The Intact Immature Rodent Uterotrophic Bioassay: Possible Effects on Assay Sensitivity of Vomeronasal Signals from Male Rodents and Strain Differences

John Ashby,[1] William Owens,[2] Jenny Odum,[1] and Helen Tinwell[1]

[1]Syngenta Central Toxicology Laboratory, Cheshire, United Kingdom; [2]Central Product Safety, The Procter & Gamble Company, Cincinnati, Ohio, USA

The vomeronasal organ in rodents is an important social and sexual signaling pathway. We have investigated whether the housing of intact immature females in close proximity to mature males would interfere with the sensitivity of the immature rodent uterotrophic bioassay as the result of vomeronasal signals transmitted by male urinary proteins. The hypothesis was that the proximity of males might induce early puberty, thereby increasing mean uterine weight and reducing the responsiveness of the assay. The hypothesis was tested in both rats and mice by housing mature males above immature females, separated only by a wire screen, for 3 days and determining possible changes in uterine weight. The results were negative. Neither the mean uterine weight nor the group mean standard deviation of the uterine weights were changed in the uterotrophic bioassay. Given that the timing of sexual maturation may vary with the strain of mouse used, we also evaluated the sensitivity of the immature mouse uterotrophic assay to diethylstilbestrol (DES) using four strains of mice. Similar sensitivity was observed for the CD-1, C57Bl6, and Alpk strains, but B6CBF$_1$ mice were marginally less sensitive to DES than were the other strains. These findings add to earlier data indicating the robustness of the rodent uterotrophic assay protocol. *Key words:* **puberty, sexual development, strain differences, uterine weight, uterotrophic assay, vomeronasal.** *Environ Health Perspect* **111:1568–1570 (2003). doi:10.1289/ehp.5981 available via** *http://dx.doi.org/* **[Online 23 January 2003]**

The standardization and validation of the uterotrophic bioassay in immature rodents is of current interest (Kanno et al. 2001, 2003a, 2003b; Odum et al. 1997), especially the level of accuracy in detecting weak estrogen agonists and antagonists. The need is for a responsive assay with a large dynamic range where uterine weights in vehicle control animals are both low and uniform to detect modest changes in uterine weights. For the immature rodent assay, the animals must be prepubertal, that is, before the rise in endogenous estrogen levels that occurs at approximately postnatal day (pnd) 30. The use of animals near puberty may have increased mean uterine weights and greater interindividual variability (leading to increased group mean standard deviations and reduced assay sensitivity (Christian et al. 1998; Schlumpf et al. 2001; Thigpen et al. 1987). Consequently, factors that may affect pubertal timing, and potentially interfere with the assay sensitivity, are worthy of study.

Sexual development and maturity can be modulated by vomeronasal signals in rodents, particularly in mice. The presence of adult females may delay the onset of puberty in prepubertal females and suppress estrous cyclicity in grouped females [the Lee–Boot effect (van der Lee and Boot 1955)]. Adult males can also induce estrus in adult females [the Whitten effect (Whitten 1956)] and accelerate puberty in prepubertal females [the Vandenbergh effect (Vandenbergh 1969)]. The acceleration of puberty may be determined by earlier first estrus or vaginal opening (Vandenbergh 1969) or by increased uterine weight (Price and Vandenbergh 1992).

The vomeronasal system is not a true olfactory organ. Rather, it is a blind cavity in the nasal epithelium that may require direct sniffing to capture particles or droplets or oral licking to transfer sufficient compounds into the vomeronasal organ (Greene and Kipen 2002). The literature indicates that the female responses are due to a class of major urinary proteins (MUPs) produced by the male that are members of the lipocalin protein family (Flower 1994; Sansom et al. 1994). The MUPs appear to be species, sex, and strain specific and may include individual polymorphisms (Clissold and Bishop 1982; Johnson et al. 1995). The MUPs bind to vomeronasal receptors in the female. The binding first leads to molecular events in the vomeronasal organ and the generation of neural signals that must be transmitted along intact nerve pathways to brain centers to impact behavior or neuroendocrine function (Brennan et al. 1999; Dudley et al. 1996; Mucignat-Caretta et al. 1995; Vandenbergh et al. 1975). These reports (Brennan et al. 1999; Dudley et al. 1996; Mucignat-Caretta et al. 1995; Vandenbergh et al. 1975) suggest that the female vomeronasal system is unlikely to be sensitive to highly volatile compounds that might come from males in the same facility, or even housed in the same room. Rather, the female must be in contact with a sufficient amount of contaminated bedding or the male himself.

