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Neonatal Exposure to Coumestrol, a Phytoestrogen, Does Not Alter Spermatogenic Potential in Rats

Caleb A. Awoniyi,[1] Dawn Roberts,[1] Varadaraj Chandrashekar,[2] D. N. Rao Veeramachaneni,[3] Bradley S. Hurst,[1] Kathleen E. Tucker,[1] and William D. Schlaff[1]

[1]Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, University of Colorado Health Sciences Center, Denver, CO; [2]Department of Physiology, Southern Illinois University, Carbondale, IL; and [3]Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO

The objective of this study was to determine the effects of neonatal exposure to phytoestrogens on male reproductive function as adults. Male rats were injected either with 100 μg coumestrol or DMSO (controls) daily during their first 5 d of life. Pituitary gland, testes, sex accessory organs, and blood were collected on d 60 of life. Serum testosterone, LH, and FSH levels were determined by RIA. Levels of steady-state mRNA for gonadotrophin subunits (LHβ and FSHβ were determined by Northern blot analysis and quantified by a scanning densitometer. Coumestrol had no effect on weights of testes and sex accessory organs, or sperm count. Similarly, there were no significant differences among serum concentrations of testosterone, LHβ and FSH of coumestrol-treated rats and those of controls. Whereas steady state levels of LHβ mRNA in coumestrol-treated rats did not differ from those of controls, steady state levels of FSHβ mRNA increased (37%) in treated animals. However, the augmented FSHβ mRNA expression in coumestrol-treated rats did not negatively affect reproductive potential in male rats. We conclude that neonatal exposure to coumestrol does not alter reproductive organ structure or spermatogenic potential in male rats.

Key Words: Coumestrol; spermatogenesis; testis; gonadotrophin subunits.

Introduction

Recent reports have shown that the incidence of abnormalities of male genitalia and disorders of spermatogenesis is on the rise, and that the sperm counts have been falling over the past 50 yr in human populations in several countries *(1–3)*. The relatively short period during which these changes have occurred in human populations is evidence that the cause is environmental rather than genetic. Estrogens or estrogen-like chemicals have been implicated as one of the causative agents for testicular abnormalities and decline in sperm counts *(4)*.

Estrogenic xenobiotics, such as isoflavonoids, abound as natural constituents of plants, and, therefore, are in diets. These phytoestrogens include coumestans, isoflavones, and retinoids *(5)*. These forms of plant estrogens are found in alfalfa, coffee, wheat, corn, and soybeans *(6,7)*. These ingredients/staples form a large part of the human diet and their estrogenic constitutents can be detected in the urine of herbivores, including humans *(8)*. Some of these naturally occurring compounds have been shown to mimic estradiol and diethylstilbestrol (DES) *(9)*, resulting in reproductive anomalies. For example, coumestrol (a phytoestrogen) has been shown to induce persistent vaginal cornification, hemorrhagic ovarian follicles, premature vaginal opening, decreased ovulation rates, and increased embryo degeneration in mice *(10–12)*. None of the reports cited above addresses the possibility that neonatal coumestrol exposure might adversely affect the male reproductive system. This is particularly surprising, since estrogenic compounds, such as DES, which causes testicular perturbations, could be expected to have their greatest impact on the male reproductive system, especially if exposure occurs early in development.

In light of considerable evidence that neonatal exposure to estrogens and estrogenic chemicals, including mycotoxins, results in alterations of structural and functional differentiation of the reproductive system *(13–15)*, we sought to investigate the long-term (postpubertal) sequelae in male rats following neonatal exposure to coumestrol. We evaluated the steady-state mRNA levels of gonadotrophin subunits (LHβ and FSHβ), serum concentrations of gonadotrophins, gametogenic potential (total sperm count/testis), and weights of testes and accessory sex organs of 60-d-old rats that were exposed daily to 100 μg of coumestrol during the first 5 d of life.

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Author to whom all correspondence and reprint requests should be addressed: Caleb A. Awoniyi, Division of Reproductive Endocrinology, Department of Obsterics and Gynecology, University of Colorado Health Services Center, Denver, CO, 80262. E-mail: caleb.awoniyi@uchsc.edu

Table 1
Effects of Neonatal Exposure of Male Rats to Coumestrol on Testis Weight, Total Sperm/Testis and Accessory Sex Organ Weights

Group	Testis weight, g	Total sperm/ testis, million	Seminal Epididymis, g	Ventral vesicle, g	Prostate, g
Control (n=8)	1.5 ± 0.05	159 ± 17	0.29 ± 0.01	0.83 ± 0.05	0.34 + 0.03
Coumestrol-treated (n = 8)	1.4 ± 0.09	159 ± 16	0.30 ± 0.01	0.87 ± 0.04	0.33 ± 0.03

Results

There were no significant differences between weights of testes and accessory sex organs (epididymis, seminal vesicle, and ventral prostate) or testicular sperm content of coumestrol-treated rats and those of controls (Table 1).

