Estradiol-Type Activity of Coumestrol in Mature and Immature Ovariectomized Rat Uterotrophic Assays

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Makaverich et al. [*Environ Health Perspect* 103:574–581 (1995)] reported that the uterotrophic activity of the phytoestrogen coumestrol in the immature ovariectomized rat was atypical in that it was not associated with increased uterine hyperplasia and DNA content. We previously reported that coumestrol gave a typical estradiol-type uterotrophic response in the immature intact rat, yielding increases in uterine epithelial cell height, glandular formation, cell labeling, and DNA content. These papers did not answer the question of whether there is a basic difference between the ovariectomized and the intact rat uterotrophic assays. In this paper, we report that coumestrol gives a typical estradiol-type uterotrophic response in uterotrophic assays using immature intact, immature ovariectomized, and mature ovariectomized rats. We concluded that the uterotrophic activity of coumestrol is typical of the natural estrogen estradiol. *Key words*: endocrine disruption, immature rats, ovariectomized rats, phytoestrogens, sexual development. *Environ Health Perspect* 108:631–634 (2000). [Online 31 May 2000]

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Makaverich et al. (*1*) reported that the phytoestrogen coumestrol gives an atypical positive response in the ovariectomized immature rat uterotrophic assay. Although an increase in uterine weight was observed, they reported no accompanying increase in uterine cell division or DNA content. This led the authors to suggest that the positive uterotrophic response for coumestrol was mechanistically distinct from that of estradiol, which is known to be accompanied by concomitant increases in uterine cell division and DNA content. Subsequently, we reported (*2*) that the uterotrophic activity of coumestrol in the immature intact rat was indistinguishable from that of estradiol benzoate (E_2B); both induced uterotrophic responses that were accompanied by increases in uterine epithelial cell height, gland formation, cell division, and DNA content. We did not repeat the original immature ovariectomized rat uterotrophic assay reported by Makaveric (*1*) due to the scarcity and expense of coumestrol; thus, it was not clear if this difference in assay outcome between Makaverich et al. (*1*) and our study (*2*) was absolute, or a reflection of an intrinsic difference between the uterotrophic assay in the immature rat versus the ovariectomized rat. In this paper, we describe the results of uterotrophic assays of E_2B and coumestrol using immature intact, immature ovariectomized, and mature ovariectomized rats. We used the same dose levels (milligrams per kilogram) of the two test agents for each of the test protocols in order to compare the relative sensitivities of the protocols. We used the same dose level of coumestrol as in our earlier study, the same dose estimated by Makaverich et al. (*1*) to have been used in their original studies (60 mg/kg/day).

Materials and Methods

Chemicals. Coumestrol, estradiol benzoate (E_2B), and arachis oil were as previously described (*2*). We obtained 5-bromodeoxyuridine (BrdU), 3,3′-diaminobenzidine, and orcinol from Sigma Chemical Company (Poole, Dorset, UK). All antibodies were supplied by DAKO Ltd (High Wycombe, UK).

Animals. Three groups of female Alpk:AP rats were obtained from the breeding unit at AstraZeneca (Alderley Park, Macclesfield, Cheshire, UK). As in our previous uterotrophic assays (*3*), the first group of female rats (intact weanlings) was 21–22 days of age and had body weights of 38–48 g. The second group of female rats [ovariectomized (OVX) weanlings] was ovariectomized at 21–22 days of age and dosed between 29 and 30 days of age, as described by Makaverich et al. (*1*). The final group of animals (OVX matures) consisted of female rats that were ovariectomized at 6–8 weeks of age and dosed at 7–9 weeks of age, as previously described (*3*). All animals were housed and maintained as previously described (*2*). Diet and water were available *ad libitum*. Intact weanlings were acclimatized for 24 hr before being dosed, and all OVX females were acclimatized for 4 days before dosing. All animals were dosed over the same 3-day period. The dose levels of coumestrol used by Makaverich et al. (*1*) varied between experiments and were not always clearly stated. However, the authors noted that uterine weight was doubled when rats were exposed to 100 µg coumestrol/mL drinking water; they estimated that this exposure yielded 60 mg coumestrol/kg body weight/day (*1*). Therefore, we used the dose of 60 mg/kg/day coumestrol for the present experiments.