Another factor that may affect the timing of puberty is the strain of animal used (Nelson et al. 1990). Different strains may also show differing responses to treatment. For example, Spearow et al. (1999) detected a 16-fold difference in susceptibility to disruption of male sexual development in four strains of male mice exposed to estradiol.

In this study, we have investigated the possible effects of vomeronasal signals and strain differences on the performance of the uterotrophic assay. To test the effect of vomeronasal signals, we housed mature males in close proximity to immature females and determined the effects on uterine weight after 3 days. We used both rats and mice in these experiments. The mature males were housed directly above the immature females with only a wire screen separating the sexes. This would allow any vomeronasal signals in male urine to be transmitted to the females. We also used standard uterotrophic assays to investigate the sensitivity of four strains of immature mice to diethylstilbestrol (DES).

Materials and Methods

Chemicals. DES (> 99% pure) and arachis oil were obtained from Sigma Chemicals (Poole, Dorset, UK).

Animals. Alpk (Alpk:ApfSD, Wistar-derived) rats and Alpk (Alpk:AP$_f$CD-1, Swiss-derived) and B6CBF$_1$ (C57BL/6J-Alpk X CBA/Ca-Alpk) mice were obtained from the AstraZeneca breeding unit (Alderley Park, Macclesfield, Cheshire, UK). CD-1 [CD-1 Crl:CD-1 (ICR) BR] mice were obtained from Charles River UK (Margate, Kent, UK). C57BL6J (C57BL/6JolaHsd) mice were obtained from Harlan UK (Bicester, Oxon, UK). Female rats were 18–19 days of age on arrival (body weights ≤ 45 g); male rats were 10–12 weeks of age. Female mice were 19–20 or 20–21 days of age on arrival, and male mice were 10–12 weeks of age. Immature animals were selected randomly from a large number of litters by the suppliers and were

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Address correspondence to J. Ashby, Syngenta Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, UK. Telephone: 44 0 1625 512833. Fax: 44 0 1625 590249. E-mail: john.ashby@syngenta.com

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already weaned at delivery. Animals were allowed 24 hr acclimatization before the start of the uterotrophic assays. Female animals were housed (up to five per cage) in metal cages with wire mesh bases and were supplied with shredded paper bedding. Males were not supplied with bedding to allow the females maximum contact with the male urine. Rat and Mouse No. 1 diet (Special Diet Services Ltd., Witham, Essex, UK) and water were available *ad libitum*. Animal care and procedures were conducted according to in-house standards as described previously (Odum et al. 1999).

Uterotrophic assays. Immature rat and mouse uterotrophic assays were conducted in an identical manner.

The effect of the close proximity of mature males on female uterine growth was tested in Alpk rats and Alpk mice that were 19–20 days of age (pnd 19–20) and 20–21 days of age (pnd 20–21), respectively, at the start of treatment. Untreated females were housed for 3 days in groups of three in a cage placed directly under a cage containing a mature (untreated) male. The base of the male's cage was open wire mesh, thus allowing direct contact with the male's urine. Control untreated animals were housed for 3 days in a separate room containing no male animals. DES (5 µg/kg) or vehicle (arachis oil) were administered by subcutaneous (sc) injection (dosing volumes for rats and mice were 2.5 and 5 mL/kg, respectively) daily for 3 days to two additional groups of animals housed in the same room as the control untreated animals (i.e., in the absence of males).

The impact of strain on the sensitivity of the mouse uterotrophic assay was tested in Alpk, C57BL6J, B6CBF$_1$, and CD-1 mice. C57BL6J and B6CBF$_1$mice were 20–21 days of age (pnd 20–21) at the start of treatment, whereas the CD-1 mice were 21–22 days of age (pnd 21–22) at the start of treatment (animals 20–21 days of age were unavailable). Three experiments were carried out in which Alpk mice were tested alongside C57BL6J, B6CBF$_1$, or CD-1 mice. Alpk mice were present in each experiment and of the same age as the strain against which they were being compared. DES (1 or 10 µg/kg) or vehicle (arachis oil) were administered by sc injection (dosing volume 5 mL/kg) daily for 3 days.

In all cases, on the fourth day animals were killed by an overdose of halothane (AstraZeneca plc), followed by cervical dislocation. The abdomen was opened and the uterus removed. The cervix and ovaries were then removed, and the uterus was trimmed free of fat. Blotted uterine weights and dried uterine weights were determined as described earlier for the immature rat uterotrophic assay (Odum et al. 1997).

