used as a detergent, emulsifier and wetting agent. It is generally accepted that it acts as a weak estrogenic substance when evaluated in in vitro and in vivo short-term screening assays. The sensitivity of animal species (mouse versus rat), strain (inbred versus outbred) has been a matter of concern when selecting assay type for testing of estrogenicity of chemicals. The present study was designed to investigate whether the choice of different animal strain, could affect the outcomes of studies. Fischer and Wistar adult male rats were exposed to vehicle or 400 mg/kg bw of 4-tert-octylphenol administrated orally by gavage. Estradiol benzoate, at a dose of 40 mug/kg bw, was
used as positive control agent. Treatment with estradiol benzoate decreased serum levels of testosterone, LH, FSH, inhibin and increased prolactin. Additionally, estradiol benzoate decreased the weight of all investigated reproductive organs, decreased sperm production and increased seminiferous tubular degeneration in both strains. More progressive effects on testis weight and histopathology were observed in the Fischer rats. Oral administration of octylphenol at 400 mg/kg bw to both rat strains increased prolactin levels but had no effect on LH, FSH, testosterone or inhibin. In the octylphenol-treated Fischer rats the weights of the seminal vesicles and the levator ani/bulbocavernous muscle were significantly decreased, whereas only the levator ani/bulbocavernosus muscle was affected in Wistar rats. The weights of all other reproductive organs and sperm count were unaffected. It is concluded that there might be an organ specific difference in sensitivity between the two strains with the Fischer rat being the most sensitive rat model as demonstrated mainly by the more progressive effects on testis weight and histopathology in estradiol benzoate-treated Fischer rats but also by the decrease in seminal vesicle weight in octylphenol-treated rats.

**General information**

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**The combined effects of vinclozolin and procymidone do not deviate from expected additivity in vitro and in vivo**

The combination effects of the well-known antiandrogenic fungicides, vinclozolin and procymidone, were tested both in vitro and in vivo. In vitro both vinclozolin and procymidone significantly inhibited the binding of agonist to the androgen receptor with the concentration that resulted in 50% inhibition (IC50) values of 0.1 and 0.6 μM, respectively. By applying the isobole method, the effect of combining the two pesticides in vitro was found to be additive. In castrated testosterone-treated rats the administration of vinclozolin starting at 10 mg/kg led to a decrease in organ weight of all tested reproductive organs. The levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) were increased significantly with doses of 100 mg/kg vinclozolin and above. Expression of the androgen-responsive gene, TRPM-2, was increased starting at 100 mg/kg vinclozolin. For procymidone, reproductive organ weights were diminished at 10 mg/kg and LH was increased at a concentration of 25 mg/kg and above, compared to the testosterone-treated controls. FSH was significantly increased only at 25 mg/kg procymidone. The studied gene expressions were changed by 100 mg/kg procymidone. Dosing the animals with a combination of a 1:1 mixture of vinclozolin and procymidone resulted in a weight reduction in the reproductive organs and an increase of serum LH and FSH as early as with 10 mg/kg combined dose. The relative expressions of TRPM-2 and PBP C3 were changed compared to controls at 100 mg/kg. The level of 5-HT in the rat brain was increased after a dose of 10 mg/kg. Using the isobole method, comparisons of the observed and predicted effects assuming additivity on reproductive organ weights, hormone levels, and gene expression showed agreement and thus the combination effects are suggested to be additive in vivo as well as in vitro.

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Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity in vitro
Twenty-four pesticides were tested for interactions with the estrogen receptor (ER) and the androgen receptor (AR) in transactivation assays. Estrogen-like effects on MCF-7 cell proliferation and effects on CYP19 aromatase activity in human placental microsomes were also investigated. Pesticides (endosulfan, methiocarb, methomyl, pirimicarb, propamocarb, deltamethrin, fenpropathrin, dimethoate, chlorpyrifos, dichlorvos, tolchlofosmethyl, vinclozolin, iprodion, fenarimol, prochloraz, fosetylaluminum, chlorothalonil, daminozid, paclobutrazol, chlormequat chloride, and ethephon) were selected according to their frequent use in Danish greenhouses. In addition, the metabolite mercaptodimethur sulfoxide, the herbicide tribenuron-methyl, and the organochlorine dieldrin, were included. Several of the pesticides, dieldrin, endosulfan, methiocarb, and fenarimol, acted both as estrogen agonists and androgen antagonists. Prochloraz reacted as both an estrogen and an androgen antagonist. Furthermore, fenarimol and prochloraz were potent aromatase inhibitors while endosulfan was a weak inhibitor. Hence, these three pesticides possess at least three different ways to potentially disturb sex hormone actions. In addition, chlorpyrifos, deltamethrin, tolclofos-methyl, and tribenuron-methyl induced weak responses in one or both estrogenicity assays. Upon cotreatment with 17beta-estradiol, the response was potentiated by endosulfan in the proliferation assay and by pirimicarb, propamocarb, and daminozid in the ER transactivation assay. Vinclozolin reacted as a potent AR antagonist and dichlorvos as a very weak one. Methomyl, pirimicarb, propamocarb, and iprodion weakly stimulated aromatase activity. Although the potencies of the pesticides to react as hormone agonists or antagonists are low compared to the natural ligands, the integrated response in the organism might be amplified by the ability of the pesticides to act via several mechanism and the frequent simultaneous exposure to several pesticides.

