Increasing the Sensitivity of the Rodent Uterotrophic Assay to Estrogens, with Particular Reference to Bisphenol A

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The gravimetric uterotrophic assay is currently the most well-established, short-term rodent estrogenicity assay. Increasing attention is being paid to the extent to which use of morphometric or molecular changes in the uterus could act as surrogates for the gravimetric end point of the assay, thereby perhaps increasing the sensitivity of the assay. In this paper I discuss the available data, paying particular attention to studies on bisphenol A (BPA) because it offers the largest database for consideration. I conclude that the case has yet to be made for augmenting the gravimetric end point of the uterotrophic assay. To resolve this important question, it will be necessary to conduct detailed dose–response studies where the no-observed-effect level (NOEL) for the proposed surrogate end points are compared with the NOEL for the gravimetric end point. Currently, few such studies exist, and among those that do no clear message emerges. The general trend to increasing use of molecular assays in toxicology (multigene microarrays and real-time polymerase chain reaction) emphasizes the need for clear criteria for comparing the performance of individual markers of toxicity. *Key words*: bisphenol A, lactoferrin, no-effect level, NOEL, surrogates for the uterotrophic assay. *Environ Health Perspect* 109:1091–1094 (2001). [Online 10 October 2001] *http://ehpnet1.niehs.nih.gov/docs/2001/109p1091-1094ashby/abstract.html*

The rodent uterotrophic assay is currently being validated as a regulatory screening assay for estrogenicity by the Organisation for Economic Co-operation and Development (OECD) (*1*). The assay determines growth of the uterus gravimetrically in response to administration of a chemical to either sexually immature or ovariectomized rodents. Historically, both rats and mice and a variety of routes of administration of test chemicals have been used for the assay. I have assumed here that substantial equivalence exists between the different ways of conducting the assay, but individual cases of one species of rodent being more sensitive than the other can be anticipated, just as the use of different routes of administration may modulate some assay responses due to differences in the adsorbtion, distribution, metabolism, and excretion of the chemical. In fact, these variables, and their possible influence on the routine use of the uterotrophic assay, are being considered in phase II of the OECD validation study (*1*).

A positive uterotrophic response is not, of itself, an adverse toxicologic response. Rather, it provides information on the ability of a chemical to influence an estrogen-responsive process in living animals. Recently, several research groups evaluated whether the sensitivity of the assay can be increased by the use of surrogate markers, such as changes in the expression of estrogen-regulated uterine genes, advancement of vaginal opening in immature animals, or changes in uterine pathology or cellular growth. The objective of these efforts is that the lowest-effective-dose level (LOEL) of an active agent may be reduced by increasing the sensitivity of the assay, which in some cases may result in activity for an agent that was previously found to be inactive. Underlying these efforts is the assumption that a cascade of molecular and biological events leads to uterine growth and that any of the presumed precursor events can act as surrogates for an increase in uterine weight. In this paper I explore three issues: *a*) whether such surrogate markers are, in fact, more sensitive than gravimetric analysis of the uterus, *b*) whether they are needed to predict the estrogenicity of chemicals, and *c*) whether they are reliable and suitable for general adoption.

One difficulty with these issues is that few investigators have rigorously compared markers of uterine growth with changes in uterine weight in the same study. In a recent study, Freyberger et al. (*2*) reevaluated the reported lack of uterotrophic activity of resveratrol (*3*) and failed to demonstrate any activity using the variety of markers of uterine growth evaluated. However, the largest database available relates to multiple evaluations of bisphenol A (BPA). Most of these studies were initiated in an attempt to reconcile reports of the weak uterotrophic activity of BPA and its lack of effects in multigenerational rodent studies, with conflicting reports of its ability to influence the sexual development and maturation of rodents at much lower doses (*4,5*). In particular, in a recent evaluation of BPA, Markey et al. (*5*) noted that the induction of premature vaginal opening by BPA occurred at a dose 1,000 times lower than the dose at which a uterotrophic response was seen. This led these investigators to question the usefulness of the uterotrophic assay. In this paper, I will discuss many of the available BPA assays in the uterus, beginning with the rat studies.

