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Uterotrophic Assay Using Ovariectomized Female Rats with Sub-cutaneous Administration

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Abstract - The objective of this study was to prevalidate the Organization for Economic Cooperation and Development's (OECD) rodent uterotrophic assay as a test method for screening of potential endocrine disrupting chemicals (EDCs). This study was conducted exactly as described in the OECD protocol documents. A positive control substance, 17α -ethinyl estradiol (EE), was administered daily for three days to ovariectomized (OVX) Sprague-Dawley rats at various doses for determine the dose-response curve. Additionally, a pure anti-estrogenic chemical, ZM189,154 was administered to OVX rats at the same time EE to determine the effectiveness of the material against blocking the estrogenic effects of EE. At higher concentration of EE (10 μg/kg), a statistically significant difference in body weight gain and food consumption was observed compared to vehicle controls. In uterine responses, EE produced a dose-related increase in uterus weights compared to vehicle control. These increases were statistically significant at the >1.0 μg/kg doses. However, a similar dose-response relationship was not observed in vagina weight. A comparison of the two groups receiving ZM189,154 (0.1 and 1.0 mg/kg) with 0.3 μg/kg of EE and the group receiving only 0.3 μg/kg of EE showed dose-related decreases in uterus weights. However, statistical significance was shown in 1.0 mg/kg of ZM189,154. In conclusion, administration of EE produced a dose-related increase in uterine (wet and blotted) weights. Additionally, the 1.0mg/kg dose of ZM189,154 was effective in blocking the estrogenic activity of EE. These data suggest 3-day uterotrophic assay using OVX rats may serve as a good tool for EDCs screening.

Keywords uterotrophic assay, ovariectomized, 17-ethinyl estradiol, ZM 189,154.

INTRODUCTION

The rodent uterotrophic assay evaluates the ability of a chemical to show biological activities consistent with agonism or antagonism of natural estrogens. Uterine responses correlated directly with estrogen activity have been used predominantly by the pharmaceutical company to identifying estrogen-like activity of potential therapeutic compounds (Clark and Peck, 1979). Recently, these assays have been recommended in several test protocols for the screening of endocrine disrupting chemicals (EDCs)(O'Connor *et al.*, 1996; Odum *et al.*, 1997; Fail *et al.*, 1998). Uterotrophic is a term used to describe an increased growth of tissue of the uterus. Thus, a chemical causing an increase in uterine weight suggests that it has endogenous estrogen-like activity. The most commonly measured uterine response is a change in uterine weight. However, alteration in uterine weight does not represent just a single response, since increased uterine weight reflects a variety of processes including alterations in vascular permeability, water retention, and cell proliferation (Clark and Peck, 1979; Reel *et al.*, 1996).

This protocol was proposed as the first step in an Organization for Economic Cooperation and Development's (OECD) project to validate the rodent uterotrophic assay. This study was designed First, to demonstrate dose-response relationship of uterine weight in adult OVX rats following sub-cutaneous injection of the reference estrogen, 17-ethinyl estradiol (EE). Second, to enable variation between laboratories to be investigated and protocol refinement to be proposed. Third, to enable comparison of the results from similar protocols, i.e. the comparison of this protocol with two similar ones in which immature female rats are used with exposure by oral gavage and one in which immature female rats are used with exposure by sub-cutaneous injection. Forth, to assist in selecting the appropriate reference dose of EE to use in a subsequent protocols for investigating chemicals of unknown

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estrogenic activity. In addition, this protocol is intended to confirm the anti-estrogenic effects of ZM 189,154, a pure estrogen antagonist. This is important as anti-estrogens in the later steps of the OECD validation work.