Screening of food samples for dioxin levels – comparison of GC/MS determination with the CALUX bioassay

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Toxicity testing and chemical analyses of recycled fibre-based paper for food contact

Food-contact materials, including paper, have to comply with a basic set of criteria concerning safety. This means that paper for food contact should not give rise to migration of components, which can endanger human health. The objectives of this pilot study were, first, to compare paper of different qualities as food-contact materials and to perform a preliminary evaluation of their suitability from a safety point of view, and, second, to evaluate the use of different in vitro toxicity tests for screening of paper and board. Paper produced from three different categories of recycled fibres (B-D) and a raw material produced from virgin fibres (A) were obtained from industry, and extracts were examined by chemical analyses and diverse in vitro toxicity test systems. The products tested were either based on different raw materials or different treatments were applied. Paper category B was made from 40% virgin fibres, 40% unprinted cuttings from newspapers, and 20% de-inked newspapers and magazines. Paper categories C and D were based on newspapers and magazines. However, paper D was de-inked, whereas C was not. To identify constituents of the papers with a potential to migrate into foodstuff, samples of the paper products were extracted with either 99% ethanol or water. Potential migrants in the extracts were identified and semi-quantified by GC-1R-MS or GC-HRMS. In parallel to the chemical analyses, a battery of four different in vitro toxicity tests with different endpoints were applied to the same extracts: (1) a cytotoxicity test using normal human skin fibroblasts. The test was based on measurements of the reduction of resazurin to resorufin by cellular redox processes and used as a screening test for acute or general toxicity; (2) a Salmonella/microsome assay (Ames test) as a screening test for mutagenic and potentially carcinogenic compounds; (3) a recombinant yeast cell bioassay as a screening test for compounds with oestrogenic activity; (4) an aryl hydrocarbon (Ah)-receptor assay (CALUX assay) as a screening test for compounds with dioxin-like activity. In addition, the papers were tested for microbial content and, in general, the microbiological load was quite low. The following microorganisms were counted and identified on both surface and homogenized pulp samples: the total number of aerobic bacteria, the number of aerobic and anaerobic spore formers, the number of Bacillus cereus/thuringiensis, and the number of yeast and moulds. The chemical analyses showed a significantly higher amount and different composition pattern of chemicals extracted with ethanol compared with water. Analyses of the ethanol extracts showed a distinctly smaller number and lower concentrations of chemicals in extracts prepared from sample A compared with extracts of samples B-D. The compounds identified in B-D were similar, but the amounts were lower in B compared with C and D. In accordance with the chemical analyses, the water extracts were less cytotoxic than the ethanol extracts. The extract prepared from virgin fibres was less cytotoxic than the extracts prepared from paper made from recycled fibres, and extracts prepared from C was the most cytotoxic. None of the extracts showed mutagenic activity. No conclusion about the oestrogenic activity could be made, because all extracts were cytotoxic to the yeast cells. Ethanol extracts of A and B showed a negligible positive response in the Ah-receptor assay at the highest nontoxic concentration, whereas C and D showed a more pronounced effect with C being the most potent. A comparable weak effect of water extracts of samples B-D was.

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Effect of dietary antioxidants and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) on biomarkers for redox and hormonal status and enzyme detoxification capacity in blood and major organs of male F344 rats

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Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity

Polychlorinated biphenyls (PCBs) are ubiquitous environmental persistent contaminants giving rise to potential health hazard. Some PCBs exert dioxin-like activities mediated through the aryl hydrocarbon receptor. Although reports on interaction with other nuclear receptors are sparse, some congeners are hypothesized to possess endocrine disruptive potential. Here we present evidence that the three PCBs most abundant in biological extracts, 2,2',3',4,4',5-hexachlorobiphenyl (PCB # 138), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB # 153), and 2,2',3,4,4',5,5'-heptachlorobiphenyl (PCB # 180) have pleiotropic effects on the estrogen- and androgen-receptor. In MCF-7 cells a slightly increased cell proliferation was observed at low concentrations (1-10 nM) in cells co-treated with 0.01 nM 17 beta -Estradiol. whereas the compounds inhibited cell growth significantly at 1 and 10 muM. In reporter gene (ERE-tk-CAT) analysis the three congeners exhibited a significantly estrogen receptor-ligand mediated decrease of the chloramphenicol transferase activity in both control and 10 nM 17 beta -estradiol induced MCF-7 cells. In addition, PCB # 138 elicited a dose-dependent antagonistic effect on androgen receptor activity in transiently co-transfected Chinese Hamster Ovary cells with an IC50, of 6.2 muM. In summary, this study indicate that the di-ortho, multiple-chloro substituted biphenyls, PCB # 138, PCB # 153 and PCB # 180, can compete with the binding of the natural ligand to two nuclear receptors and thus possess the ability to interfere with sexual hormone regulated processes.

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In utero reproductive study in rats exposed to nonylphenol

Alkylphenol ethoxylates are widely used non-ionic surfactants. Nonylphenol ethoxylate constitutes 82% of the production of all alkylphenol ethoxylates and the breakdown product of nonylphenol ethoxylate, nonylphenol (NP) has been shown to be estrogenic in both in vitro and in vivo screening assays. To determine the potential reproductive toxicity of NP, a one-generation in utero study was conducted. Rats were dosed from gestation day 11 through 18 with NP at 3, 15, or 75 mg/kg/day or diethylstilbestrol (DES) at 30 µg/kg/day. DES was used as a positive control. Both substances were given orally by gavage. Male offspring were sacrificed at postnatal day (PND) 11, 21, or 110 and reproductive parameters were evaluated. Pup birth weight and body weight and percent motile sperm at age of 110 day were significantly reduced by DES. The absolute weight of the right epididymis was significantly reduced in the DES group. The absolute weight of the right epididymis were also significantly decreased in the animals exposed to 75 or 15 mg/kg/day NP, effects which disappeared when organ weight was related to body weight. This study showed a dose-dependent effect of nonylphenol on male reproductive development at doses of 75 and 15 mg/kg bw/day based on absolute epididymal weight.

Quantification of antiandrogen effect determined by Lightcycler technology

During the last decade, the possible effects of xenobiotics on male reproductive health have resulted in great concern. More recently, evidence of antiandrogen effect in vivo by certain chemicals has been reported. The classical Hershberger in vivo assay determining organ weight changes can be improved by measuring hormone levels as well as determining changes in gene expression of androgen-responsive genes. A real-time RT-PCR method using LightCycler technology (Roche) suitable for quantitative determination of gene expression is described. The technique combines rapid thermocycling with online fluorescence detection of PCR product formation. In this study, investigation of expression of prostate specific binding protein polypeptide C3 (PBP C3) and testosterone-repressed prostastic message 2 (TRPM-2) in the ventral prostate was performed in 60-days-old castrated Wistar rats treated daily with testosterone with or without addition of flutamide or vinclozolin for 7 days in total. We show that we can quantify the level of gene expression by use of LightCycler technology, supported by changes in reproductive organ weights as well as in hormone levels, and that analysis of gene expression levels is an even more sensitive endpoint.
Using the CALUX bioassay for screening and determination of dioxin-like compounds in human milk

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Identification and quantification of estrogenic compounds in recycled and virgin paper for household use as determined by an in vitro yeast estrogen screen and chemical analysis

The use of recycled paper for the manufacture of food contact materials is widespread, but very little is known about the presence of potential contaminants in the paper. The purpose of this study was to assess the worst-case migration of estrogenic active compounds using extracts of paper for household use. Twenty different brands of kitchen rolls, nine of which were made from recycled paper and the remainder from virgin paper, were obtained from retail shops. Paper extracts were subjected to (a) determination of the total estrogenic activity by using an in vitro estrogen screen based on yeast cells stably transfected with the human estrogen receptor alpha and (b) chemical analysis and quantification by GC/MS, GC/FTIR/MS, and GC/FID for detection of a variety of estrogenic compounds. A marked estrogenic response was observed in nine of the extracts, seven of which were made from recycled paper and two from virgin paper. The chemical analysis revealed that extracts made from recycled paper contained levels of bisphenol A ranging from 0.6 to 24 mg/kg of kitchen roll, whereas extracts from virgin paper contained no bisphenol A or only negligible amounts. In contrast, 4-tert-octylphenol, 4-nonylphenols, and di-n-butyl and diisobutyl phthalate were present to a varying degree in both recycled and virgin paper with no apparent preferable distribution between the two paper types. The estrogenic response of the two extracts made from virgin paper appeared to be due partly to the presence of the preservative propyl paraben. Diisopropynaphthalene, which turned out to be weakly estrogenic active in vitro (EC50 = 53 μM), was detected in minor amounts in most of the extracts without the major part, ranging from 0.3 to 4.7 mg/kg of paper, found in recycled paper. Our findings that recycled kitchen rolls contain bisphenol A and other xenoestrogens may apply to other types of recycled paper used for food packaging and emphasize the importance of identifying this and other contaminants in recycled paper in general. These data indicate that bisphenol A may be useful as a purity indicator for recycled paper.