Uterotrophic assays. The uterotrophic activities of coumestrol (60 mg/kg) and E_2B (80 µg/kg) were investigated as described previously (*2*). The test agents were dissolved (E_2B) or homogeneously suspended (coumestrol) in arachis oil and administered by a single oral gavage on each of three successive days using a dosing volume of 5 mL/kg body weight. Twenty-four hours after the final dose, animals were killed by an overdose of Fluothane (AstraZeneca Pharmaceuticals) followed by cervical dislocation. At the time of death, we recorded vaginal opening (weanling intact and immature OVX females) or took vaginal smears (mature OVX females). Test groups, daily dose levels, and animal group sizes are shown in Table 1. We administered BrdU in the drinking water (0.8 mg/mL) for the duration of the experiment beginning 24 hr before the gavage treatments, as described previously (*2*).

The reproductive tract was excised and handled as previously described (*2*) to yield the individual weights of the blotted uterus, the vagina, and the cervix. The uterus was then cut so the left horn of the uterus, together with the junction to the cervix, could be processed for histopathology (*2*). The right horn was flash frozen in liquid nitrogen and stored at -70°C for possible future use.

Determination of uterine hyperplasia. The left uterine horn, together with its junction to the cervix, from each animal was fixed in 10% formal saline and processed to paraffin wax. Longitudinal sections were stained to reveal BrdU as described previously (*2,4*). We determined the labeling index for epithelial cells of the uterine lumen as well as for the endometrial glands and stroma (500 cells/animal were assessed where possible) (*2*).

Determination of uterine hypertrophy. Uterine sections were stained with hematoxylin and eosin. We calculated the mean endometrial and epithelial cell height on the basis of 10 locations measured per animal (*2*).

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Table 1. Activity of coumestrol (60 mg/kg), E_2B (80 µg/kg), and arachis oil (5 mL/kg) in the rodent uterotrophic assay using immature intact, immature OVX, or mature OVX rats.

	Tissue weight (mg)							
	Intact uterus (mg)		Blotted uterus		Vagina		Cervix weight	
Group	Individual	Mean ± SD	Individual	Mean ± SD	Individual	Mean ± SD	Individual	Mean ± SD
Intact weanling								
Arachis oil	34.4		27.5		28.8		8.7	
	24.1		18.8		37.5		3	
	27.3		23.4		25.4		5.8	
	23.4		17.9		21.4		6.8	
	22		19.2		44.8		6.7	
	38.5	28.3 ± 6.7	30.5	22.9 ± 5.2	27.6	30.9 ± 8.6	5.2	6.0 ± 1.9
Coumestrol	109.6		90.5		79		25.8	
	151.6		125.8		67.7		21.6	
	79.1	**	70.6	**	49.5	**	21.8	**
	79.3		70.8		41.5		20.6	
	112.5		94.3		61.3		26	
	87.8	103.3 ± 27.8	75.2	87.9 ± 21.2	52.6	58.6 ± 13.5	20.7	22.8 ± 2.5
E_2B	161.1		101.9		80.6		21.1	
	113.8		93.8		61.3		27.9	
	88.1	**	74.3	**	30.1	**	23.9	**
	68.1		71.6		58.7		38.7	
	72		57		59.8		18.1	
	82.2	97.6 ± 35.1	74.5	78.9 ± 16.3	40.5	55.2 ± 17.7	17.2	24.5 ± 8.0
Weanling OVX								
Arachis oil	20.7		15.3		46.7		8.9	
	17.1		12.5		34.3		6.1	
	15.5		10.8		46.7		6.5	
	22.9		9.1		55.9		2.9	
	20.9		13.2		36.2		4.4	
	17.9		12.3		29.7		5.1	
	18.9	19.1 ± 2.5	11.8	12.1 ± 1.9	45	42.1 ± 9.0	8.3	6.0 ± 2.1
Coumestrol	83.7		71.2		48.3		19.1	
	80.7		67.8		85		23.4	
	73.1	**	63.5	**	59.7	**	30.6	**
	119.9		96.9		68		28.6	
	61.7		53.6		55.8		10.1	
	75.2		65.6		83		15.1	
	75.5		65.4		87.4		27.1	
	84.3	81.8 ± 17.0	71.9	69.5 ± 12.4	77.1	70.5 ± 14.8	26.8	22.6 ± 7.2
E_2B	83.6		71.6		69.1		17	
	50.7		40.7		56.5		9.9	
	43.7	**	33.6	**	53.3	**	8.9	**
	67.9	61.5 ± 17.9	57.1	50.8 ± 17.0	89.3	67.1 ± 16.3	28.3	16.0 ± 8.9
Mature OVX								
Arachis oil	106.6		91.6		81.8		24.9	
	111.6		95.6		103.9		33.5	
	88.6		77		86.3		37.6	
	120.9		100.8		156.9		30.5	
	86		66.2		106.1		21.6	
	101.1		83.4		130.6		24.9	
	101.6		89.1		114.4		41.5	
	73.2	98.7 ± 15.4	62.7	83.3 ± 13.7	92.6	109.1 ± 24.9	20.2	29.3 ± 7.7
Coumestrol	181.4		158		176.9		48.8	
	144.7		126.3		137.3		36.6	
	146	**	127	**	170.6	**	33.3	**
	163.3		145.8		207.2		45.5	
	207.4		178.8		128.5		57.1	
	148.4		114.2		148.3		45.5	
	121.2		106.2		122.7		46.6	
	168.1	160.1 ± 26.3	142.9	137.4 ± 23.9	197	161.1 ± 31.7	68.6	47.8 ± 11.1
E_2B	101.6		88.5		135		23.7	
	97.7		76.8		130.7		26.5	
	112.5		98.3		146.4		29.5	
	118.5		95		90.2		28.8	
	93.4		72.1		143.8		25.4	
	90.9		77.6		131.1		20	
	98.8		83.1		111.6		24.2	
	84.1	99.7 ± 11.3	70.4	82.7 ± 10.4	114.2	125.4 ± 18.9	18.7	24.6 ± 3.8