Uterine responses have been measured in several strains of rats under a variety of assay conditions. Immature (prior to initiation of estrus cycling) and OVX animals have been tested frequently. The routes of administration have been included oral gavage, subcutaneous injection, and intraperitoneal injection with several vehicles. Exposure period and end point measuring time after last treatment have also varied. These variations can significantly influence the results of the uterotrophic assay. In our study, EE was administered by s.c. injection to adult OVX rats for three consecutive days. Two satellite groups of rats were used to confirm the anti-estrogenic activity of ZM189,154. Twenty-four hours after the last administration, the rats were humanely killed and uterine and vagina weight were recorded

MATERIALS AND METHODS

Test Substances

Estrogen agonist (17α-ethinyl estradiol, Batch/Lot 29052944) and estrogen antagonist (ZM189,154, Y10423/002/001) were supplied by Schering Pharmaceutical Co. (AG, D-13342 Berlin, Germany) and Zeneca Pharmaceutical PLC (Stanhope Gate, London, W1Y 6LN, UK), respectively. Corn oil (C-5677) and 95% ethanol (E-9987) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All substances were shipped and stored in glass containers at room temperature.

Test animals

Adult female Sprague-Dawley Crl:CD rats (Charles River Laboratories, Japan) were obtained from KFDA Laboratory Animal Resources under SPF-conditions. They were scheduled to arrive when they are 5 weeks old. All animals were assigned and checked for overt signs of ill health and anomalies. They were acclimatized for 1 week prior to ovariectomy.

Animal maintenance

All animals were housed in polycarbonate cages with solid bottom in controlled environment with a 12h light-dark cycle. The ambient air temperature was controlled at 23±2°C and the relative humidity was maintained at 50±10%. Bedding materials used in this study were autoclaved Elm tree. Prior to the experimental start date, the animals were allocated to the various treatment groups by random sort in accordance with the body weight. The mean body weight variation per group was within ±5 g. This study was identified with a unique study number, group number, and individual rats, respectively. Individual animals per cage were identified by tail tattoos, and each group of rats was coded by a color. Also, each cage number was labeled to show the laboratory code for the group. Prior to and at the end of the study, the cages and other materials that the animals may touch were cleaned with appropriate agents as specified in the laboratory standard operating procedure.

Feed and drinking water

All animals were maintained on PMI® Certified Rodent LabDiet (Nutrition International, Richmond, Indiana, USA), and filtered tap water was supplied using glass bottles. Diet and water were available ad libitum. We did maintain frozen samples of the rodent diet used in this study so that the diet can be further analysed, if necessary, for phytoestrogens and isoflavones.

Procedure for ovariectomy

The essential procedure was based on the OECD protocol (OECD Document 4, 1999). All animals were housed in polycarbonate cages and acclimatized to laboratory condition for at least 1 week before ovariectomy. The ovariectomy was performed on 6-week old animals. The dorso-lateral abdominal wall was cut 1cm length ways at mid point between the costal inferior border and the illiac crest, and a few millimeters lateral to the lateral margin of the lumbar muscle. The ovary was pulled out and disconnected at the junction of the oviduct and the uterine body. After confirming that no massive bleeding is occurred, abdominal wall and the skin were closed by autoclips, respectively. All animals were allowed to acclimatized after surgery for at least 1 week.

Administration of the test substances

Approximately 1 week after ovariectomy, animals were dosed by subcutaneous injection. Date and time of each injection were recorded exactly. The test substance was administered once a day for three consecutive days. The amount administered was calculated on the body weight of the animal on treatment day. All test materials were prepared daily prior to injection. The EE and ZM189.154 were dissolved in a minimal amount of 95% ethanol and diluted to final working concentration with corn oil (final concentration of 95% ethanol was 2.5%). When testing the estrogen antagonist ZM189,154,