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Identification and Quantification of Estrogenic Compounds in Recycled and Virgin Paper for Household use as determined by an in vitro Yeast Estrogen Screen and Chemical Analysis

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Rapid and sensitive reporter gene assays for detection of antiandrogenic and estrogenic effects of environmental chemicals

Reports on increasing incidences in developmental abnormalities of the human male reproductive tract and the recent identifications of environmental chemicals with antiandrogenic activity necessitate the screening of a larger number of compounds in order to get an overview of potential antiandrogenic chemicals present in our environment. Thus, there is a great need for an effective in vitro screening method for (anti)androgenic chemicals. We have developed a rapid, sensitive, and reproducible reporter gene assay for detection of antiandrogenic chemicals. Chinese Hamster Ovary cells were cotransfected with the human androgen receptor expression vector and the mouse mammary tumour virus (MMTV)(2)-luciferase vector using the new nonliposomal transfection reagent FuGene. Stimulation of the cells for 24 h with the synthetic androgen receptor agonist, R1881 (10 nM), resulted in a 30- to 60-fold induction of luciferase activity. The classical antiandrogenic compounds hydroxy-flutamide, bicalutamide, spironolactone, and cyproterone acetate together with the pesticide(metabolite)s, vinclozolin, p,p'-DDE, and procymidone all potently inhibited the response to 0.1 nM R1881. Compared to the traditional calcium phosphate transfection method, this method has the advantage of being more feasible, as the assay can be scaled down to the microtiter plate format. Furthermore, the transfection reagent is noncytotoxic, allowing its addition together with the test compounds thereby reducing the hands-on laboratory time. This assay is a powerful tool for the efficient and accurate determination and quantification of the effects of antiandrogens on reporter gene transcription. To extend the application of FuGene, the reagent was shown to be superior compared to Lipofectin for transfecting MCF7 human breast cancer cells with an estrogen response element-luciferase vector. Thus, FuGene may prove to be valuable in diverse reporter gene assays involving transient transfections for screening of potential endocrine disrupters for (anti)androgenic and (anti)estrogenic properties.

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Screening of selected pesticides for oestrogen receptor activation in vitro

Twenty pesticides were tested for their ability to activate the oestrogen receptor in vitro using an MCF7 cell proliferation assay and a Yeast Oestrogen Screen. The fungicides fenarimol, triadimefon, and triadimenol were identified as weak oestrogen receptor agonists, which at 10 μM induces a 2.0, 2.4, and 1.9-fold increase in proliferation of human MCF7 breast cancer cells (E3 clone). The relative proliferation efficiency (RPE) was 43-69%, indicating partial agonism at the oestrogen receptor. Several pesticides did not have any effect on the proliferation response after 6 days of exposure, including chlorpyrifos, diuron, iprodion, linuron, pentachlorphenol, prochloraz, propiconazol, propyzamine, quintozene, tetrachlorvinphos and tetradiion. Some pesticides resulted in a negligible proliferation response, which was not statistically significant under the present experimental conditions. These were bromopropylate, chlorfenvinphos, chlorobenzilate, dicofol, heptachlor, and imazalil. Fenarimol and dicofol also gave rise to a positive oestrogenic response in yeast cells transfected with the oestrogen receptor alpha, whereas the remaining compounds resulted in a negative response due either to biological inactivity or cytotoxicity to the yeast cells. The EC50 for fenarimol has estimated to be 13 μM in the yeast cells, compared with an EC50 of 3 μM in the MCF7 cells, indicating higher sensitivity of the latter assay. No in vivo data for fenarimol, triadimefon or triadimenol have previously been published that support oestrogenic activity in the intact animal. Thus, from the present results, we suggest that oestrogen receptor activation may not be an important mode of action for these compounds. The need to include at least two bioassays in a screening procedure and for combining in vitro and in vivo data is emphasized.

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Screening af plastemballage til slik og chokoladevarer for østrogen aktivitet

The effects of n-butanol vapour on respiratory rate and tidal volume.

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The effects of n-butanol vapour on respiratory rate and tidal volume.

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