Neonatal exposure to coumestrol had no effect on spermatogenesis as evidenced by number of homogenization-resistant spermatids per testis and subjective evaluations of testicular histology. There was no obvious treatment effect on organization of seminiferous epithelium or morphology of cells in the tubular cross-sections. Testes from both coumestrol-treated and control rats manifested similar tubular architecture, representing all stages of the cycle of seminiferous epithelium (not shown), without missing generations or a noticeably different number of cells. The appearance of Leydig cells in the coumestrol-treated animals was also similar to that of controls.

Steady-state levels of mRNA of LHβ and FSHβ in the pituitary glands are shown in Fig. 1. At 60 d of age, the level of LHβ-subunit mRNA in coumestrol-treated rats was similar to that of controls. However, there was a significant increase (37%) in the level of FSHβ-subunit mRNA in rats exposed to coumestrol compared to controls.

We compared these results observed at the pretranslational levels with those observed at the secretory level. Serum concentration of neither LH nor FSH was affected by coumestrol treatment; LH: 0.40 ± 0.06 ng/mL in treated rats vs 0.44 ± 0.16 ng/mL in controls; FSH: 8.93 ± 0.95 in treated rats vs 9.88 ± 0.45 ng/mL in controls. Serum concentration of testosterone (T) also was not affected by coumestrol treatment; 2.42 ± 0.7 ng/mL in treated rats vs 2.12 ± 0.3 ng/mL in controls (Table 2).

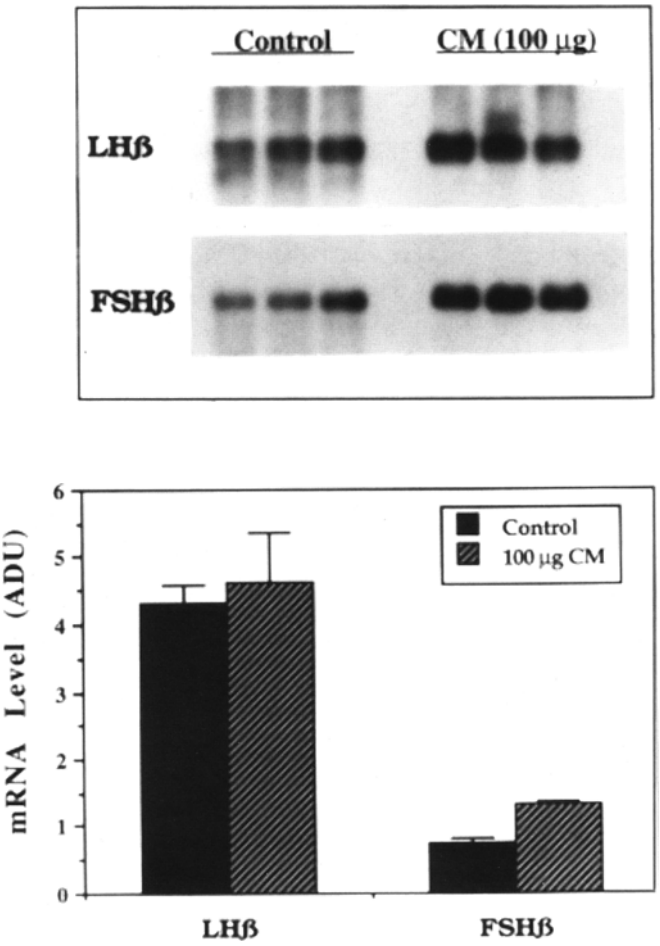

Fig. 1. Top panel: Effects of neonatal exposure (d 1–5 of life) on male rats to DMSO (control) or 100 µg coumestrol (CM) on the steady-state mRNA levels of LHβ and FSHβ at d 60 of life. Total RNA was isolated from individual pituitary gland, and subunit mRNA levels were determined by Northern blot. The results for 3 representative rats/group are shown. Bottom panel: Each bar represents the mean arbitrary densitometric units (ADU) ± SEM of eight autoradiographic bands of Northern blots. The membranes were rehybridized with an 18S rRNA probe to control for loading. The specific probe mRNA signal or optical density was corrected for RNA loading using the 18S signal optical density and expressed as relative optical density. Asterisk (*) represents significant difference at $p < 0.05$.