BPA has been established as a weak uterotrophic agent in most of the studies conducted in the rat (*6–12*). The first study in which surrogate markers were evaluated was by Gould et al. (*10*), who reported that orally administered BPA gave a negative uterotrophic response in the immature rat when tested to the limit of its solubility in oil (150 mg/kg/day; Figure 1). However, Gould et al. (*10*) observed changes in the levels of estrogen-responsive uterine protein peroxidase (Per) and the progesterone receptor (PR) at several doses of BPA that were evaluated. The combined data reported by Gould et al. (*10*), together with the positive uterotrophic activity of BPA in immature rats concomitantly reported by Ashby and Tinwell (*9*), are shown in Figure 1. The two sets of uterotrophic data are probably consistent with each other and form part of the same dose–response relationship. The increases in Per and PR seen at the higher doses also seem to be a part of the same uterotrophic dose response, but the induction patterns of these two proteins at lower doses of BPA are ambiguous. First, the Per activity shows a significant reduction at the lowest dose tested, in contrast to the increases seen at the upper two doses, an effect that may be due to chance. Second, the induction of PR was significantly elevated across a plateau embracing the five lower doses of BPA evaluated. This absence of a dose response for PR induction is inexplicable and raises questions as to its biological significance and its no-observed-effect level (NOEL).

Steinmetz et al. (*11*) studied the ability of BPA to increase hyperplasia and the expression of c-*fos* in the uterus of the rat after the intraperitoneal injection of BPA. The NOELs for increases in uterine weight and uterine hyperplasia (both determined 20 hr after the final administration of BPA) were 100 mg/kg and 30 mg/kg, respectively; the NOEL for c-*fos* expression was 3 mg/kg (determined 2 hr after the final administration of BPA). However, c-*fos* induction suffers from being a nonspecific marker, as

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Received 5 March 2001; accepted 25 April 2001.

shown by its failure to correlate with the strain-specific induction of vaginal DNA synthesis in BPA-exposed rats (*13*). More recently, Laws et al. (*6*) reported positive uterotrophic activity for doses of 200 and 400 mg/kg BPA (oral gavage) in immature rats and a negative uterotrophic response for 100 mg/kg BPA. Laws et al. (*6*) also reported no advances in vaginal opening in immature rats exposed to doses of 100–400 mg/kg BPA. These data indicate that uterotrophic activity for BPA can consistently be detected in the rat only at doses > 100 mg/kg and that effects on uterine c-*fos* gene expression and peroxidase activity can be detected at doses up to approximately 30 times lower. However, the reproducibility, consistency, and biological significance of the changes in effects of c-*fos* expression and peroxidase activity remain to be established.

In the first uterotrophic assay of BPA in the mouse, Coldham et al. (*14*) used immature CFLP mice and subcutaneous (sc) injections of the test agent; results were negative. However, four additional and more extensive mouse uterotrophic assays of BPA were reported in isolation of each other over the past year (*5,15–17*). Papaconstantinou et al. (*15*) used ovariectomized B6C3F$_1$ mice and reported results for the dose range of approximately 1–400 mg/kg BPA (sc injection; doses expressed per mouse, with no body weights provided). Positive uterotrophic activity was observed at ≥ 40 mg/kg BPA. At the uterotrophic dose of 200 mg/kg BPA, uterine stromal and myometrial thickness and epithelial cell height were increased (*15*). However, the absence of data for these histopathologic parameters at the lower doses precludes assessment of their sensitivity relative to changes in uterine weight. In the earliest of the three immature mouse uterotrophic assays, Mehmood et al. (*16*) treated CD-1 mice with a range of potent synthetic estrogens and compared the uterotrophic responses of the mice to the induced changes in the expression of lactoferrin and Per and in the incorporation of bromodeoxyuridine (BrdU) into the luminal epithelium of the uterus. For most of these reference estrogens, the cellular end points studied were of similar or greater sensitivity than changes in uterine weight. However, Mehmood et al. (*16*) concluded that BrdU labeling of the stromal myometrium is difficult to quantify and that it is probably not a practical assay end point. In addition, they noted occasional unexpected increases in BrdU labeling in the uterus of some of their control animals, which they suggested may reflect the early onset of puberty. They endorsed the use of lactoferrin as a practical assay end point, but presented only pooled data for lactoferrin induction, making it impossible to assess the data statistically. Nonetheless, this marker seemed to have similar sensitivity to the gravimetric end point (*16*). Mehmood et al. (*16*) also studied orally administered BPA over the dose range 0.01–1,000 mg/kg/day and concluded that it failed to *a*) increase uterine weight, *b*) reproducibly alter the level of expression of Per and lactoferrin, or *c*) increase BrdU incorporation into the uterus.

