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Ovulation Rates and Embryo Degeneracy in Female Mice Fed the Phytoestrogen, Coumestrol[1] (41156)

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Abstract. Two trials were conducted to determine the effects of dietary coumestrol upon ovulation rates and embryo degeneracy in mice. When female mice, strain B6 D2 F1, were fed 150, 300, or 900 ppb (parts per billion) coumestrol, ovulation rates were reduced 50, 59, and 66%, respectively. In contrast, the incidence of degenerate embryos increased from 4% for controls to 26, 49, and 81%, respectively. When female mice, strain ICR, were fed 50, 100, 200, or 400 ppb coumestrol, ovulation rates were reduced 30, 36, 42, and 54%, respectively, compared to controls. The incidence of degenerate embryos increased from 3 to 46% with 400 ppb dietary coumestrol. Fertilization rate was not affected by dietary coumestrol in the first trial, but was reduced in the second trial. Uterine weights were increased with all levels of added coumestrol in both trials. Maternal weights were not affected by 900 ppb coumestrol in the first trial, but were significantly reduced by 400 ppb coumestrol in the second trial. Differences may exist among strains of mice in body weight response to dietary coumestrol. Dietary coumestrol markedly reduced ovulation rates and increased embryo degeneracy in female mice treated with PMSG and HCG and fed 50 ppb coumestrol or greater.

Many plants contain substances with estrogenic activity. These substances, termed phytoestrogens, are found in alfalfa, coffee, licorice, sunflowers, wheat, barley, corn, rye, oats, apples, cherries, plums, potato tubers, peas, beans, brussel sprouts, and many other foods (1, 2). Phytoestrogens are categorized into two classes: the isoflavones which include formononetin, diadzein, genistein, and Biochanin A; and the coumestans, which include coumestrol. Coumestrol is 30 to 100 times more active than the other major phytoestrogens using mice uterine weight as a bioassay for estrogenic activity (3).

Impairment of animal reproduction by dietary phytoestrogens has been demonstrated in several animal species. Delayed estrus and reduced fertilization rate occurred in ewes grazing legume pastures at time of breeding (4, 5). Coop and Clark (6) reported a 15% decrease in conception rate and a 10% reduction in twinning rate in ewes fed lucerne prior to mating. Smith *et al.* (7) showed a 34% reduction in ovulation rate of ewes fed 100 ppm coumestan. Female mice fed ladino meal had a conception rate of less than 10% compared to 57% in control mice (8). Uterine growth and vaginal changes also occurred in mice fed phytoestrogens (9).

Few reports are available on the specific cause of infertility in animals fed coumestrol. Likewise, the minimum level of dietary coumestrol affecting reproduction is not known. The objective of this experiment was to establish a dose–response curve of coumestrol on ovulation rate and embryo degeneracy in mice.

Materials and Methods. *Coumestrol extraction.* Coumestrol was isolated from alfalfa according to Knuckles, *et al.* (10) and quantitated by thin-layer chromatography. A coumestrol standard[2] was used to prepare working standards of 5, 10, 20, 50, and 100 ng coumestrol/μl. Five microliters of each standard and sample extract was applied to a G-1500 silica gel plate. The plate was placed in a chromatography tank and developed in a solution of chloroform and methyl alcohol, 8/1 (v/v), at room temperature. Immediately after drying the plate

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TABLE I. COMPOSITION OF BASAL DIET

Basal diet composition	Percentage of diet
Casein, 35% protein	20.0
DL-Methionine	0.3
Cornstarch	15.0
Sucrose	50.0
Choline bitartrate	0.2
Cellulose	5.0
Fat (Corn oil)	5.0
Mineral mix[a]	3.5
Vitamin mix[b]	1.0

[a] AIN-76 mineral mix, percent of mix: $CaHPO_4$, 50.0; NaCl, 7.4; potassium citrate–H_2O, 22.0; K_2SO_4, 5.2; MgO, 2.4; manganous CO_3, 0.35; ferric citrate, 0.60; $ZnCO_3$, 0.16; $CuCO_3$, 0.03; KIO_3, 0.001; Na_2SeO_3–$5H_2O$, 0.001; $CrK(SO_4)_2$–$12H_2O$, 0.055; sucrose, powdered, 11.8.