Table I. Details of experimental groups and dose levels

Grous	N	Dose		Route	Maximum total s.c. volume/day/rat
		EE (μg/kg)	ZM189,154 (mg/kg)		
1 (Untreated control)	6	0	0	Not applicable	Not applicable
2 (Vehicle control)	6	0	0	s.c.	4 ml/kg/day
3	6	0.01	0	s.c.	4 ml/kg/day
4	6	0.03	0	s.c.	4 ml/kg/day
5	6	0.10	0	s.c.	4 ml/kg/day
6	6	0.30	0	s.c.	4 ml/kg/day
7	6	1.00	0	s.c.	4 ml/kg/day
8	6	3.00	0	s.c.	4 ml/kg/day
9	6	10.0	0	s.c.	4 ml/kg/day
10	6	0.3	0.1	s.c.	4 ml/kg/day
11	6	0.3	1.0	s.c.	4 ml/kg/day

it was administered first by s.c. injection and then the EE was administered also by s.c. within a 15 min as possible. The total amount of injection per rat was 4 ml/kg/day. The study was comprised eleven groups of six females each, as shown in Table I including 7 doses of EE, one untreated control group, one vehicle control group and two satellite groups for ZM189,154 (0.1 mg/kg and 1.0 mg/kg)

Observations

Clinical signs

Through the test period, each animal was observed at least once a day. On working days, all cages were checked in the morning and afternoon for dead or moribund animals. All abnormalities were recorded and included in the study report.

Body weight and food consumption

The body weight of each rat was recorded daily to the nearest 0.1g, starting just prior to initiation of treatment i.e. when the animals are allocated into groups. The amount of food consumed during the treatment period was measured per cage by weighing the feeders. The food consumption results are expressed in grams per rat per day.

Measurement of uterus and vagina weight

Twenty-four hours after the last treatment, the rats were humanely killed by cervical dislocation in the same sequence as the test substance was administered. The uterus was carefully dissected, trimmed free of fat to avoid loss of luminal contents. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with ovaries. The vagina was removed from the uterus at the level of the uterine cervix. The uterus was transferred to a uniquely marked and weighed container (e.g., a petri-dish) with care to avoid desiccation before weighing. The uterus was weighed with the luminal contents (wet weight) to the nearest 0.1 mg. Each uterus was individually processed to open the uterine wall and carefully blot the excess fluid. For example, both uterine horns was pierced or cut longitudinally and then placed on moistened filter paper (Whatman No. 3) and gently pressed to absorb the luminal fluid.

Statistical Analysis

All values were expressed as mean±SD. Statistical analysis of the data was performed using the one-way analysis of vari-

Table II. The effects of 17-ethinyl estradiol and ZM 189,154 on clinical signs of ovariectomized rats administered by s.c. injection

Treatments	Control		EE (μg/kg/day)							EE 0.3 μg/kg+ ZM(mg/kg/day)	
Dosage	-	+	0.01	0.03	0.1	0.3	1	3	10	0.1	1.0
No. of animals examined	6	6	6	6	6	6	6	6	6	6	6
No. of animals survival/death	6/0	6/0	6/0	6/0	6/0	6/0	6/0	6/0	6/0	6/0	6/0
No. of animals with normal findings	6	6	6	6	6	6	6	6	6	6	6
No. of animals with abnormal findings	0	0	0	0	0	0	0	0	0	0	0
Decrease in locomotor activity	0	0	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	0	0	0	0	0	0	0
Increase in locomotor activity	0	0	0	0	0	0	0	0	0	0	0
Convulsion	0	0	0	0	0	0	0	0	0	0	0
Hair loss	0	0	0	0	0	0	0	0	0	0	0
Red urine	0	0	0	0	0	0	0	0	0	0	0
Diarreha	0	0	0	0	0	0	0	0	0	0	0

- ; untreated control, + ; vehicle control (corn oil)

ance (ANOVA) and the Bonferroni t-test. p values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Clinical signs

Mortality, evidence of overt toxicity, and treatment-related clinical signs were not observed during the study period (Table II).

Body weight and food consumption

The effects of EE and ZM189,154 on body weight changes in ovariectomized female rats are shown in Table III. Compared to untreated control group, the overall body weights gain of 10 μg/kg EE group was significantly decreased as treatment period goes by. The body weights gain at treatment day 3 and 4 were 89.6% and 86.6% of untreated control, respectively. However, no statistically significant differences in body weights or body weight changes were observed in <10 μg/kg of EE treatment groups. Similarly, Zacharewski *et al.*(1998) demonstrated that EE (1.0 mg/kg) significantly decreased body weight gain in OVX Sprague-Dawley rats by oral administration for 4 days.