In the second immature mouse uterotrophic assays of BPA reported, Tinwell et al. (*17*) described the results of nine independent uterotrophic assays in the AP mouse covering the dose range 0.02–300 mg/kg BPA (eight assays using sc injection and one using oral gavage). The reason so many experiments were performed was that signs of weak uterotrophic activity were observed for BPA at some dose levels, but in general, these observations were not confirmed in repeat experiments. In three of the experiments, uterine cell height was assessed and BrdU labeling of the uterus was performed; the results of these studies are shown in Table 1. Study 8 (Table 1) showed statistically significant, but weak, uterotrophic activity for a dose of 5 mg/kg BPA (21% increase in uterine weight) and for doses of ≥ 50 mg/kg BPA (up to 26% increases), but not at 10 mg/kg BPA (8% increase). There was some support for these activities from the BrdU-labeling data, but not from the cell height data. Increases in BrdU labeling were observed for 200 and 300 mg/kg BPA in the oral study (study 9; Table 1), but in the absence of a significant uterotrophic response (maximum increase in uterine weight of 14% at 300 mg/kg BPA). Tinwell et al (*17*) observed an isolated, weak, but statistically significant increase in the BrdU labeling index of the stromal layer for 5 mg/kg BPA (Study 9; Table 1). Overall, these mouse data for BPA (*15–17*) do not support the measurement of uterine cellular compartment dimensions or labeling indices