Discussion

Although it has been postulated that environmental estrogens may account for the putative fall in sperm counts in men over the last 50 yr *(4)*, the health risk of hormone-modulating pollutants, naturally occurring estrogens, and other chemicals in the environment remains controversial *(16,17)*. By using DES, a highly potent synthetic estrogen, several studies indicate that *in utero* exposure to this compound caused adverse effects in male offspring *(18)*. As such, the adverse developmental effects of DES has been used in various arguments to serve as a prototype for concern about the potential reproductive toxic effects of environmental estrogens.

This study was performed to determine if neonatal exposure to coumestrol exerts any deleterious effects on male

Table 2
Effects of Neonatal Exposure of Male Rats to Coumestrol on Serum LH, FSH, and Testosterone Concentrations (ng/mL)

Group	LH	FSH	Testosterone
Control ($n = 8$)	0.44 ± 0.16	9.88 ± 0.45	2.12 ± 0.3
Coumestrol-treated ($n = 8$)	0.40 ± 0.06	8.93 ± 0.95	2.42 ± 0.7

reproductive function of the male as an adult and to identify the site in the pituitary-gonadal axis where such effect might be. We exposed rats neonatally to coumestrol during the critical period of CNS and goandal differentiation, and analyzed the effects of this exposure on pituitary function, and spermatogenesis. Notwithstanding the fact that coumestrol has binding affinity for both α- and β-estrogen receptors in vitro *(19)*, we found that the regimen of neonatal exposure to coumestrol as used in our current study did not have any negative impact on the pituitary gonadotrophin gene expression, gonadotrophin secretion, or spermatogenesis.

These results do not indicate involvement of dietary estrogens, such as coumestrol, in causing deterioration of reproductive potential of the male. In fact, other investigators have identified potential beneficial, rather than adverse, effect of consuming diets that are rich in phytoestrogens *(20–23)*. For example, Japanese men who consume a diet high in soy products have a very low mortality from prostate cancer *(24)*, suggesting that high isoflavonoid levels may inhibit the development or growth of prostate cancer.

At the pituitary level, several studies have reported changes in pituitary responsiveness to GnRH in rats treated with various phytoestrogens *(25–28)*. In the present study, we found only an augmentation in the steady-state mRNA levels of FSHβ in the coumestrol-treated rats with no change in LHβ mRNA levels or serum LH and FSH concentrations. The precise mechanisms of the observed differential regulation of the FSHβ-subunit and LHβ-subunit mRNAs by neonatal exposure to coumestrol are unknown, and may involve differences in transcription rates, mRNA splicing, and/or differential stability of these mRNAs. However, because there is no evidence that coumetrol has an extended half-life in the body, our data suggest that brief neonatal exposure to coumestrol at a vulnerable period of sexual development may have altered the imprinting for the FSHβ gene in these animals. The implications of the increase in FSHβ mRNA expression with regard to long-term reproductive performance in these animals, as well as the possible impact of chronic exposure, are as yet uncertain.

Previous studies have reported supression of spermatogenesis of adult rats treated with 17β-estradiol. These changes have been shown to be associated with impaired LH (and/or FSH) secretion and consequent changes in intratesticular testosterone *(29–32)*. The absence of an effect on LHβ mRNA and serum LH and FSH levels in this study indicates that under the conditions of this study, i.e., 5 d of early neonatal coumestrol exposure, there is no effect on pituitary gonadotropes. This conclusion is supported by our observation that there was no difference among weights of testis and sex accessory organs, serum T, testicular sperm counts, or histological features of testis of coumestrol-treated and control animals.

One of the key factors that determines the number of spermatozoa produced is the number of Sertoli cells contained in each testis. The number of Sertoli cells per testis is determined during fetal and neonatal life, and is under the control of FSH *(33,34)*. In this study, we presume that alteration of the imprinting of FSHβ occurred during the neonatal period when exposure occurred. Augmentation of FSHβ and lack of effect on FSH secretion then may have increased or maintained the number of Sertoli cells per testis during prepubertal period, resulting in no negative impact on spermatogenesis,. This will be an important factor that requires further study.

In summary, the present observations that acute neonatal exposure to coumestrol did not negatively affect sperm production suggest that the hypothesis of a correlation between potential decrease in gametogenic potential of the males and exposure to phytoestrogens should be viewed with caution. Because of the potentially important health benefits associated with the consumption of food containing phytoestrogens *(20,23,24)*, further studies using dietary levels that mimic human exposure are needed to demonstrate conclusively negative effects on sperm output and fertility of the mature animal.

