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Postweaning dietary genistein exposure advances puberty without significantly affecting early pregnancy in C57BL/6J female mice

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ABSTRACT

An epidemiological study indicates higher plasma level of genistein in girls with earlier puberty. This study tests the hypothesis in C57BL/6J mice that postweaning (peripubertal) dietary genistein exposure could result in earlier puberty in females assessed by vaginal opening, estrous cyclicity, corpus luteum and mammary gland development. Newly weaned female mice were fed with 0, 5, 100, or 500 ppm genistein diets. Decreased age at vaginal opening, increased length on estrus stage, and accelerated mammary gland development were detected in 100 and 500 ppm genistein-treated groups. Increased presence of corpus luteum was found in 5 ppm genistein-treated group at 6 weeks old only. Increased expression of epithelial-specific genes but not that of ERα or ERβ was detected in 500 ppm genistein-treated mammary glands at 5 weeks old. No significant adverse effect on embryo implantation was observed. These data demonstrate causal effect of dietary genistein on earlier puberty in female mice.

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1. Introduction

Genistein is a phytoestrogen abundant in soy [1]. High levels of genistein are found in traditional soy food, such as soy milk, tofu, miso, etc., as well as a variety of processed food, such as meatless burger, energy bar and soy yogurt, etc. [2]. The estimated daily intake of genistein in US adults is ∼0.6 mg/day based on National Health and Nutrition Examination Survey 1999–2002 data [3], and ∼6–19 mg/day in Asian people [4–6]. Since US FDA approved the health claims of soy diet on reducing coronary disease in 1999 [7], soy consumption in US has been steadily increasing [8].

Genistein could have different effects. The beneficial effects of genistein include relieving menopausal symptom, protecting cardiovascular system, preventing breast cancer, etc. [9–12]. Since genistein is a weak estrogen [13,14], its potential endocrine disruptive effects have also been identified in many studies and recognized in the NTP-CERHR Expert Panel Report [15]. For example, genistein has been widely regarded as a contributing agent for a trend of earlier puberty in US and European girls [16–20]. Puberty is the physical development process of an immature body to an adult body capable of reproducing under the regulation of sexual hormones, such as estrogen [21]. A longitudinal study in UK including 1920 girls shows a positive correlation between soy formula intake during infancy and earlier menarche age [22]. Since menarche is an indicator of puberty [16] and genistein is the major phytoestrogen in the infant plasma after soy formulate consumption [23], it is most likely that genistein contributes to the puberty advancement upon infant soy formulate consumption. A case–control study of 150 6–12 years old precocious girls and 90 age-matched control girls in Korea reveals a significantly higher plasma level of genistein in the precocious group [24], implying that increased prepubertal exposure to genistein is associated with early puberty.

The majority of the human population is mainly exposed to genistein from food after infancy when non-milk food is added to the diet, equivalent to postweaning dietary exposure in rodents. We hypothesized that postweaning exposure to genistein in the diet could lead to earlier puberty in females. This hypothesis was tested in C57BL/6J female mice using human relevant exposure levels (5 ppm, 100 ppm, and 500 ppm genistein diets). It was reported that rats fed with 5 ppm and 100 ppm genistein diets could produce

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plasma levels of genistein similar to that in Western and Asian people, respectively [25], while 500 ppm genistein diet could be found in soy products, e.g., soy bacon [2]. These doses were also used in the multi-generational studies of genistein by the National Toxicology Programs (NTP) [26]. Vaginal opening, estrous cyclicity, ovulation initiation, and mammary gland development were monitored as indicators for puberty development in this study.

2. Materials and methods

2.1. Animals

C57BL/6J is a sensitive mouse strain to endocrine disruptors [27–29] and was selected as an *in vivo* model in this study. C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained on phytoestrogen-free AIN-93G diet (Bio-Serv, Frenchtown, NJ) in Coverdell animal facility at the University of Georgia. The mice were housed in polypropylene cages with free access to food and water on a 12 h light/dark cycle (0600–1800) at 23 ± 1 °C with 30–50% relative humidity. All methods used in this study were approved by the University of Georgia IACUC Committee (Institutional Animal Care and Use Committee) and conform to National Institutes of Health guidelines and public law.

2.2. Treatment

The genistein diets were prepared following the similar procedure as described previously [29]. Briefly, 0 g, 0.0025 g, 0.05 g, or 0.25 g genistein were dissolved in 150 ml 70% ethanol. Each solution was well mixed with 500 g AIN-93G diet in a glass bowl to attain 0 ppm (control), 5 ppm, 100 ppm, and 500 ppm genistein diets, respectively. Food pellets were hand squeezed, air dried at room temperature, and kept at 4 °C in the dark. Fresh diets were prepared every two weeks. Breeding females were on phytoestrogen-free AIN-93G diet ad libitum throughout pregnancy and lactation. Newly weaned (postnatal day 21) female pups were randomly assigned into four groups and fed with 0 ppm, 5 ppm, 100 ppm, or 500 ppm genistein diet, respectively, until sacrificed for tissue collection/determination of pregnancy status. Food and water consumption were monitored weekly. The numbers of mice included in different experiments were indicated under each experiment. Newly weaning males were sacrificed without further study.

