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# Effects of genistein in the maternal diet on reproductive development and spatial learning in male rats

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ABSTRACT

Endocrine disruptors, chemicals that disturb the actions of endogenous hormones, have been implicated in birth defects associated with hormone-dependent development. Phytoestrogens are a class of endocrine disruptors found in plants. In the current study we examined the effects of exposure at various perinatal time periods to genistein, a soy phytoestrogen, on reproductive development and learning in male rats. Dams were fed genistein-containing (5 mg/kg feed) food during both gestation and lactation, during gestation only, during lactation only, or during neither period. Measures of reproductive development and body mass were taken in the male offspring during postnatal development, and learning and memory performance was assessed in adulthood. Genistein exposure via the maternal diet decreased body mass in the male offspring of dams fed genistein during both gestation and lactation, during lactation only, but not during gestation only. Genistein decreased anogenital distance when exposure was during both gestation and lactation, but there was no effect when exposure was limited to one of these time periods. Similarly, spatial learning in the Morris water maze was impaired in male rats exposed to genistein during both gestation and lactation, but not in rats exposed during only one of these time periods. There was no effect of genistein on cued or contextual fear conditioning. In summary, the data indicate that exposure to genistein through the maternal diet significantly impacts growth in male offspring if exposure is during lactation. The effects of genistein on reproductive development and spatial learning required exposure throughout the pre- and postnatal periods.

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The endocrine disruptor hypothesis proposes that exogenous compounds can interfere with endocrine function by altering the binding, release, or metabolism of endogenous hormones (Baskin et al., 2001; Colborn et al., 1993). Evidence in favor of this hypothesis has included rodent studies of plant-derived estrogenic compounds termed phytoestrogens. For example, studies of the soy-derived phytoestrogen genistein showed that exposure of pregnant females causes decreased birth weight, reduced anogenital distance (AGD), delayed puberty, altered mass of reproductive organs, and altered reproductive behavior in male offspring (Levy et al., 1995; Nagao et al., 2001; Santell et al., 1997; Wisniewski et al., 2003; Wisniewski et al., 2005).

Exposure of males to genistein not only impacts their reproductive development and function but also alters several sexually dimorphic behaviors including learning and memory. Sex-based differences in behavior are thought to be established by the organizational effects of gonadal hormones on brain development and morphology (Isgor and Sengelaub, 1998; Isgor and Sengelaub, 2003; Joseph et al., 1978; Leret et al., 1994; Lucion et al., 1996; Roof and Havens, 1992; Williams et al., 1990). Interestingly, studies have shown that exposure to estrogen or estrogen-like compounds (such as genistein) perinatally or in adulthood can diminish sex differences in behavior (Gupta et al., 2001; Lephart et al., 2002; Lephart et al., 2004; Leret et al., 1994; Lund and Lephart, 2001a; Lund et al., 2001; Pan et al., 2000). These data support the idea that sexually dimorphic behavioral traits are sensitive to endocrine disruption.

The sensitivity of sexually dimorphic traits to gonadal steroids is well-understood to depend on the developmental stage of the animal. In rodents, for example, gestational exposure to androgens masculinizes the external genitalia (Faber and Hughes, 1992; Grady et al.,

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1965) and pre- and postnatal exposure (up to postnatal day 10) masculinizes the CNS in rodents (Isgor and Sengelaub, 1998; Isgor and Sengelaub, 2003; Roof and Havens, 1992). With these findings in mind, we undertook the current study to examine whether the effects of genistein on reproductive development and sexually dimorphic behavior require exposure throughout gestation and lactation, as done by Wisniewski et al (Wisniewski et al., 2003; Wisniewski et al., 2005), or whether these effects can be seen when exposure is limited to either critical period.

## Materials and methods

### *Animals*

Male and female Sprague–Dawley rats were obtained from the Drake University Breeding Colony. All animals had access to food and water *ad lib*. Animals were maintained on 12:12 light-dark cycle with lights on at 0600 h CST in a temperature-controlled vivarium. Adequate measures were taken to minimize pain or discomfort of the animals. Experiments were conducted in accordance with international standards on animal welfare and were in compliance with local and national regulations. All procedures were approved by the Drake University IACUC.

### *Diets*

Genistein (4′,5,7-trihydroxyisoflavone) was purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ) and mixed with a casein-based diet (5K96, Purina Mills, Richmond, IN) that contained less than 1 ppm phytoestrogens at a concentration of 5 mg/kg of feed. The 5K96 diet is similar to the standard NIH-31 formula except that soy and alfalfa proteins are replaced with casein. 5K96 diet without genistein added served as the control diet in all experiments. All diets were sterilized by irradiation.