As similar to body weight changes, food consumption was significantly decreased by 10 μg/kg EE treatment compared to the untreated controls. The food consumption on days 2~3 in >1.0 μg/kg EE was significantly lower than those of the untreated controls. However, no consistent differences in food consumption were observed in the <0.3 μg/kg EE-treated groups compared to the untreated controls (Table IV). The effects of estrogens on the growth and body weight of animals have been reported in long-term studies with most estrogens (Heywood and Wadsworth, 1980; Hart, 1990).

The effects of estrogens on the growth and body weight of rodents involve decreases in food and water intake (Finkelstein, 1986) and adiposity (Wade *et al.*, 1985). In decreasing appetite in the rats, estrogen appears to act centrally at the

Table IV. The effects of 17-ethinyl estradiol and ZM 189,154 on food consumption of ovariectomized rats administered by s.c. injection

Groups	Dosage	Day 1 (g/rat)	Day 2 (g/rat)	Day 3 (g/rat)
Untreated control	0	18.2 ± 0.88	21.9 ± 0.38	21.4 ± 0.49
Vehicle control	0	23.7 ± 3.78	22.8 ± 0.60	23.6 ± 3.51
EE (μg/kg)	0.01	21.5 ± 3.72	21.0 ± 1.31	22.0 ± 2.63
	0.03	20.2 ± 0.05	21.6 ± 0.11	22.0 ± 0.49
	0.1	19.7 ± 0.11	20.3 ± 0.44	20.3 ± 0.11
	0.3	20.0 ± 0.71	19.4 ± 1.26*	21.4 ± 0.11
	1.0	18.8 ± 1.04	18.9 ± 0.16*	18.3 ± 0.88*
	3.0	16.8 ± 0.01	18.6 ± 0.44*	18.7 ± 0.93*
	10.0	12.7 ± 0.99**	16.8 ± 0.99**	14.7 ± 0.27**
EE (0.3 μg/kg) + ZM189,154 (mg/kg)	0.1	18.6 ± 1.92	18.3 ± 0.66**	18.8 ± 2.08
	1.0	19.4 ± 1.10	18.8 ± 0.44**	18.9 ± 0.01

Data are presented as mean±SD. Vehicle control received corn oil containing 2.5% ethanol. Six animals were used per treatment group. All animals were ovariectomized on day 6 week. After 1 week, the test substance was administered sub-cutaneously once a day for three consecutive days. The animals were weighed and sacrificed by cervical dislocation 24 h after the last treatment. Significantly different from untreated controls at *p<0.05. and **p<0.01.

Table III. The effects of 17-ethinyl estradiol and ZM 189,154 on body weight change of ovariectomized rats administered by s.c. injection

Groups	Dosage	Day 1 (g)	Day 2 (g)	Day 3 (g)	Day 4 (g)
Untreated control	0	195.3 ± 2.32	201.6 ± 2.75	208.8±3.10	211.7 ± 2.17
Vehicle control	0	195.0 ± 2.01	203.6 ± 3.63	209.8±5.22	215.5 ± 3.89
EE (μg/kg)	0.01	196.2 ± 3.30	203.0 ± 3.27	209.7±1.96	213.9 ± 1.87
	0.03	195.3 ± 2.82	202.6 ± 4.14	210.5±4.62	213.8 ± 5.67
	0.1	194.6 ± 2.94	198.5 ± 5.82	206.9±6.08	210.9 ± 7.46
	0.3	196.3 ± 3.11	203.2 ± 5.95	207.4±7.14	212.6 ± 6.01
	1.0	195.9 ± 2.98	200.5 ± 2.58	205.8±3.72	209.3 ± 5.21
	3.0	196.2 ± 4.15	198.7 ± 5.46	202.2±5.22	205.4 ± 4.88
	10.0	193.3 ± 3.58	192.2 ± 6.25*	194.7±6.23**	195.5 ± 5.62**
EE (0.3 μg/kg) + ZM189,154 (mg/kg)	0.1	197.7 ± 3.31	210.3 ± 5.52*	216.2±7.82	221.5 ± 8.92
	1.0	196.0 ± 3.23	201.5 ± 6.48	204.4±6.45	208.6 ± 7.21