[b] AIN-76 vitamin mix, per killigram of vitamin mix (g); Thiamine–HCl, 0.6, riboflavin, 0.6; pyridoxine–HCl, 0.7; niacin, 3.0; Ca-pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; vitamin B_{12}, 1 mg; vitamin A, 400,000 IU; vitamin E, 5000 IU; vitamin D_3, 2.5 mg; vitamin K, 5.0 mg; sucrose, powdered, to make 1000 g.

was placed in a chromatographic viewing chamber under short ultraviolet light (200–300 nm). Intensities of the sample spots were compared to standards to determine the amount of coumestrol on the plate.

Preparation of the diet. The diet used in these trials (Table I) met all the known nutrient requirements for mice (11). Additions of coumestrol to the diet were standardized by reducing the extract volume containing a known amount of coumestrol to approximately 3 ml then increasing it to 20 ml with 95% ethanol. The extract was added to the basal diet and mixed for 10 min.

Experimental procedure. Trial 1. One hundred and sixty female mice, 6 weeks old, strain B6 D2 F1,[3] were randomly assigned among four dietary treatments containing 0, 150, 300, and 900 parts per billion coumestrol on Day 1 of the trial and fed *ad libitum* for a 14-day period. Female mice were superovulated by interperitoneal injection of 10 IU of pregnant mare serum gonadotropin (PMSG) at 10:00 AM on Day 9 and 10 IU of human chorionic gonadotropin (HCG) at 2:00 PM on Day 11. After the HCG injection, females were placed with males for 24 hr. On Day 14, female mice were weighed and sacrificed. The oviducts and uteri were removed, and embryos recovered from the oviduct by flushing with Whitten's medium (12). Ova were collected to determine ovulation rates and the embryos were examined by phase-contrast (200×) microscopy for fertilization, stage of development, and gross abnormalities. Uterine and maternal weights also were recorded.

Trial 2. This trial was to evaluate reduced levels of coumestrol on reproductive parameters. Two hundred female mice, 6 weeks old, strain ICR,[4] were randomly assigned to dietary treatments of 0, 50, 100, 200, and 400 ppb coumestrol and fed *ab libitum*. The experimental procedure was as described above.

Evaluation of data. Data were compiled by the following parameters:

Number ovulated	= number of ova ovulated per mouse
Number fertilized	= number of ova fertilized per ovulating mouse
Percentage fertilized	= (number of fertilized ova/ number of ova ovulated) × 100 (per mouse)
Number degenerate	= average number of degenerate ova in those mice having fertilized embryos
Percentage degenerate	= (number of degenerate embryos/number of fertilized ova) × 100 (per mouse)

Only fertilized ova were evaluated for degeneracy. Embryos were classified as degenerate if any of the following characteristics were observed: unequal cell size, ruptured cells, uneven cytoplasm, fragmentation, or differences in estimated state of embryonic development from ovulation.

Statistical analysis. Data were analyzed by analysis of variance and treatment means compared using Duncan's new multiple range test (13).