Materials and Methods

Experimental Design

Sprague Dawley rats purchased from SASCO (Omaha, NE) on day 17 of gestation were housed individually in a room with a 14:10 h (light/dark) photoperiod and temperature of 22°C with a relative humidity of 40–50%. Animals were provided free access to rat chow and water ad libitum.

Treatment

Beginning from the day of birth, all pups within a litter were subcutaneously injected once daily for 5 d with either 0.05 ml of DMSO or 100 μg coumestrol (Eastman Kodak Company, Rochester, NY) dissolved in 0.05 mL DMSO. Day of birth was considered as d 1 of age if delivery occurred before 1200 h. The dose of coumestrol was chosen based on earlier studies of Medlock et al. *(9)* in which a 100-μg coumestrol injection into female rats on d 1–5 of life resulted in biochemical and morphological toxicity much like that of DES. Each dose was given to two litters, and all male pups in each litter represent experimental animals. Pups were weaned on d 21. On d 60, animals were euthanized by decapitation, and trunk blood, pituitary glands and reproductive organs were collected. Pituitary

glands were snap frozen in liquid nitrogen and stored at −70°C prior to RNA isolation. Serum was separated and stored at −20°C for determination of concentrations of LH, FSH, and testosterone. Weights of the testes, epididymides, ventral prostate, and seminal vesicles were determined. One testis from each rat was used for the determination of number of homogenization-resistant elongated spermatids using methods described by Robb et al. *(35)*. The contralateral testis of each rat was fixed in Bouin's solution and processed for histology. Tissues were embedded in paraffin, and 5-μm thick sections were cut and stained with hematoxylin and eosin or PAS and hematoxylin.

Hormone Assays

A previously described, RIA procedure was used to determine serum T concentration in duplicate samples *(36)*. The T antiserum GDN S250, generously supplied by G. D. Niswender, was used in this assay; the crossreactivity of this antiserum has been reported *(37)*. All serum samples were measured in the same assay to avoid interassay variance. The intra-assay coefficient of variation was 8.6%.

Serum levels of LH and FSH were determined in duplicate samples (100 μL) by specific homologous RIA as described by Niswender et al. *(38)*, with minor modifications *(39)*. In brief, using reagents supplied by NIDDK, a preparation consisting of a hormone standard or serum samples, EDTA phosphosaline buffer, and the specific antiserum suitably diluted to bind the specific hormone ^{125}-I trace was mixed and incubated overnight. Twenty-four hours after the addition of the antibody and trace, a goat antirabbit γ-globulin was added to precipitate the antigen–antibody complex. Following an additional 20–24 h of further incubation, bound and free hormone were separated by centrifugation. Final values, expressed in terms of the NIDDK reference preparations, represent weighted mean potency estimates given by linear regression analysis of the combined log–logit response curve of the reference preparation and unknown samples. The minimal amount of the hormone detected per assay tube was 0.25 ng Rat FSH-RP-2 and 0.025 ng Rat-LH-RP-2. All samples were measured at one time. The intra-assay coefficient of variation was <10% for both LH and FSH.

RNA Extraction, Northern Blots, and Probes Used for Hybridization

Total RNA was isolated from pituitary gland and testis according the methods of Chomczynski and Sacchi *(40)*. Aliquots of total RNA (5 μg) per pituitary gland or equal amount (0.2%) from the pituitary gland were eletrophoresed through a denaturing 1.2% agarose gel and subsequently blotted by capillary action to a nylon filter *(41)*. Each pituitary blot was sequentially hybridized with LHβ and FSHβ probes (graciously supplied by Joseph L. Roberts, and Richard Maurer, respectively), using conditions previously described *(42,43)*. After hybridization with LHβ, and FSHβ probes, the membranes were stripped and reprobed with a synthetic oligonucleotide probe recognizing the 18S ribosomal RNA (rRNA) sequence. Autoradiographs were standardized to account for differences in loading by the 18S rRNA signal. The relative amount of each of the gene transcripts was determined by densitometric scanning of each autoradiograph using an imaging densitometer (Bio-Rad, Model GS-670, Hercules, CA). The amount of RNA in each lane (5 μg by OD_{260}) was internally standardized within a blot by determining the amount of 18S rRNA signal and then correcting it for reproted LHβ and FSHβ mRNA levels.

Statistical Analysis

All data were analyzed using a one-way analysis of variance (ANOVA) on PROC GLM from SAS. Post hoc tests were conducted when the overall ANOVA was significant at the $p < 0.05$ level. Scheffe's test *(44)* was used to identify differences between groups.

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