Data are presented as mean±SD. Vehicle control received corn oil containing 2.5% ethanol. Six animals were used per treatment group. All animals were ovariectomized on day 6 week. After 1 week, the test substance was administered sub-cutaneously once a day for three consecutive The animals were weighed and sacrificed by cervical dislocation 24 h after the last treatment. Significantly different from untreated controls at *p<0.05. and **p<0.01.

hypothalamus. However, administration of 17β,-estradiol (at 0.12 and 0.24 ppm) in the diet for 90-day to Wistar rats did not produce any effects on body weight or food consumption, while diethylstilbestrol (0.12 ppm) administration showed the decreases in body weight and food consumption (Ferrando and valette, 1975).

Measurement of uterus and vagina weight

The effects of sub-cutaneous administration of EE and ZM189,154 on uterus weight are summarized in Table V. The higher doses of EE (>1.0 μg/kg) gave an approximately 6.75-10.57 fold increase in uterus wet weights when injected with s.c. in OVX rats, while low doses of EE (<0.1 μg/kg) did not show any significant difference compared to vehicle controls. Although the peak of uterotrophic response showed at EE 10 μg/kg, these values showed steady state level from 1.0 μg/kg to 10 μg/kg EE. Also, statistical analysis showed that the uterine blotted weight was significantly, dose-dependently increased at concentration of 1.0 μg/kg EE and above. Mean uterine blotted weight increased 3.24 to 3.79 fold compared to untreated controls at doses from 1.0 μg/kg to 10 μg/kg. Also, the ratio of increase in uterine blotted weight was lower than that of uterine wet weight. However, the vagina weight were significantly increased at >0.3 μg/kg EE and showed peak (186% of vehicle control) at 3.0 μg/kg EE (Table VI).

In this study, the increase of uterine wet and blotted weight

Table VI. The effects of 17-ethinyl estradiol and ZM 189,154 on vagina weight of ovariectomized rats administered by s.c. injection

Groups	Dosage	Vagina weight (mg)	Vagina wt (mg)/ 100g body wt.
Untreated control	0	71.1 ± 7.8	33.6 ± 3.9
Vehicle control	0	69.0 ± 11.8	32.0 ± 5.7
EE (μg/kg)	0.01	64.0 ± 16.3	30.0 ± 7.8
	0.03	74.3 ± 6.3	34.8 ± 3.4
	0.1	72.2 ± 19.2	34.3 ± 9.8
	0.3	122.7 ± 8.2**	58.1 ± 2.7**
	1.0	126.7 ± 16.9**	60.5 ± 7.2**
	3.0	132.2 ± 17.0**	64.6 ± 7.7**
	10.0	129.4 ± 15.5**	66.6 ± 8.8**
EE (0.3 μg/kg) + ZM189,154(mg/kg)	0.1	106.9 ± 14.4**	48.2 ± 5.5**
	1.0	73.2 ± 5.9	35.0 ± 3.1†

Data are presented as mean±SD. Vehicle control received corn oil containing 2.5% ethanol. Six animals were used per treatment group, but 5 animals were used at 0.3 μg/kg EE + ZM189,154 1.0mg/kg treatment group. All animals were ovariectomized on day 6 week. After 1 week, the test substance was administered sub-cutaneously once a day for three consecutive days. The animals were weighed and sacrificed by cervical dislocation 24 h after the last treatment. The uteri and vagina were quickly removed, excised of connective tissue, and weighed. Significantly different from untreated controls at **p<0.01. Significantly different from EE 0.3 μg/kg at +p<0.05.

