Endocrine Disruptors and Hypospadias: Role of Genistein and the Fungicide Vinclozolin

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OBJECTIVES The phytoestrogen (plant estrogen) genistein, present in soy products, is of interest because in utero exposure to genistein can cause hypospadias in our mouse model and maternal consumption of soy is prevalent in human populations. Another compound of interest is the fungicide vinclozolin, which also causes hypospadias in the mouse and rat and can occur concurrently with genistein in the diet as a residue on exposed foods. A study in the United Kingdom found no relationship between a maternal organic vegetarian diet and hypospadias frequency, but women who consumed nonorganic vegetarian diets had a greater percentage of sons with hypospadias. Because nonorganic diets can include residues of pesticides such as vinclozolin, we sought to assess the interaction of realistic daily exposures to genistein and vinclozolin and their effects on the incidence of hypospadias.

METHODS Pregnant mice were fed a soy-free diet and orally gavaged from gestational days 13 to 17 with 0.17 mg/kg/day of genistein, 10 mg/kg/day of vinclozolin, or genistein and vinclozolin together at the same doses, all in 100 μL of corn oil. The controls received the corn oil vehicle. The male fetuses were examined at gestational day 19 for hypospadias, both macroscopically and histologically.

RESULTS We identified no hypospadias in the corn oil group. The incidence of hypospadias was 25% with genistein alone, 42% with vinclozolin alone, and 41% with genistein and vinclozolin together.

CONCLUSIONS These findings support the idea that exposure to these compounds during gestation could contribute to the development of hypospadias. UROLOGY 70: 618–621, 2007. © 2007 Elsevier Inc.

A longitudinal pregnancy study in the United Kingdom found that the sons of vegetarian mothers had an odds ratio for hypospadias of 4.99 (95% confidence interval 2.1 to 11.88)[1]; however, if the vegetarian mothers ate only organic produce, no hypospadias developed, even though a low frequency of cases would have been expected. These findings raised the question of whether pesticide residues on fruits and vegetables could be involved in this increased frequency, rather than the phytoestrogens, which are known to be estrogenic. The study also found that mothers who drank soy milk and ate soy products delivered a larger proportion of boys with hypospadias, although this result was not statistically significant. The mothers who were vegetarians before their pregnancies but became omnivores throughout pregnancy were no more likely to have a son with hypospadias than those mothers who were never vegetarians.[1]

A compound that derails normal endocrine signaling, especially during development, falls into the category of an endocrine disruptor. Vinclozolin has been identified as an anti-androgenic endocrine disruptor that produces malformations related to androgen inhibition in rats.[2–4] This fungicide is used on food crops (soft fruits and vegetables)[5] and is thus potentially a prominent residue in inorganic vegetarian diets. Genistein is a phytoestrogen found in soy. The two can occur together in a diet that includes soy and foods that have been exposed to vinclozolin. Our goal was to model in utero exposure using vegetarian diets with and without the presence of a pesticide to determine the effects, if any, on genital tubercle development and the incidence of hypospadias. We used the mouse model of hypospadias to explore these outcomes.[6]

MATERIAL AND METHODS

Animals

The committee on animal research at the University of California, San Francisco, approved the scientific protocol. Timed-pregnant CD1 mice (Charles River, Wilmington, Mass) were received on gestational day (GD) 8 and housed in separate animal cages until GD 13. All animals were housed one per cage (20 × 25 × 47 cm) with laboratory-grade pine shavings (heat-treated to remove resins) as bedding. They were acclimated to 20° to

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Submitted: January 20, 2007; accepted (with revisions): May 14, 2007

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0090-4295/07/$32.00
doi:10.1016/j.urology.2007.05.004

23°F and 40% to 50% relative humidity on a reversed light schedule (14 hours light and 10 hours dark) and received a special diet (soy-free diet chow) and tap water.