Data were assessed for statistical significance by ANOVA.
**$p < 0.01$ as compared to controls.

Statistical assessment of data. We assessed statistical difference of all uterotrophic (Table 1) and hypertrophy data (Table 2) by analysis of variance (ANOVA) (*5*). We analyzed the hyperplasia data (Table 3) using ANOVA after a double arcsine transformation (*6*). Comparisons were carried out separately for all observations between the control group and all test groups.

Results

Uterotrophic assays. The results of the uterotrophic assays performed using the three test protocols are shown in Table 1. The data generated for coumestrol and E_2B in the intact weanlings confirmed previously published observations (*2*). Both compounds induced comparable and significant increases in blotted uterine weight, vaginal weight, and cervical weight (Figure 1).

The OVX weanling females also had significantly increased uterine, vaginal, and cervical weights after exposure to both compounds. Coumestrol and E_2B induced similar responses and the magnitude of these responses was comparable to those observed in the intact weanling females (Figure 1).

Coumestrol induced significant increases in tissue weights in the OVX mature females. However, the magnitude of these responses was lower than those observed in the two weanling groups. Specifically, the average fold increase in uterine and cervical weights for the weanling intact and weanling OVX females was ~3- to 4-fold, whereas those for the mature OVX females was only ~1.6-fold. Only the fold increase in vaginal weight was similar across the three groups of females (~1.8-fold; Figure 1). E_2B did not significantly increase the uterine or cervical weights in the mature OVX uterotrophic assay, but we observed a small increase in the vaginal weight (Table 1, Figure 1). This is consistent with earlier observations (*3*) and was due to the use of a lower E_2B dose than is optimum for this chemical in oral uterotrophic assays (enforced by our decision to use standardized dose levels across the three test protocols).

Measurement of hypertrophy. Treatment with coumestrol led to a highly significant increase in the height of the uterine epithelium in all three groups of females (Table 2; Figure 2A). The effect was most prominent in 21–22-day-old intact weanlings and least prominent in mature OVX animals, as observed with the tissue weight data. A highly significant increase in the height of the endometrium was observed in immature OVX and intact immature animals (OVX > intact) (Figure 2B). However, there was no effect in the mature OVX females, which is consistent with the lack of tissue weight gain.

Table 2. Effect of coumestrol (60 mg/kg), E_2B (80 µg/kg), and arachis oil (5 mL/kg) on the height (mean ± SD) of the uterine epithelium and endometrium.

Group	Compound	No. of females	Epithelium height (µm)	Endometrium height (µm)
Immature intact	Arachis oil	6	11.0 ± 1.4	193.6 ± 39.6
	Coumestrol	6	41.0 ± 7.6**	305.8 ± 40.5**
	E_2B	6	34.7 ± 6.2**	235.1 ± 39.8
Immature OVX	Arachis oil	7	9.5 ± 1.2 (6)	123.2 ± 24.1 (6)
	Coumestrol	8	26.5 ± 5.0**	299.5 ± 49.8**
	E_2B	4	14.7 ± 2.8** (3)	245.4 ± 43.9**
Mature OVX	Arachis oil	8	9.6 ± 1.2	275.1 ± 97.9
	Coumestrol	8	20.1 ± 5.0** (7)	365.7 ± 85.1* (7)
	E_2B	8	13.6 ± 3.6*	282.7 ± 51.0

Numbers in parentheses indicate the number of observations upon which the group mean is based. Data were assessed for statistical significance by ANOVA.
*$p < 0.05$; **$p < 0.01$.