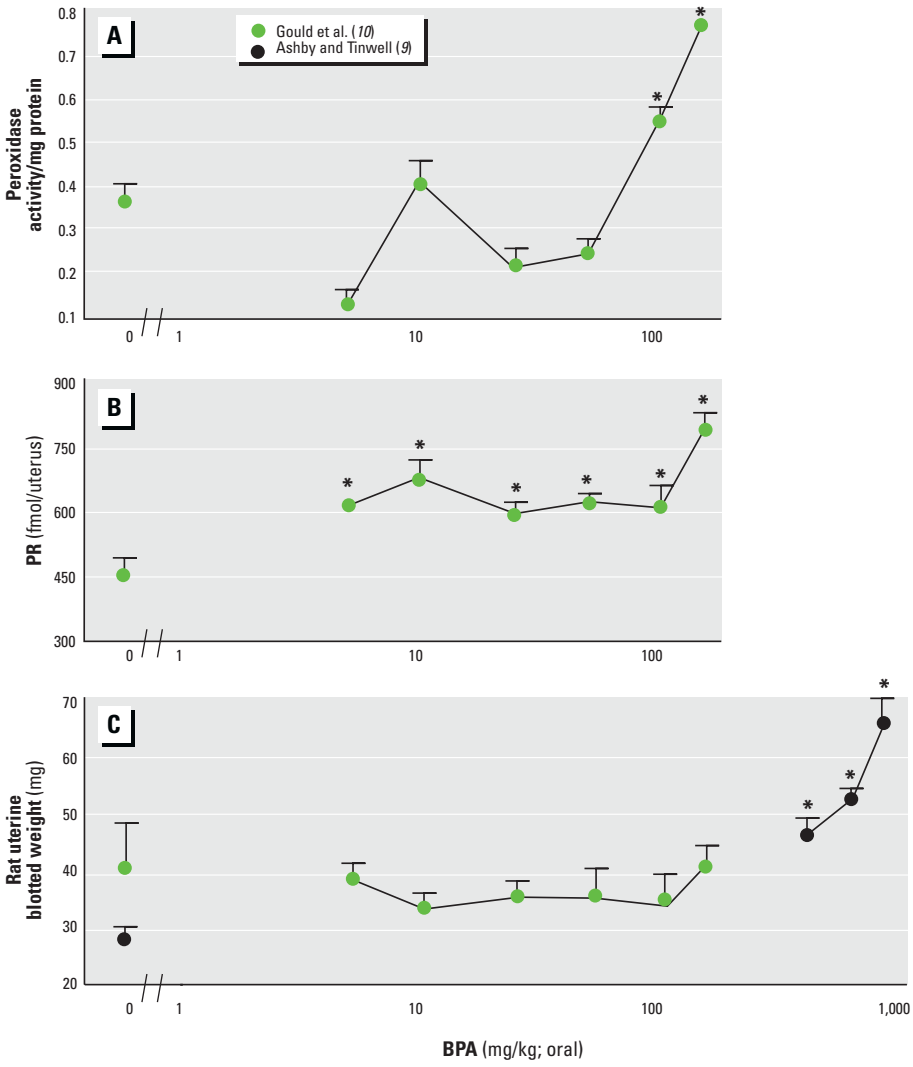

Figure 1. Data for BPA in the rat uterus as reported by Gould et al. (*10*) and Ashby and Tinwell (*9*).
*Statistically significantly different from control values.

as providing major improvements in the sensitivity of the uterotrophic assay for BPA.

The most recent immature mouse uterotrophic assay of BPA was reported by Markey et al. (*5*) in the CD-1 mouse. The chemical was delivered by osmotic pumps implanted into the subcutis; this method was estimated to yield exposures of between 0.1 and 100 mg/kg/day BPA (data summarized in Figure 2 and Table 2). The authors concluded that although BPA was only active in the uterotrophic assay at the 100 mg/kg dose, it induced cellular and other changes at lower doses. There was general concordance between the gravimetric uterotrophic data and the changes in epithelial cell height, luminal and glandular labeling [determined using proliferating cell nuclear antigen (PCNA), a method that the authors believe is not as accurate as BrdU], and lactoferrin expression over the dose range of 75–100 mg/kg BPA; isolated morphometric effects were observed at 5 mg/kg BPA (Figure 2, Table 2).

Markey et al. (*5*) also reported an advance in the mean day of vaginal opening for the lowest and the highest doses of BPA evaluated (Figure 2, Table 2); however, these effects are probably not biologically significant. First, the group sizes for were low (five and six mice observed, respectively). Second, a surprisingly high proportion of the control mice had open vaginas at the end of the study (19/48; 40%) and the mean control uterine weights were unusually high (19 mg), both of which may be explained by the study starting at the unusually late time of postnatal day 23. Together, these data indicate that the mice used by Markey et al. (*5*) were entering puberty by the end of their study, which weakens the conclusions drawn. Further, if BPA did not advance vaginal opening in the mouse, it would be consistent with the absence of such an effect in the rat (*6*). Thus, none of the effects that Markey et al. (*5*) reported for BPA in the mouse uterus give unequivocal and consistent indications that the surrogate markers used were more sensitive indicators of estrogencity than are changes in uterine weight.

The first issue I raised in this paper was whether the uterine cellular or molecular surrogate markers currently under study can provide a more sensitive indicator of the estrogenicity of chemicals than do changes in uterine weight. This remains an important area of investigation; however, further detailed dose–response studies using reference estrogens such as diethylstilbestrol and genistein will be needed before this question can be answered. The data discussed herein for resveratrol and BPA do not support the need for surrogate markers, but a larger database is required. The fact that all of the surrogates evaluated to date are expensive and cumbersome increases the need to answer this question unequivocally.

The second issue I raised was the relevance and need for surrogate markers. One way to assess this is to compare the uterine tissue responses to a chemical to the chemical's activity in multigenerational studies. In the case of BPA, no reproductive or developmental effects were observed at doses < 500 mg/kg BPA in dietary or gavage multigenerational studies in the rat (*18,19*), which accords well with the weak uterotrophic activity of BPA in the rat. However, comparing the LOEL of BPA in the uterotrophic assay (approximately 100 mg/kg) to reports of its endocrine activity at much lower doses (*4,5*) shows that there is a need for potentially more sensitive surrogates for the uterotrophic assay, a need not met by the markers considered in this analysis. In the case of BPA, therefore, the surrogate markers evaluated have not fulfilled their promise.

The third issue I raised was the reliability and practicality of the surrogate markers currently under study. As a general point,

Table 1. Data for three mouse uterotropic assays of BPA in which markers of uterine growth were also evaluated.