The pregnant mice were separated into four groups (n = at least 8/group): group 1 received corn oil-vehicle control (0.1 mL/day); group 2 received genistein only (0.17 mg/kg/day in 0.1 mL corn oil); group 3 received vinclozolin only (10 mg/kg/day in 0.1 mL corn oil); and group 4 received genistein (0.17 mg/kg/day) plus vinclozolin (10 mg/kg/day in 0.1 mL corn oil). The dosages were chosen on the basis of the realistic daily exposure levels of genistein, which we determined according to the amounts of genistein in various foods. The dose of vinclozolin was determined from the U.S. Environmental Protection Agency's report of the lowest observed effect level of 11.5 mg/kg/day for acute exposure.[7] On GD 13, when the genital tubercle differentiation is beginning, we began daily gavaging of the pregnant mice. Gavaging ended on GD 17. On GD 19, as established for this model[6] and when the development of the genital tubercle is clearly differentiated, the fetuses were harvested by cesarean section, and the sex was determined by examination of the gonads under a dissection microscope. The determination of hypospadias was made by expressing the bladder contents and noting where they emerged from the urethral opening. An opening below the tip of the genital tubercle, the normal location in male fetuses at this stage, was identified as hypospadic.

Histologic Examination

After the sex was identified, we dissected out the genital tubercle for about one half of the specimens and fixed the tissue in formalin, followed by processing for paraffin embedding. Coronal serial sections of the genital tubercle from GD 19 embryonic male and female mice were taken and stained with hematoxylin-eosin. The slides were analyzed microscopically using a 10× magnification and photographed using a Nikon 900 digital camera (Melville, NY). The main criterion for a urethral abnormality was the co-occurrence of an open urethra and a visible corpus cavernosum, a structure that occurs in the shaft of the genital tubercle. In normal E19 male genital tubercles, coronal sections show a closed urethra distally, well before any structures of the shaft become visible.[6]

Statistical Analysis

The hypospadias frequencies among the groups were compared using Fisher's exact analysis of two-by-two contingency tables.

RESULTS

After macroscopic identification, we analyzed the slide sections histologically. Figure 1 shows sections of genital tubercles from males from each group, including a corn-oil control male (no hypospadias) and males from each treatment group that were identified as having hypospadias. We found that the frequency of hypospadias overall was 0% in the control group (n = 30); 25% in the genistein group (n = 24); 42% in the vinclozolin group (n = 26); and 41% in the combination group (n = 29; Fig. 2).

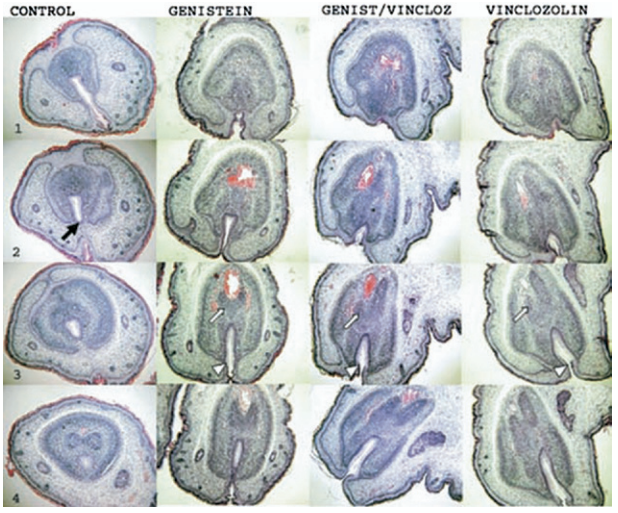

Figure 1. Histologic analysis of genital tubercles in males. Columns represent different treatments, as labeled. Rows 1 through 4 are distal to proximal coronal sections, beginning at tip of genital tubercle and moving toward body. A normal E19 male mouse genital tubercle is closed near the distal tip (column 1, row 2, black arrow). In males with hypospadias, the urethra remains open well into the shaft; the presence of the butterfly-shaped corpus cavernosum indicates sections well into the penile shaft (columns 2 to 4, white arrows). Co-occurrence of open urethra (columns 2 to 4, arrowheads) and corpus cavernosum indicates abnormally proximal urethral opening, or hypospadias.

Table 1 shows the different comparisons and the *P* values from Fisher's exact analysis of frequency data. We found that genistein alone, vinclozolin alone, and the combination of the two significantly increased the frequency of hypospadias. Vinclozolin and the combination of the two produced a greater frequency than did genistein alone.