Results

The data from two studies to investigate female uterine growth in the presence of mature males are shown in Table 1; the first study was conducted in rats and the second in mice. Each study was performed with four groups of animals: untreated immature females with mature males housed directly above and separated only by a wire screen to allow exposure to MUPs and other possible vomeronasal signals; untreated immature females with no males housed in the same room; a vehicle control group; and a positive control group treated with DES. For animal welfare considerations, the vehicle control and DES groups were limited to three animals. For power, the groups housed in proximity to mature males and with males absent were enlarged to 9 or 10 animals. Blotted and oven-dried uterine weights were recorded, as well as body weight at termination.

In both studies, the DES-treated group had significantly increased uterine weights and the vehicle control group had uterine weights consistent with previous results in each species (Odum et al. 2002; Tinwell et al. 2000). As shown in Table 1, the mean blotted and dry uterine weights of females in close proximity to males were similar to those of untreated females in the absence of males and those of the vehicle controls. Further, the standard deviations of females in close proximity to males were also similar to the controls. Therefore, we detected no impact of mature males in close proximity to immature females in the uterotrophic bioassay using either rats or mice.

The second series of experiments was designed to investigate the response of different strains of mice to DES. All strains showed a highly significant uterotrophic response to DES at 10 µg/kg/day, although the uterine weight increase varied from 64 mg in the Alpk to 35 mg in the B6CBF$_1$ strain (Table 2).

Table 1. The effect of males on uterine weight of immature females (mean ± SD) in Alpk rats and Alpk mice.

Treatment/condition	No.	Uterine blotted weight (mg)	Uterine dry weight (mg)	Body weight at termination (g)
Rats[a]				
Untreated females, close proximity to males	9	21.3 ± 2.3	4.1 ± 0.5	49.2 ± 5.9
Untreated females (males absent)	10	22.8 ± 1.7	4.5 ± 0.4	48.3 ± 6.1
Arachis oil, 5 mL/kg/day (males absent)	3	23.6 ± 5.8	4.4 ± 1.1	48.0 ± 4.6
DES, 5 µg/kg/day (males absent)	3	107.2 ± 7.6*	18.6 ± 0.9*	51.5 ± 4.6
Mice[b]				
Untreated females, close proximity to males	9	11.7 ± 2.9	2.5 ± 0.6	16.1 ± 2.4
Untreated females (males absent)	10	10.8 ± 3.6	2.4 ± 0.6	15.9 ± 2.8
Arachis oil, 5 mL/kg/day (males absent)	3	12.2 ± 3.7	2.8 ± 0.61	16.3 ± 2.8
DES, 5 µg/kg/day (males absent)	3	70.8 ± 11.5*	11.3 ± 1.6*	17.9 ± 2.9

[a]19–20 days of age at the start of dosing. [b]20–21 days of age at the start of dosing. *Significantly different from the appropriate vehicle control group ($p < 0.01$).

Table 2. Uterine weight (mean ± SD) of different strains of immature female mice exposed to DES.

Experiment/strain/compound	Age (days) at start of dosing	Uterine blotted weight (mg)	Body weight at termination (g)[a]
Experiment 1			
Alpk			
Arachis oil, 5 mL/kg/day	20–21	11.0 ± 2.8	16.0 ± 1.7
DES, 1 µg/kg/day		25.7 ± 8.6*	16.6 ± 1.5
DES, 10 µg/kg/day		64.2 ± 8.1*	17.1 ± 1.7
C57BL6J			
Arachis oil, 5 mL/kg/day	20–21	8.8 ± 1.3	11.3 ± 1.1
DES, 1 µg/kg/day		20.3 ± 8.0*	11.2 ± 1.0
DES, 10 µg/kg/day		45.7 ± 6.2*	11.7 ± 1.6
Experiment 2			
Alpk			
Arachis oil, 5 mL/kg/day	20–21	9.6 ± 3.2	17.4 ± 0.7
DES, 1 µg/kg/day		16.3 ± 3.1*	17.4 ± 1.5
DES, 10 µg/kg/day		51.7 ± 7.4*	18.5 ± 1.5
B6CBF$_1$			
Arachis oil, 5 mL/kg/day	20–21	8.8 ± 1.6	10.3 ± 0.9
DES, 1 µg/kg/day		11.7 ± 2.6	10.6 ± 1.6
DES, 10 µg/kg/day		34.6 ± 5.0*	10.7 ± 1.0
Experiment 3			
Alpk			
Arachis oil, 5 mL/kg/day	21–22	10.9 ± 1.9	15.4 ± 1.2
DES, 1 µg/kg/day		26.8 ± 5.9*	16.0 ± 1.1
DES, 10 µg/kg/day		45.1 ± 8.6*	15.6 ± 2.2
CD-1			
Arachis oil, 5 mL/kg/day	21–22	21.0 ± 6.8	15.4 ± 1.2
DES, 1 µg/kg/day		29.6 ± 7.3	14.9 ± 1.0
DES, 10 µg/kg/day		60.9 ± 9.0*	15.1 ± 1.5