Experiment No.	Assay parameter	BPA (mg/kg) 0.02	0.2	0.5	1	5	10	50	100	200	300
6 (sc)	Uterine blotted wt	–	–							**	
	Epithelial LI	–	–							**	
	Glandular LI	–	–							**	
	Stromal LI	–	–							**	
	Epithelial height	–	–							–	
	Endometrial height	–	–							–	
8 (sc)	Uterine blotted wt		–	–	–	**	–	**	**	**	
	Epithelial LI		–	–	–	*	–	**	**	**	
	Glandular LI		–	–	–	–	–	**	*	**	
	Stromal LI		–	–	–	–	–	*	–	–	
	Epithelial height		–	–	–	–	–	–	–	*	
	Endometrial height		–	–	–	–	–	–	–	–	
9 (oral)	Uterine blotted wt			–	–	–	–	–	–	–	–
	Epithelial LI			–	–	–	–	–	–	**	**
	Glandular LI			–	–	–	–	–	–	–	**
	Stromal LI			–	–	*	–	–	–	**	**
	Epithelial height			–	–	–	–	–	–	–	–
	Endometrial height			–	–	–	–	–	–	–	–

Abbreviations: –, no change; LI, labeling index as measured in BrdU-stained cells; wt, weight. Empty boxes represent values not determined. Data from Tinwell et al. (*17*).
*$p < 0.05$; **$p < 0.01$.

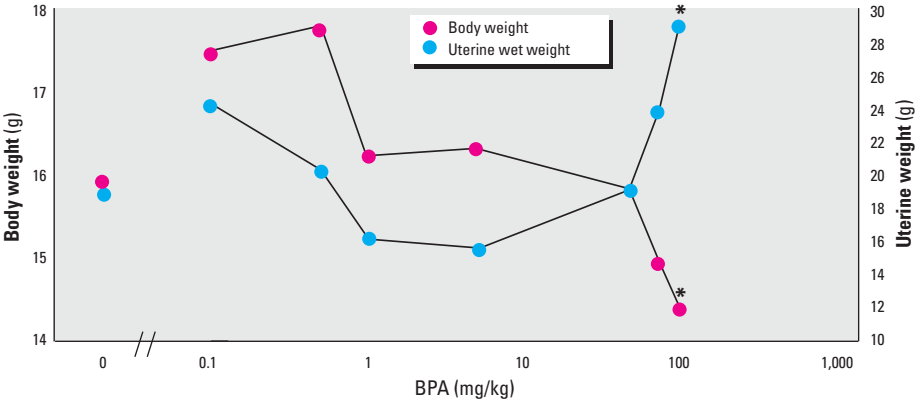

Figure 2. Mouse body weight and uterine wet weight data for BPA as reported by Markey et al. (*5*).
*Statistically significant changes.

Table 2. Mouse uterine data for BPA as reported by Markey et al. (*5*).

Parameter	BPA (mg/kg) 0.1	0.5	1	5	50	75	100
Vaginal opening	+	–	–	–	–	–	+
Epithelial cell height	–	–	–	+	–	+	+
Lamina propria area	–	–	–	+	–	–	–
Luminal and glandular epithelial LI	–	–	–	–	–	+	+
Lactoferrin expression	–	–	–	–	–	+	+

Abbreviations: –, no change; +, change; LI, labeling index (PCNA).

technical considerations complicate the use of many of the markers. For example, although protein levels (such as for PR using Western blotting or competitive binding) can be determined using uterine tissue collected up to 24 hr after the final administration of the test agent (as is usual with the uterotrophic assay), the optimum time of determination of mRNA levels will be influenced by the half-lives of individual messages; this may be as early as 2 hr after dosing (*11,13*). In fact, there may be no single optimum sampling time that embraces all of the possible surrogate end points for the uterotrophic assay. In addition, the accurate quantification of protein/mRNA levels adds a further level of complexity, which in the case of mRNA changes will involve the use of real-time polymerase chain reaction. Set against these problems is the fact that gravimetric analysis of the uterus sometimes also yields conflicting or weak responses, as illustrated by the results observed with the nine mouse uterotrophic assays of BPA reported by Tinwell et al. (*17*).

