



# PERINATAL EXPOSURES TO PHYTOESTROGENS ALTER STEROID HORMONE RECEPTOR GENE EXPRESSION IN ACCESSORY SEX GLANDS OF ADULT RATS

Karen Hancock, Jessica Sherrill and Benson Akingbemi

Department of Anatomy, Physiology and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849



## ABSTRACT

The presence of genistein and daidzen, predominant isoflavones in soybeans, in the amniotic fluid of second trimester fetuses have been reported, and high dietary exposures occur in infants fed soy-based formula. Genistein and daidzen are known to possess estrogenic activity. Accessory sex glands in the male express estrogen receptors (ER $\alpha$ , ER $\beta$ ) and androgen receptors (AR), and are subject to regulation by steroid hormones. To test the hypothesis that exposures to phytoestrogens during pregnancy and nursing affect gene expression in the adult prostate and seminal vesicles, timed-pregnant dams were fed soy-based diets containing isoflavones at 0 (group 1), 5 (group 2) and 1000 mg/kg (group 3), from gestational day 12 to postnatal day 21. At weaning (21 days), blood levels of genistein and daidzen were below detection (HPLC, 9.20 nM) in groups 1 and 2 rats, and measured 86.1  $\pm$  17.2 and 117  $\pm$  17.3, respectively, in group 3 rats. Messenger RNA levels, analyzed by reverse transcription-polymerase chain reaction (RT-PCR) followed by densitometry, showed that ER $\alpha$  expression were increased in the dorsolateral prostate (DSP) and decreased in the ventral prostate (VP) of 90 day-old rats ( $p < 0.05$ ). ER $\beta$  mRNA levels were decreased both in the dorsolateral prostate and seminal vesicles at the 1000 mg/kg dose. Also, the high dose (1000 mg/kg) caused a decrease in AR gene expression in the DSP and an increase in the seminal vesicles ( $p < 0.05$ ). In agreement with mRNA levels, the levels of ER $\alpha$  protein in the VP, analyzed by Western blotting, were decreased in groups 2 and 5 rats ( $P < 0.05$ ). Because development and function of accessory sex glands is regulated by steroid hormones, studies to investigate the effects that soy-based infant diets may have on accessory sex gland function in adulthood are warranted.

## INTRODUCTION

Several biologically active chemicals have been identified in human diets of plant origin, and the high levels of isoflavones in soybeans and soy products has raised the question of whether the estrogenic properties and potential reproductive toxicity of isoflavones present in soybeans warrant attention. Hormonally active chemicals of plant origin are designated phytoestrogens because they regulate estrogen biosynthesis and estrogen receptor (ER)-mediated activity to modulate physiological events in endocrine tissues. A growing body of evidence indicates that exposures to environmental levels of estrogenic agents during the period of reproductive tract tissue differentiation cause adverse biological effects (1, 2). In this regard, approximately 750,000 infants in the United States are fed soy-based formula per year, and it is estimated that isoflavonoid concentrations are 4-6 times higher in infants fed soy-based formula than in adults eating a diet rich in soy foods (3). The endocrine disruptor (ED) hypothesis also asserts that ED-induced effects occurring in the perinatal period may persist and/or become apparent in adulthood (4). Androgens are the predominant sex hormones in the male, and are produced mostly by Leydig cells in the testis. Pituitary luteinizing hormone (LH) is the primary regulator of Leydig cells, and is secreted in response to hypothalamic gonadotropin releasing hormone (GnRH). In the present experiments, we have examined the effects of varying soybean levels in the maternal diet during the perinatal period on steroid hormone gene expression in the accessory sex glands of adult male rats. The results show that phytoestrogens have the ability to cross tissue barriers, regulate reproductive tract development in offspring and affect gene expression in the sex glands.

## MATERIALS & METHODS

### Experimental Design

Timed pregnant Long-Evans dams, obtained from Harlan-Teklad (Indianapolis, IN) were fed diets containing varying isoflavone levels (0, 5, 1000 mg/kg) from gestational day 12 to postnatal day 21 (PND 21). At weaning (PND 21), male rats were fed soy-free diets until 90 days of age. The levels of serum isoflavones in the serum (dams and male rats) and in the liver and testis (male rats) were analyzed by HPLC. Tissues were obtained from accessory sex organs at 90 days of age. Data were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison test, and significance was set at  $P < 0.05$  (Prizm 4.0 software; Graphpad, San Diego, CA).

### Measurement of Isoflavone Concentrations by HPLC

Unconjugated genistein and daidzen and the total concentration of their glucuronide metabolites were determined in serum and tissue samples using a validated analytical method based upon reverse-phase high performance liquid chromatography with ion trap mass spectrometric detection, as previously described, with minor modifications (5). All glassware used in the assay was deactivated by treatment with a 1% (v/v) solution of SurfaSil $\text{®}$  silicizing fluid (Pierce Chemicals, Rockford, IL) in HPLC grade hexanes (Fisher Scientific) and drying in an oven at 120 $^{\circ}$ C. The lower limit of detection in this assay was 9.2 nM and the mean concentration of each analyte was calculated as the geometric mean of the individual subjects.

### Gene Expression Analysis by RT-PCR and Western Blotting

Total RNA was isolated from the dorsolateral and ventral prostate and the seminal vesicles by the usual guanidine thiocyanate method and mRNA levels were semi-quantified by RT-PCR, using primers based on published sequences for genes of interest and ribosomal S16 as internal control.