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TABLE II. THE EFFECT OF LEVEL OF DIETARY COUMESTROL ON THE REPRODUCTIVE PERFORMANCE IN MICE, STRAIN B6 D2 F1[a]

Item	Level of coumestrol in diet (ppb)			
	0	150	300	900
Ovulations per mouse	$21.2^{b} \pm 1.2$	$10.6^{c} \pm 0.8$	$8.7^{d} \pm 0.6$	$7.3^{e} \pm 0.5$
Number of fertilized ova	$13.2^{b} \pm 3.0$	$6.4^{c} \pm 2.0$	$5.7^{c} \pm 1.4$	$3.9^{c} \pm 0.9$
% Fertilization	$62.3^{b} \pm 12$	$60.4^{b} \pm 11$	65.5 ± 12	$53.4^{b} \pm 11$
% Abnormal embryos	$4.4^{b} \pm 3$	$26.2^{c} \pm 6$	$48.8^{d} \pm 9$	$81.3^{d} \pm 7$

[a] Mean values ± SEM.
[b–e] Means in rows not followed by a common superscript are different ($P < 0.05$).

TABLE III. THE EFFECT OF LEVEL OF DIETARY COUMESTROL ON UTERINE AND MATERNAL WEIGHTS IN MICE, STRAIN B6 D2 F1[a]

Item	Level of coumestrol in diet (ppb)			
	0	150	300	900
Uterine weight (g)	$0.033^{b} \pm 0.002$	$0.038^{c} \pm 0.003$	$0.042^{c} \pm 0.002$	$0.040^{c} \pm 0.003$
Maternal weight (g)	$19.1^{b} \pm 0.5$	$19.9^{b} \pm 0.6$	$19.3^{b} \pm 0.4$	$19.3^{b} \pm 0.4$
$\frac{\text{Uterine weight}}{\text{Maternal weight}} \times 100$	$0.17^{b} \pm 0.01$	$0.19^{c} \pm 0.01$	$0.22^{d} \pm 0.01$	$0.21^{c,d} \pm 0.01$

[a] Mean values ± SEM.
[b–d] Means in rows not followed by a common superscript are different ($P < 0.05$)

TABLE IV. THE EFFECT OF LEVEL OF DIETARY COUMESTROL ON FERTILITY OF MICE, STRAIN ICR[a]

Item	Level of coumestrol in diet (ppb)				
	0	50	100	200	400
Ovulations per mouse	$13.0^{b} \pm 0.4$	$10.0^{c} \pm 0.3$	$8.3^{d} \pm 0.2$	$7.6^{e} \pm 0.2$	$6.0^{f} \pm 0.2$
Number of fertilized ova	$12.5^{b} \pm 0.4$	$9.0^{c} \pm 0.3$	$7.2^{d} \pm 0.2$	$6.0^{e} \pm 0.3$	$4.1^{f} \pm 0.3$
% Fertilization	$96.2^{b} \pm 1.0$	$90.0^{c} \pm 1.4$	$86.7^{c,d} \pm 1.4$	$78.9^{e} \pm 1.8$	$68.3^{f} \pm 3.3$
% Abnormal	$3.2^{b} \pm 0.8$	$10.0^{c} \pm 2.0$	$19.4^{d} \pm 2.7$	$25.0^{d} \pm 4.0$	$46.3^{e} \pm 10.0$

[a] Mean values ± SEM.
[b–f] Means in rows not followed by a common superscript are different ($P < 0.05$).

Results. *Trial 1.* Strain B6 D2 F1 female mice are highly fertile and ovulate a large number of ova. Ovulation rates were reduced significantly in mice fed 150, 300, or 900 ppb dietary coumestrol (Table II). In addition, the incidence of degenerate embryos increased with elevated levels of coumestrol in the diet. Degeneracy, expressed as a percentage of total embryos increased across all treatments from 4 to 81% as dietary coumestrol was increased from 0 to 900 ppb.

Degenerate embryos of mice fed coumestrol commonly possessed unevenly distributed cytoplasm and lack of symmetry among blastomeres. The presence of large and small blastomeres within the same ovum suggests different cleavage rates. A great deal of vacuolization was present in most ova from mice fed coumestrol at all levels.

Dietary coumestrol had no effect on the fertilization rate of mice ova (Table II). However, uterine weights were increased at all levels of coumestrol fed, but the increase in uterine weight was not dose dependent (Table III). Coumestrol had no significant effect on maternal weights of mice.