COMMENT

Hypospadias is a common congenital abnormality, affecting about 1 in every 250 boys. It is a urethral abnormality in which the urethra opens along the ventral side of the penis, rather than at the tip.[8] The final location of the urethral opening and the length of the urethra are androgen-dependent processes that can be disrupted by exposure to antiandrogens[8] or estrogens.[6] Although reports have suggested that hypospadias has increased in the past few decades,[9] these findings remain controversial.[10] The exact etiology of isolated hypospadias remains unclear, although the influences appear to be multifactorial.[8] Potential agents in this multifactorial pathway to the development of hypospadias are endocrine-disrupting compounds,[8] synthetic or naturally occurring compounds that can derail endocrine-governed developmental processes.

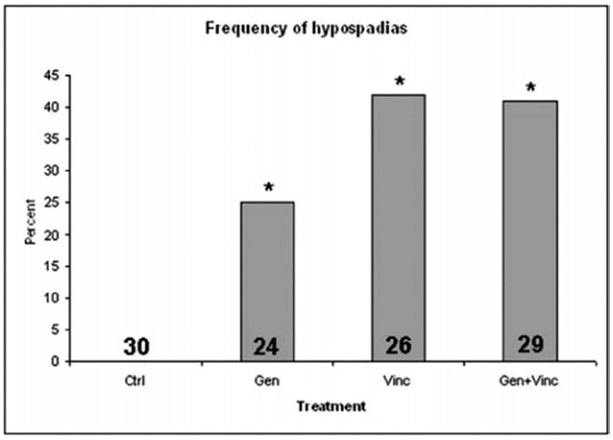

Figure 2. Frequencies of hypospadias in each treatment group. Gen = genistein treatment (0.17 mg/kg/day); Vinc = vinclozolin treatment (10 mg/kg/day); Gen+Vinc = genistein and vinclozolin treatment combined in concentrations used in single treatments. *Significant difference compared with controls. Numbers inside columns numbers of mice for each group.

Table 1. Results of Fisher's exact analysis of two-by-two contingency tables for indicated comparisons

Comparison	*P* Value
Control vs. Gen	0.0052*
Control vs. Vinc	<0.0001*
Control vs. Gen+Vinc	<0.0001*
Gen vs. Vinc	0.1607
Gen vs. Gen+Vinc	0.1530
Vinc vs. Gen+Vinc	0.56

Gen = genistein; Vinc = vinclozolin; Gen+Vinc = two combined in single treatment.
* Statistically significant.

It has already been shown that estrogens can elicit hypospadias in this mouse model[6]; thus, it is not surprising that a plant estrogen such as genistein would exert these apparently estrogenic effects. Vinclozolin is known to affect androgen receptor-mediated endpoints.[2,4,11–14] It also has recently been shown to have multigenerational effects, implying that it does pass to, and affect, the fetus.[15] In the present study, in utero exposure to genistein alone caused a significantly greater incidence of hypospadias compared with that in the controls. Vinclozolin alone also caused a significant increase in hypospadias, as did vinclozolin combined with genistein. The results suggest that a realistic daily exposure to genistein in the maternal diet could elicit hypospadias, but the addition of another endocrine disruptor such as vinclozolin, which was used at levels below that which causes observable effects in rats, will increase the possibility.

These findings add support to the suggestion that nonorganic vegetarian diets might result in greater frequencies of hypospadias than organic vegetarian diets, which presumably have lower or no levels of endocrine-disrupting pesticides. Our results have indicated that the fungicide might be the agent primarily responsible for the hypospadias incidence in these animals; it caused the same frequency of the anomaly when used alone as it did in combination. It is not uncommon for combinations of endocrine-disrupting compounds to produce less-than-additive results.[16]

The results suggest that any increased frequency of hypospadias in women consuming a vegetarian diet could be attributable to an organochlorine contaminant on the fruits and vegetables consumed if the diet is not organic. These findings appear to agree with those of the human epidemiologic study that found no specific association between maternal soy consumption and the frequency of hypospadias, but did identify a link between maternal consumption of a nonorganic vegetarian diet and the frequency of this anomaly.[1] That study also found a slight increase in hypospadias frequencies in the sons of women who drank soy milk and ate soy products during pregnancy. Although the increase was not significant, it reflects the relationship among the findings in our study.

CONCLUSIONS

Our results indicate that simultaneous maternal consumption of soy and vinclozolin, such as can occur in a nonorganic vegetarian diet, might result in an increase in hypospadias frequency.

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