2.3. Vaginal opening and estrous cycle

Vaginal opening was evaluated daily from weaning until detection of vaginal opening (N = 35 per group). Estrous cycle was monitored daily during two periods: from the day of vaginal opening for 10 days and from 5 to 8 weeks old (N = 6 per group). Some overlaps of dates were seen during these two periods, especially for 0 and 5 ppm genistein groups. Vaginal smear was collected at 0800 h. The stages of estrous cycle were determined according to the composition of nucleated, cornified cells, and leukocytes as described [29,30].

2.4. Tissue collection

All mice were dissected at estrus stage, which was determined by vaginal smear prior to dissection, by CO_2 inhalation and cervical dislocation. Only the mice on estrus stage at the selected time points (5, 6, 7, or 10 weeks old) ± 1 day were included. One side of the mammary glands was frozen, the other side of the mammary glands was used for whole mount staining or fixed for immunohistochemistry, and an ovary was fixed for histology.

2.5. Ovary histology

After fixation in formalin for 24 h, the ovaries were washed in 50%, 70%, 80%, 90%, and 95% ethanol for 30 min each, 100% ethanol for 30 min twice, and xylene for 5 min twice, then embedded in paraffin. Paraffin sections were cut at 5 μm. Serial sections of the ovaries in all four groups at 6 weeks old (N = 5–6 per group), in 0 and 500 ppm genistein-treated groups at 5 weeks old (N = 3 per group) and 7 weeks old (N = 6 per group), and in 5 ppm and 100 ppm genistein-treated groups at 7 weeks old (N = 3 per group) were evaluated. Consecutive sections were separated by 50 μm.

2.6. Mammary gland whole mount and quantification of mammary gland development

The dissected whole inguinal (the 4th) mammary gland was flattened on a slide (Fisher Scientific, Pittsburgh, PA) with weight for 24 h, then fixed in Carnoy's solution, stained by carmine alum, dehydrated through alcohol, cleared in xylene and mounted, as described [31]. Pictures were taken with an Olympus microscope BX41 with DP70 digital camera. The morphology of the mammary glands from 0 ppm, 5 ppm, 100 ppm, and 500 ppm genistein-treated groups (N = 6–10 per group) at 5, 6, 7, or 10 weeks old, respectively, was analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA). The duct length of each mammary gland was indicated by the length of the longest duct. The occupied area of each mammary gland was approximated by a polygon area that covered all the ducts.

2.7. Realtime PCR

The whole inguinal mammary gland was dissected from 5 weeks old mice in 0 and 500 ppm genistein-treated groups (N = 6–7 per group) and the lymph node was removed. Each lymph node-free mammary gland was homogenized in Trizol for total RNA isolation and cDNA synthesis using random primers (Invitrogen, Carlsbad, CA, USA) as previously described [32,33]. Realtime PCR was performed in 384-well plates using Sybr-Green I intercalating dye on ABI 7900 (Applied Biosystems, Carlsbad, CA, USA). The mRNA expression levels of amphiregulin (*Areg*), cytokeratin 5 (*CK5*), *CK8*, *CK14*, *CK18*, estrogen receptor α (*Esr1*) and *Esr2*, progesterone receptor (*PR*), and wingless-related MMTV Integration Site 4 (*Wnt4*) were determined using gene-specific primers from different exons (Integrated DNA Technology, San Diego, CA, USA). The mRNA expression levels were normalized by the expression of *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase). *Hprt1* (hypoxanthine phosphoribosyltransferase 1) served as the second house-keeping gene (Suppl Table S1).

2.8. Immunohistochemistry

Paraffin sections (5 μm) of the inguinal mammary glands from 5 weeks old mice (N = 3 per group) were used for immunohistochemistry. Sections from three mice each in 0 and 500 ppm genistein-treated groups were evaluated. After dewaxing, the slides were immunostained with rabbit anti-PR antibody (1:200, 6 μg/ml, Daco, Denmark), rabbit anti-ERα (ESR1) antibody (1:100, 5 μg/ml, Abcam), rabbit anti-phospho ERα (1:100, 10 μg/ml, Abcam), and rabbit anti-ERβ (ESR2) (1:50, 20 μg/ml, Abcam) as previously described [28].

2.9. Embryo implantation

Female mice from 0 and 500 ppm groups (N = 8–27 per group) were mated with stud males starting at three time points: right after vaginal opening, at 5 weeks old, or at 7 weeks old, respectively.

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