Alopecia about dorsal and facial areas was apparent in female mice fed diets containing 900 ppb coumestrol.

Trial 2. Strain ICR female mice used in this trial normally have smaller ovulation rates than the B6 D2 F1 females used in the preceding trial. Ovulation rates were decreased progressively by elevated levels of dietary coumestrol (Table IV). The incidence of degenerate embryos was increased significantly by all levels of dietary coumestrol; 0.4, 0.9, 1.4, 1.5, and 1.9 degenerate embryos per mouse for 0, 50, 100, 200, and 400 ppb dietary coumestrol, respectively. The percentage of degenerate embryos increased markedly with each increment of dietary coumestrol (Table IV). The fertilization rate also was decreased in mice fed all levels coumestrol.

The effects of dietary coumestrol on uterine and maternal weights are given in Table V. Uterine weights were increased significantly only when the level of coumestrol in the diet was 100 ppb or greater while maternal weights were significantly affected only at 400 ppb coumestrol. Likewise, uterine weights expressed as a percentage of maternal weights were significantly affected only by 400 ppb dietary coumestrol.

Discussion. Two strains of mice of different fecundity were used in these trials. Yet, the percentage reduction in ovulation rates caused by dietary coumestrol were very similar between these strains. Preliminary studies within strains also yielded consistent results. In these trials, dietary coumestrol levels were more accurately reflected by ovulation rates than by uterine weights. The reduction of ovulation rates of female mice treated with PMSG and HCG may be a useful bioassay for dietary phytoestrogens.

Coumestrol may effect fertility by inhibiting release of follicle stimulating hormone (FSH). Activity of FSH is inhibited in rats by estrogen injection (14). FSH activity also is postulated to be reduced by dietary coumestrol (7). Inadequate levels of FSH in the final stages of follicle development may decrease ovulation rate and delay ovulation, thereby increasing the incidence of degenerate embryos (15, 16).

TABLE V. THE EFFECT OF LEVEL OF DIETARY COUMESTROL ON UTERINE WEIGHT IN MICE, STRAIN ICR[a]

Item	Level of coumestrol in diet (ppb)				
	0	50	100	200	400
Uterine weight (g)	$0.063^{b,c} \pm 0.003$	$0.056^{b} \pm 0.003$	$0.069^{c} \pm 0.004$	$0.070^{c} \pm 0.004$	$0.083^{d} \pm 0.004$
Maternal weight (g)	$26.6^{b} \pm 0.3$	$26.0^{b,c} \pm 0.3$	$25.7^{b,c} \pm 0.3$	$25.7^{b,c} \pm 0.3$	$25.0^{c} \pm 0.3$
$\frac{\text{Uterine weight}}{\text{Maternal weight}} \times 100$	$0.24^{b,c} \pm 0.01$	$0.21^{b} \pm 0.01$	$0.27^{c} \pm 0.02$	$0.27^{c} \pm 0.02$	$0.33^{d} \pm 0.02$

[a] Mean values ± SEM.
[b–d] Means in rows not followed by a common superscript are different ($P < 0.05$).

The suggestion that coumestrol reduces growth which, in turn, disrupts ovarian function (17) was not substantiated by these trials. Maternal growth was not affected by dietary coumestrol while ovulation rates were reduced significantly at all levels of dietary coumestrol tested.

Certain varieties of peas and beans contain 400 ppb coumestrol, fresh soybean sprouts contain 71,000 ppb (2), and infected alfalfa leaves contain 600,000 ppb coumestrol (7). In this trial, reproduction in mice was significantly affected by 50 ppb dietary coumestrol. Animal species differ in their sensitivity to phytoestrogens with sheep even more sensitive than mice (18). Thus, there is need to establish the minimum level of dietary coumestrol which affects reproduction in other species of animals, including humans.

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