Table V. The effects of 17-ethinyl estradiol and ZM 189,154 on uterus wet and blotted weight of ovariectomized rats administered by s.c. injection

Groups	Dosage	Uterus wet wt. (mg)	Uterus wet wt. (mg)/100g body wt.	Uterus blotted wt. (mg)	Uterus blotted (mg)./ 100g body wt.
Untreated control	0	109.5 ± 9.4	51.8 ± 4.9	98.2 ± 9.5	46.4 ± 4.9
Vehicle control	0	107.0 ± 7.41	49.7 ± 3.6	89.3 ± 10.3	41.4 ± 4.8
EE (μg/kg)	0.01	105.7 ± 12.3	49.5 ± 6.1	88.6 ± 10.9	41.5 ± 5.3
	0.03	106.7 ± 19.5	49.9 ± 8.6	83.9 ± 20.5	39.3 ± 9.4
	0.1	109.9 ± 14.0	51.8 ± 8.2	89.7 ± 11.7	43.7 ± 7.0
	0.3	207.9 ± 57.2	98.4 ± 27.1	172.5 ± 44.7	81.7 ± 21.7
	1.0	739.5 ± 199.8**	353.8 ± 95.8**	318.6 ± 41.9**	152.6 ± 21.5**
	3.0	896.3 ± 79.9**	438.6 ± 37.3**	339.8 ± 47.6**	166.1 ± 21.6**
	10.0	1158.2 ± 183.1**	596.3 ± 104.7**	372.5 ± 32.9**	191.7 ± 20.2**
EE (0.3 μg/kg) + ZM189,154 (mg/kg)	0.1	170.0 ± 19.5	76.7 ± 7.8	137.1 ± 14.1	61.9 ± 6.8
	1.0	120.3 ± 15.6[a]	57.5 ± 7.7	101.8 ± 13.2	48.7 ± 6.91†

Data are presented as mean±SD. Vehicle control received corn oil containing 2.5% ethanol. Six animals were used per treatment group, but 5 animals were used at 0.3 μg/kg EE + ZM189,154 1.0 mg/kg treatment group. All animals were ovariectomized on day 6 week. After 1 week, the test substance was administered sub-cutaneously once a day for three consecutive days. The animals were weighed and sacrificed by cervical dislocation 24 h after the last treatment. The uteri were quickly removed, excised of connective tissue, and weighed. Significantly different from untreated controls at **p<0.01. Significantly different from EE 0.3 μg/kg at †p<0.05

at 0.3 μg/kg EE was not significantly greater than those of vehicle controls, and showed only 1.89- and 1.75-fold increase. Therefore, EE 0.3 μg/kg dose used in uterotrophic assay as a positive control might not be sufficient. Based on these results, sub-cutaneous administration of 1.0 μg/kg of EE would be adopted as the standard positive control dose for the uterotrophic assay. Also, the relationship between uterus wet, blotted, and vaginal and relative weight to the body weight was essentially consistent for all the groups in this study.

As expected, the uterine weight and vaginal weight increased by EE 0.3 μg/kg treatment were significantly ablated, in a dose-related manner, by the pure estrogen receptor antagonist ZM189,154 when the later was administered concomitantly with the same dose of EE. The uterus wet and blotted weights of OVX rats exposed to ZM189,154 (1.0 mg/kg) with EE (0.3 μg/g) were decreased by 57.8% and 59.0%, respectively, when compared to 0.3 μg/kg EE group (Table V).

These results indicated that uterotrophic assay could detect anti-estrogenic as well as estrogenic activities of unknown chemicals. In conclusion, we suggest that (1) Thc dose of positive control for uterotrophic assay would be over 0.3 μg/kg EE, (2) The measurement of blotted weight was a better endpoint than wet weight for uterotrophic assay. (3) The uterotro-phic assay is a good detection method for not only estrogenic but also anti-estrogenic activities of a certain chemical.

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