Table 3. Effect of coumestrol (60 mg/kg), E_2B (80 µg/kg), and arachis oil (5 mL/kg) on the labeling index of the uterine epithelium and endothelium.

			Labeling Index (%) ± SD		
				Endometrium	
Group	Compound	No. of females	Epithelium	Glands	Stroma
Intact weanling	Arachis oil	6	0.8 ± 0.6	3.9 ± 3.9	3.3 ± 2.8
	Coumestrol	6	36.1 ± 7.7**	61.4 ± 18.5**	31.2 ± 28.9*
	E_2B	6	9.7 ± 12.1	52.8 ± 21.9**	28.0 ± 31.3
Weanling OVX	Arachis oil	7	0.9 ± 0.5 (6)	0.9 ± 0.7	0.7 ± 0.2
	Coumestrol	8	70.6 ± 17.1**	91.6 ± 9.6**	64.6 ± 17.2**
	E_2B	4	38.3 ± 13.7** (3)	55.6 ± 14.2**	27.7 ± 14.4**
Mature OVX	Arachis oil	8	1.1 ± 0.8	3.0 ± 1.4	0.4 ± 0.1
	Coumestrol	8	36.1 ± 18.2** (7)	70.4 ± 9.4**	3.3 ± 0.2**
	E_2B	8	1.6 ± 0.8	5.0 ± 3.0	0.3 ± 0.2

Numbers in parentheses indicate the group size upon which observations were made. Data were assessed for statistical significance by ANOVA following double arcsine transformation.
*$p < 0.05$; **$p < 0.01$ as compared to controls.

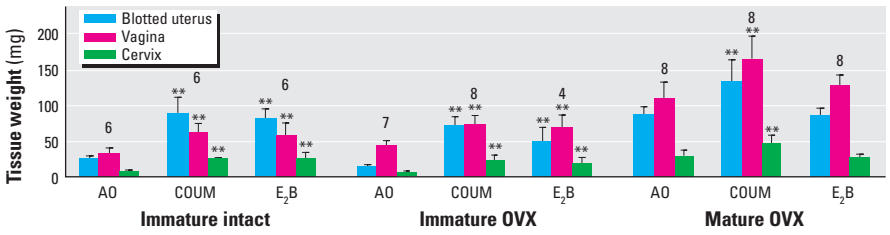

Figure 1. Activity of coumestrol (COUM; 60 mg/kg), E_2B (80 µg/kg), and arachis oil (AO; 5 mL/kg) in the rat uterotrophic assay. Data taken from Table 1 are shown as group mean ± SD and were assessed for significance by ANOVA. The numbers above columns indicate the number of animals per group.
**$p > 0.01$.

After treatment with E_2B, as with coumestrol, significant increases in the height of the uterine epithelium were observed in all treated females, with the most significant effect in intact weanlings (Figure 2A). The responses observed in both groups of OVX females were similar.

Determination of uterine hyperplasia. Coumestrol exposure led to a significant increase in the labeling index of the uterine epithelium and the glands and stroma of the endometrium in all three groups of females (Table 3, Figure 3). In all three sets of females, we observed the highest frequency of S-phase cells in the glands and the lowest frequency in the stroma. The effect of coumestrol on the labeling index of the uterus was most prominent in immature OVX animals (Figure 3).

After treatment with E_2B, there was a significant increase in the labeling index of the uterine epithelium in immature OVX animals only (Table 3). We observed increases in the labeling indices in the glands and stroma of the endometrium of the immature OVX animals and in the glands of the 21–22-day-old weanlings. E_2B induced a marginal, though significant ($p < 0.05$), increase in the total number of labeled uterine cells (Figure 3). As with coumestrol, the frequency of S-phase cells was highest in the glands and lowest in the stroma in all three groups of females.

