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Genistein and ethinyl estradiol dietary exposure in multigenerational and chronic studies induce similar proliferative lesions in mammary gland of male Sprague–Dawley rats

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ABSTRACT

Genistein and ethinyl estradiol (EE_2) were examined in multigenerational reproductive and 2-yr chronic toxicity studies with different exposure durations across generations F_0 through F_4. Sprague–Dawley rats were exposed to genistein (0, 5, 100, or 500 ppm) or EE_2 (0, 2, 10, or 50 ppb). Effects in the male mammary gland are described here. In the multigeneration studies, mammary hyperplasia was induced by both compounds; the chronic studies had a lower incidence, without proportionate neoplasia. Sexual dimorphism (predominant tubuloalveolar growth in females and lobuloalveolar in males) was retained without feminization in high dose genistein or EE_2. In the continuously exposed generations, mammary hyperplasia was sustained but not amplified, appeared morphologically similar across all generations, and was not carried over into unexposed offspring of previously exposed generations. The hyperplasia in male rats was similar whether induced by genistein or EE_2. Results substantiate and extend previous reports that mammary gland hyperplasia in the male rat is one of the most sensitive markers of estrogenic endocrine disruption.

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1. Introduction

Evidence from human and animal studies has established that exposure to diethylstilbestrol (DES) during reproductive development has a variety of adverse structural and functional reproductive effects including carcinogenesis in both sexes; some of these effects can carry over into unexposed offspring [1–4]. This has led to concerns that chemicals in the environment that have weaker estrogenic or other hormonal activities could have similar adverse effects in human and wildlife populations [5,6]. This "endocrine disruptor hypothesis" has been an area of considerable research and regulatory concern in recent years.

As part of its research effort, the National Toxicology Program (NTP) initiated multigeneration reproductive and chronic toxicity studies to address aspects of this hypothesis. The design for these studies was a modification of standard multigenerational reproductive toxicity studies, altered to determine if subtle effects observed after short-term exposure in young animals would be magnified, sustained, diminished, or reversed in subsequent generations. It would also determine any carry-over into unexposed generations, and any chronic effects, including neoplasia.

For the work reported here, the goal was to select a high dose based on results of range-finding studies that would not produce significant maternal toxicity but would induce mild reproductive tract lesions in the offspring that would not severely affect reproductive capacity in the first generation.

Genistein was selected for study as a representative phytoestrogen to which there is high human exposure in soy-based foods, dietary supplements, and infant formulas. Infants consuming soy formula, for example, have been reported to ingest 6–9 mg/kg of isoflavones-d and to have blood concentrations of total isoflavones as high as 5–10 μM [7]. Soy isoflavones, particularly genistein, interact with estrogen receptors and affect hormone synthesis and metabolism, as well as sex hormone binding proteins. Genistein inhibits multiple enzymes involved in growth regulation, including tyrosine kinases, topoisomerases, and multiple other molecular targets (see above references and [8,9–11]). Phytoestrogens, soy-containing foods, and soy components are known to produce adverse effects on reproductive processes in animals [12–15], and

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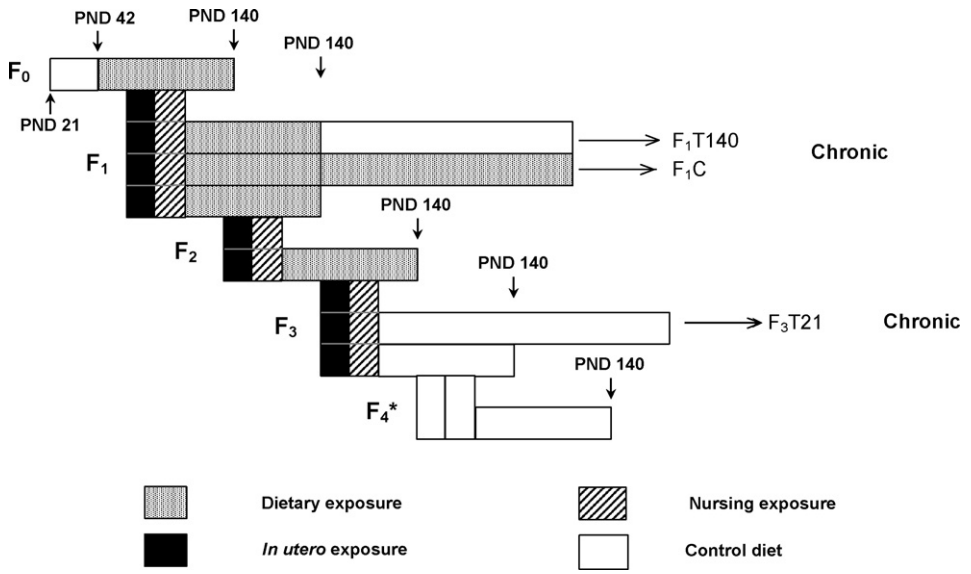

Fig. 1. Experimental design and dosing scheme for the multigenerational 140-day and 2-yr feeding studies for both genistein and EE_2. *Note*: The F_4 generation was mated following the same protocol as all previous generations to produce F_5 litters.

subcutaneous injections of genistein to neonatal female mice produce a spectrum of effects similar to those reported for DES [16–19].