### *Procedure*

For assessment of reproductive development and learning and memory of male offspring, dams were randomly assigned to one of four diet groups: control (C-C; litter $n=8$), genistein (G-G; litter $n=9$), gestation (G-C; litter $n=8$), and lactation (C-G; litter $n=8$). All dams were acclimated to their respective diets for 2 weeks prior to mating. Dams began mating at 70 days of age. Pregnancy was determined by presence of vaginal sperm plug or significant weight gain (20 g in one week). Day of birth was identified as postnatal day 1 (PND 1). Pups were exposed to genistein via gestating and/or lactating mothers consuming a 5 mg/kg dose of genistein in rat chow as described above. C-C dams were fed control diet during gestation (gestation day 1 (GD 1) through postnatal day 1 (PND 1) and during lactation (PND 2-PND 21). G-G dams were fed genistein diet during gestation and lactation. G-C dams were given genistein diet during gestation, and then switched to control diet from the day pups were born through PND 21. C-G dams were given control diet during gestation, and then switched to the genistein diet from the day that pups were born to PND 21. There were no significant group differences in the amount of food consumed during gestation and lactation (data not shown). At PND 21, pups were weaned and housed with same sex siblings and placed on control diet. On PND 70 1–4 males per litter were randomly selected for necropsy and analysis of reproductive organ weight. Remaining males (maximum of 2 per litter) were used for testing of learning and memory.

### *Measures of maternal behavior*

For assessment of maternal behavior, observations were taken from a mixture of the litters described above and similarly treated litters that were used in the reproductive anatomy and learning and memory experiments. Litter *n*'s for the assessment of maternal behavior were C-C, $n=14$; G-G, $n=13$; G-C, $n=7$; C-G, $n=8$. Observations were taken between 9:00 am and 11:00 am everyday beginning on postnatal day 3 and ending in postnatal day 21. Measurements were obtained by observing the litters 3 times for 30 s with an interval of 10 min between observations. This number of observations was chosen based on previous work by one of us (BJS) that more extensive observations do not provide new information on maternal behavior (Sanders and Gray, 1997). The specific behaviors recorded were nursing, licking of pups, and whether the dam was out of contact with the nest.

### *Measures of reproductive development and growth*

Anogenital distance (AGD) was measured on PND 2, 7, 14, 21 using a caliper with a digital readout. Using an electronic balance, pups were weighed together and averaged on PND 2, but were weighed individually on all other days. At PND 21, pups were weaned and housed by sex with siblings. At PND 70, rats were weighed and then sacrificed with $CO_2$ overdose and the wet weight of the testes, gonadal fat pad, epididymides, and seminal vesicles were obtained.

### *Assessment of spatial learning and memory in the Morris water maze*

Testing of the performance of the rats in the Morris water maze followed standard methods as previously described (Kinney et al., 2003; Wrenn et al., 2004). Rats were approximately 5 months old at the onset of testing. The maze consisted of a plastic, circular pool, with a diameter of 183 cm and a height of 76 cm. Water was added to a depth of 61 cm and rendered opaque by the addition of non-toxic white paint. Water temperature was maintained at approximately 22 °C. Rats were tested by an investigator blind to treatment group on three components of the task: hidden platform training, probe trial testing, and visible platform training. In all components of the task, video tracking of the swim path was recorded and data collected by a personal computer equipped with commercially available water maze software (Actimetrics, Evanston, IL).

Hidden platform training took place on days 1–6 and days 8–9. Probe trials took place on day 7 and day 10, and there was a 2-day break between day 4 and day 5; thus hidden platform testing spanned 12 days with the hidden protocol performed a total of 8 times. Each rat received four trials per day with each trial consisting of placing the rat, facing the wall of the pool, in a pseudorandomly selected start position. Rats were allowed a maximum of 60 s to reach a hidden platform (circular, 15 cm in diameter) which was submerged 2 cm below the waterline in a fixed spatial location. Rats were removed from the platform after 30 s and given a 30-s rest period between consecutive trials. Performance measures recorded included latency to find the platform and swim speed.

In order to shed light on how the rats were reaching the platform, we supplemented our latency data by identifying the predominant swim strategy used on the first trial of each training day. To identify swim strategies we used the following decision algorithm which is modified from previously published methods (Brody and Holtzman, 2006; Graziano et al., 2003; Janus, 2004; Wolfer and Lipp, 2000):

(a) If >50% of the swim path was within 10 cm of the edge of the pool and the latency to find the platform was>15 s, the strategy was identified as *thigmotaxis.*
(b) If the swim path did not meet the criteria for *thigmotaxis*, was predominantly circular, and the latency to find the platform was >15 s, the strategy was identified as *circling.*
(c) If the swim path did not meet the criteria for *circling,* entered all three quadrants after leaving the start quadrant, did not remain in any one quadrant for >40% of the trial, and the

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