These primers were: 5'-GCTCCAATTCTGACAATCGAC-3' and 5'-TTTCGTATCCCGCCTTTCATC-3' (ER $\alpha$ , 308 bp), 5'-AACCTCAAAGAGTCCTTGGTGTG-3' and 5'-AACACTTGCGAAGTCGGCAG-3' (ER $\beta$ , 327 bp), 5'-CCCATCGACTATTCTCCACC-3' and TTCTCCTTCTCCTGTAGTTGA-3' (AR, 270 bp), 5'-AAGTCTCGGACGCAAGAAA-3' and 5'-TTGCCAGAAGCAGAACAG-3' (S16, 150 bp).

Differences in mRNA levels were determined by gel analysis followed by densitometry (BioDoc-It Imaging System, Upland, CA). Whole cell lysate was obtained after disruption of cells using an extraction buffer (Pierce, Rockford IL). SDS-PAGE was performed according to standard procedures and an antibody specific for the ER $\alpha$  (Sc-7207, Santa Cruz Biotechnology).

Table 1. Serum levels of free (unconjugated) genistein and daidzen in 21 day-old male rats and their dams (in parentheses)\*

Isoflavone content of maternal diet (mg/kg)	Genistein (nM)	Daidzen (nM)
0	ND	ND
5	ND	ND
50	18.2 $\pm$ 5.9	26.3 $\pm$ 12.5
500	48.9 $\pm$ 6.6 (17.3 + 26.7)	68.9 $\pm$ 7.1 (55.8 + 29.8)
1000	86.1 $\pm$ 17.2 (22.8 + 18.8)	117 $\pm$ 17.3 (60.6 + 33.8)

ND = Below limit of assay detection (9.2 nM).

\*Pregnant dams were fed diets from gestational day 12 to postnatal day 21.

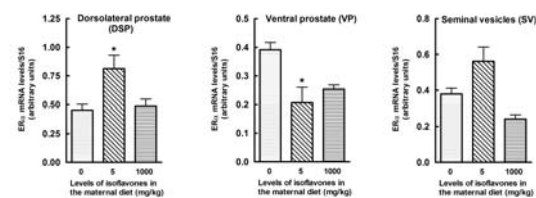


Fig. 1. Messenger RNA (mRNA) levels for the estrogen receptor-alpha (ER $\alpha$ ) in sex accessory organs of adult male rats after exposure to phytoestrogens in the maternal diet from gestational day 12 to postnatal day 21. \* $p < 0.05$  compared to control.

• Exposures to the low phytoestrogen dose (5 mg/kg) increased ER $\alpha$  mRNA levels in the dorsolateral prostate but caused a decrease in the ventral prostate; ER $\alpha$  mRNA levels in seminal vesicles were unchanged.

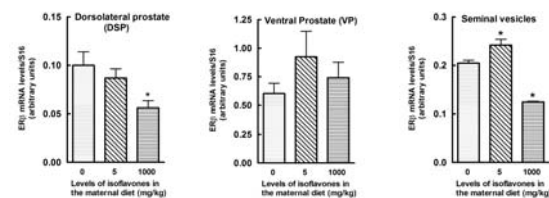


Fig. 2. Messenger RNA (mRNA) levels for the estrogen receptor-beta (ER $\beta$ ) in sex accessory organs of adult male rats after exposure to phytoestrogens in the maternal diet from gestational day 12 to postnatal day 21. \* $p < 0.05$  compared to control.

• Exposures to the high phytoestrogen dose (1000 mg/kg) decreased ER $\beta$  mRNA levels in the dorsolateral prostate and seminal vesicles, and the 5 mg/kg dose increased ER $\beta$  mRNA levels in the seminal vesicles; ER $\beta$  mRNA levels in the ventral prostate were unchanged.

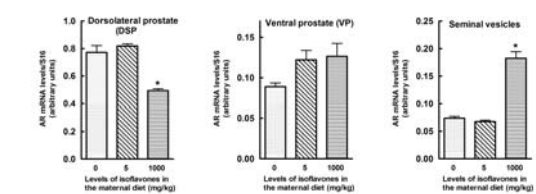


Fig. 3. Messenger RNA (mRNA) levels for the androgen receptor (AR) in sex accessory organs of adult male rats after exposure to phytoestrogens in the maternal diet from gestational day 12 to postnatal day 21. \* $p < 0.05$  compared to control.

• Exposures to the high phytoestrogen dose (1000 mg/kg) decreased AR mRNA levels in the dorsolateral prostate but caused an increase in seminal vesicles; AR mRNA levels in the ventral prostate were unchanged.

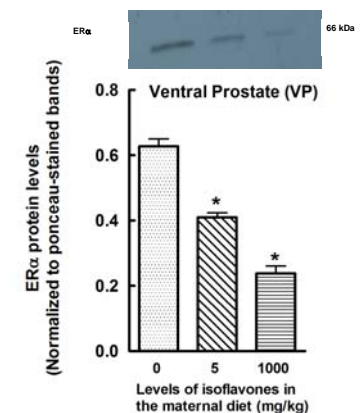


Fig. 4. Protein levels for the estrogen receptor-alpha (ER $\alpha$ ) in the ventral prostate of adult male rats after exposure to phytoestrogens in the maternal diet from gestational day 12 to postnatal day 21. \* $p < 0.05$  compared to control.

Exposures to low (5 mg/kg) and high phytoestrogen doses (1000 mg/kg) both decreased ER $\alpha$  protein levels in the ventral prostate.

## CONCLUSIONS

- Perinatal exposures of male rats to phytoestrogens affect steroid hormone receptor gene expression in accessory sex glands in adulthood.
- Down or up-regulation of steroid hormone receptor gene expression potentially affects development and function of accessory sex glands, which have implications for male fertility
- Further studies are warranted to investigate dose-dependent phytoestrogen-mediated effects on prostate hyperplasia, carcinogenesis and chemoprevention.

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