[a]$n = 10$ animals per group. *Significantly different from the appropriate vehicle control group ($p < 0.01$).

DES at 1 μg/kg/day produced a significant uterine weight increase in the Alpk and C57BL6J mice but a nonsignificant increase in the other two strains. In the case of the CD-1 strain, this may have been due to the high uterine weight in controls (21 mg compared with ~10 mg in the other strains). The CD-1 mice were 1 day older than the other strains at the start of dosing, which may have contributed to the increased uterine weight in controls, although Alpk mice of this age showed a similar response to mice that were 1 day younger.

Discussion and Conclusions

The rodent uterotrophic assay is currently undergoing validation by the Organisation for Economic Co-operation and Development (OECD) as a tier 1 screening bioassay to identify estrogen agonists and antagonists that may warrant testing for adverse effects (Kanno et al. 2001, 2003a, 2003b). The gravimetric weight of the uterus in response to chemical administration can be assayed in sexually immature or ovariectomized rodents, and either rats or mice can be used. Recently, several experiments have been conducted on the possible influence of several variables on the responsiveness of the bioassay, including the age of immature animals (Yamasaki et al. 2001), diet (Ashby et al. 2000, 2001; Degen et al. 2002; Owens et al. 2003; Yamasaki et al. 2002), and vehicle (Yamasaki et al. 2001). One interest here was to define whether certain animal husbandry conditions may lead to early puberty and decreased assay responsiveness. A second interest was to determine possible strain differences in the response to estrogen agonists such as DES.

Both the group mean of blotted and dry uterine weights and the standard deviations of these group means were unaffected by proximity of males for either rats or mice. These data, therefore, provide no evidence that the presence of mature males will alter the results of the uterotrophic bioassay or lead to conditions that will interfere with the responsiveness of the bioassay. The absence of an effect in these studies is probably due to the young age of the animals (24 days of age at termination). Colby and Vandenberg (1974) showed accelerated first estrus in mice 24–29 days of age at the time of exposure to male urine, an effect that is absent in younger animals.

Strain differences were not a major variable in the OECD validation of the rat uterotrophic assay. No differences were observed between Sprague-Dawley and Wistar rat strains among several laboratories using ethinyl estradiol as a potent reference and also five weak estrogen agonists (Kanno et al. 2001, 2003a, 2003b). However, strain differences in response to estrogen receptor agonists have been observed with male mice exposed to estradiol, where marked differences in the effects on testes and sperm were evident (Spearow et al. 1999). Strain differences in the response of rats to bisphenol A (Long et al. 2000) have also been reported, with F344 rats more sensitive than Sprague-Dawley rats, although we found no difference in the magnitude of the uterotrophic effect of nonylphenol in Sprague-Dawley and Alpk rats (Odum et al. 1999). The work of Thigpen et al. (1987) and Schlumpf et al. (2001) clearly demonstrate the impact of the early events of prepuberty in mice and rats, respectively. In both species, an increase in mean uterine weight and a rapid increase in the standard deviations of a group occurs. Thus, the timing of puberty must be taken into account with each species and each strain. In this investigation, $B6CBF_1$ mice were less sensitive to the action of DES, and control CD-1 mice had relatively high uterine weights. However, in each of the strains, DES was clearly detected as an estrogen. Although the possibility remains that the response to vomeronasal signals may differ with strain, the age of the immature animals appears to be the primary determinant of sensitivity, in agreement with others (Thigpen et al. 1987; Schlumpf et al. 2001). Our chosen strain was unaffected.

These findings add to earlier data (Kanno et al. 2001, 2003a, 2003b) indicating the robustness of the rodent uterotrophic assay protocol, and they raise the possibility that some strains of animal or the conditions under which they are used may lead to differences in sensitivity to the action of estrogens. This supports the efforts of Kanno et al. (2001, 2003a, 2003b) to standardize the age of their animals and of Owens et al. (2003) to investigate the influence of dietary phytoestrogens.

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