EE_2 has long been the predominant estrogen in oral contraceptives because of its significantly greater oral bioavailability in women relative to 17β-estradiol. Pregnancies do occur in women who are taking oral contraceptives; postconception use of oral contraceptives has been reported in 0.4–2.5% [20], and 4–8% [21] of users. The risks of obvious defects detectable at birth resulting from inadvertent exposure of the fetus to oral contraceptives appear to be low [22,23], although potential subtle long-term consequences of such exposures have not been addressed rigorously.

Detection of EE_2 as an environmental contaminant has recently raised concerns about potential effects in aquatic wildlife [24]. While reports on the adverse effects of *in utero* and neonatal exposure to EE_2 are fewer than those of DES, EE_2 is reported to produce adverse effects following developmental as well as adult exposure in both sexes of mice and rats similar to those caused by DES [25–35].

This manuscript summarizes data regarding genistein- or EE_2-induced hyperplastic and neoplastic changes in the mammary gland of male rats in which cohorts were fed one of four doses, for different durations at various ages. The complete study reports, which include data not reported here, are contained in NTP Reports 539, 545, 547, and 548, which are available at the NTP website (http://ntpserver.niehsnih.gov/index.cfm?objectid=084801F0-F43F-7B74-0BE549908B5E5C1C). The major nonneoplastic effects of genistein and EE_2 in females and neoplastic effects in other organs of both sexes will be presented separately.

The doses used in these studies were established from results of prior short-term dose range-finding reproductive studies using the same facilities, staff, feed, test animal system, and test articles as in the present studies. The complete results of this range-finding work and the rationale used to set the dose ranges for the present studies are detailed elsewhere [36–39].

2. Materials and methods

All animal procedures were conducted under protocols approved by the NCTR Institutional Animal Care and Use Committee.

2.1. Background isoflavone content of the diet

The base diet used for the current study was an irradiated soy- and alfalfa-free rodent feed (5K96, Purina Mills, Inc., Richmond, IN) in order to maintain consistently low background exposure to phytoestrogens. This feed maintains the nutritional specifications of the NIH-31 feed, which is the standard breeding and maintenance diet used in the NCTR rodent colony, and contains casein in place of the soy and alfalfa protein (www.labdiet.com). This feed was routinely assayed for total isoflavone content after acid hydrolysis, using HPLC/MS methods that indicated levels of genistein and daidzein of ≤0.5 ppm each [40]. Animals consuming the control feed were thus ingesting a concentration of genistein approximately 10-fold lower than that of the groups exposed to the low dose (5 ppm) in the genistein studies. This background concentration is consistent with the isoflavone intake of humans consuming typical Western diets. The 5K96 diet underwent routine analyses for nutrient and contaminant levels according to standard NCTR procedures.

2.2. Test compounds and dose formulations

Genistein (Toronto Research Chemicals, Inc., North York, Ontario, Canada) and EE_2 (Sigma–Aldrich Corporation, St. Louis, MO) were determined to have purities of greater than 99% and 98.5%, respectively. Dosed feed was blended on an as needed basis, but not more than every 5 (genistein) or 9 (EE_2) weeks, in a Patterson–Kelley twin-shell blender. Genistein dosed feed was prepared by dry mixing, while a solution of EE_2 in 95% ethanol was directly injected into Purina 5K96 in the blender to prepare the 10 and 50 ppb EE_2 diets. Ethanol was removed from the EE_2-dosed feed and from the control feed that was mixed with 95% ethanol alone by heating under vacuum in the blender. The 2 ppb EE_2 dose formulations were prepared by 1:5 dry dilutions of the 10 ppb dose feed. Blended dosed feed formulations were stored in stainless steel cans at 4 °C. Purity of the bulk chemical was periodically confirmed during the course of the studies. Homogeneity of the blended dosed feed was confirmed, and stability in stainless steel storage cans was confirmed for up to 16 (EE_2) or 17 (genistein) days at ambient temperature and for up to 24 (EE_2) or 32 (genistein) weeks at 2 ± 8 °C. Because of the very low exposure concentrations used in the EE_2 study, the technical difficulties associated with measurements of such concentrations in the complex diet matrix were recognized, and a somewhat higher degree of variability than would be seen in studies with higher exposure concentrations was anticipated and accepted prior to the start of the studies. Analysis of blended dosed feeds indicated that genistein concentrations were ±10% of the target dose, while EE_2 concentrations were ±30% of the target. These values were within the established study specifications.

2.3. Experiment design, multigeneration EE_2 and genistein reproductive studies

The multigenerational reproductive toxicology and chronic study designs are outlined in Fig. 1. The F_0 generation was exposed to dosed feed from 6 weeks of age until termination at PND 140, while the F_1 and F_2 generations were exposed continuously from conception through termination at PND 140. The F_3 generation was exposed from conception to weaning (PND 21) and then placed on control feed for the remainder of the study. The F_4 generation was not directly exposed to dosed feed and was included to test for transgenerational effects of compound exposure.

An additional consideration beyond the main study design was to examine mammary gland effects after both continuous exposure and potential recovery after treatment was stopped in younger male rats, ages PND 50 and 90. For this multigenerational 90-day feed study, a subset of male pups from the F_1 and F_2 generations of the EE_2 study were utilized. One male from each of 18 litters in F_1 was sacrificed