Discussion

The present data supplement those described earlier (*2*) and confirm that coumestrol gives a classical estradiol-type uterotrophic response in the three protocols used here for the rat uterotrophic assay (Table 4). As noted earlier (*2*), the claim of Markaverich et al. (*1*) that coumestrol was an atypical uterotrophic agent in the immature ovariectomized rat was based on limited data; the present observations seem to override those earlier data. It is critical to know, for purposes of human hazard estimation, if phytoestrogens such as coumestrol act differently in the uterotrophic assay than do natural or

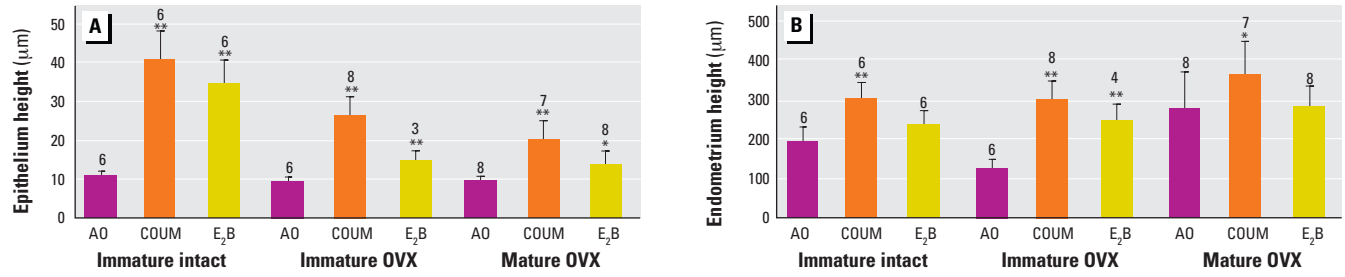

Figure 2. Effect of coumestrol (COUM; 60 mg/kg), E_2B (80 µg/kg), and arachis oil (AO; 5 mL/kg) on the height (in micrometers) of (*A*) the uterine epithelium and (*B*) the endometrium. Data were assessed for statistical significance by ANOVA. The number above each bar indicates the number of females in each group from which measurements could be made.
*$p > 0.05$; **$p < 0.01$.

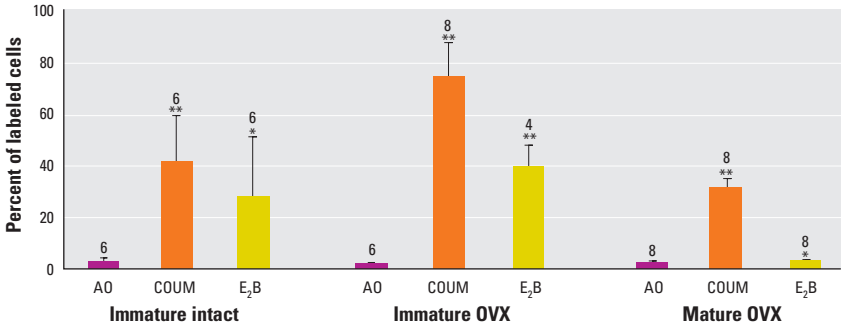

Figure 3. Effect of coumestrol (COUM; 60 mg/kg), E_2B (80 µg/kg), and arachis oil (AO; 5 mL/kg) on the labeling index of the uterus (epithelium and endometrium combined). Data were assessed for statistical significance by ANOVA following a double arcsine transformation.
*$p > 0.05$; **$p > 0.01$.

Table 4. Qualitative outcome for each parameter measured after exposure to coumestrol (60 mg/kg) or E_2B (80 µg/kg).

	Immature intact		Immature OVX		Mature OVX	
End point	Coumestrol	E_2B	Coumestrol	E_2B	Coumestrol	E_2B
Blotted uterine weight	**	**	**	**	**	–
Vaginal weight	**	**	**	**	**	–
Cervical weight	**	**	**	**	**	–
Epithelium height	**	**	**	**	**	*
Endometrium height	**	–	**	**	*	–
Uterine labeling index[a]	**	*	**	**	**	–

–, Not significant. Data were assessed for statistical significance by ANOVA (following double arcsine transformation in the case of the labeling index).
[a]Combined epithelium and endometrium. *$p < 0.05$; **$p < 0.01$.

synthetic estrogens. The present data do not support that claim.

The uterotrophic activity of a fixed dose level of coumestrol was similar across the three test protocols, whereas that of a fixed dose level of E_2B was much reduced in mature animals. This may reflect accelerated metabolic clearance of E_2B by the mature animals (but apparently not of coumestrol) due to their more well developed hepatic metabolic competence.

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