Finally, the future use of cellular or molecular markers will focus the potential difference between the no-observed-adverse-effect level (NOAEL) of a chemical and its NOEL. In time, it may be possible and necessary to differentiate the expression of estrogen-regulated genes associated with the eventual appearance of an adverse effect from the expression of those that merely give nonspecific evidence of chemical exposure. In this connection, the assumption that all estrogen-dependent changes induced in the uterus by a chemical form part of a continuous cascade, ending in uterine growth, is challenged by the observation that certain synthetic estrogens, but not estradiol, induce the expression of lactoferrin in uterine epithelial cells of the estrogen receptor (ER)-knockout mice (*20,21*). This observation indicates an estrogen-signaling pathway in the mouse uterus that is independent of both ERα, and ERβ (*20,21*). Until such uncertainties are resolved, I suggest that the gravimetric uterotrophic assay should remain a reference tier-1 assay for assessment of the estrogenic activity of xenobiotic chemicals. The practicality of the assay remains its primary strength, and although estrogenic effects of a different magnitude may be produced by chemicals in tissues other than the uterus [due to possession by the chemical of selective estrogen receptor modulating (SERM) activity], it is interesting to note that raloxifene, a SERM reported not to affect the uterus (*22*), gives a positive uterotrophic response (*23*). Nonetheless, all biological end points, including the uterotrophic assay, have intrinsic fragility, as evidenced by the demonstration that changes in the nutritional status of immature rodents can initiate premature uterine growth via centrally mediated mechanism (*24*).

Perhaps the greatest current need is for agreement on criteria for concomitantly comparing the relative sensitivity of individual assay end points. For example, although Newbold et al. (*25,26*) recently concluded that assessment of gland number and lactoferrin expression in the mouse uterus enhances the sensitivity of the uterotrophic assay, it was unclear about which of the data sets presented were concomitantly determined and which were mechanistically coupled (*27*).

References and Notes

1. Kanno J, Onyon L, Haseman J, Fenner-Crisp P, Ashby J, Owens W. The OECD program to validate the rat uterotrophic bioassay to screen compounds for *in vivo* estrogenic responses: Phase 1. Environ Health Perspect 109:785–794 (2001).
2. Freyberger A, Hartmann E, Hildebrand H, Krotlinger F. Differential response of immature rat uterine tissue to ethinylestradiol and the red wine constituent resveratrol. Arch Toxicol 74:709–715 (2001).
3. Ashby J, Tinwell H, Pennie W, Brooks AN, Lefevre PA, Beresford N, Sumpter JP. Partial and weak oestrogenicity of the red wine constituent resveratrol: consideration of its superagonistic activity in MCF-7 cells and its suggested cardiovasular protective effects. J Appl Toxicol 19:39–45 (1999).
4. Ashby J. Getting the problem of endocrine disruption into focus: the need for a pause for thought. APMIS 108:805–813 (2000).
5. Markey CM, Michaelson CL, Veson EC, Sonnenschein C, Soto AM. The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A. Environ Health Perspect 109:55–60 (2001).
6. Laws SC, Carey SA, Ferrel JM, Bodman GJ, Cooper RL. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol Sci 54:154–167 (2000).
7. Dodds EC, Lawson W. Synthetic oestrogenic agents without the phenanthrene nucleus. Nature 137:996 (1936).
8. Ashby J, Odum J, Paton D, Beresford NA, Sumpter JP. Re-evaluation of the first synthetic estrogen and bisphenol-A using both the ovariectomised rat model used in 1933 and additional assays. Toxicol Lett 115:231–238 (2000).
9. Ashby J, Tinwell H. Uterotrophic activity of bisphenol A in the immature rat. Environ Health Perspect 106:719–720 (1998).
10. Gould JC, Leonard LS, Maness SC, Wagner BL, Conner K, Zacharewski T, Safe S, McDonell DP, Gaido KW. Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. Mol Cell Endocrinol 142:203–214 (1998).
11. Steinmetz R, Mitchner NA, Grant A, Allen DL, Bigsby RM, Ben-Jonathon N. The xenoestrogen bisphenol A induces growth differentiation and c-fos gene expression in the female reproductive tract. Endocrinology 139:2741–2747 (1998).
12. Mathews JB, Twomey K, Zacharewski TR. In vitro and in vivo interactions of Bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. Chem Res Toxicol 14:149–157 (2001).
13. Long X, Steinmetz R, Ben-Jonathan N, Caperell-Grant A, Young PCM, Nephew KP, Bigsby RM. Strain differences in vaginal responses to the xenoestrogen bisphenol A. Environ Health Perspect 108:243–247 (2000).
14. Coldham NG, Dave M, Sivapathasundaram S, McDonnell DP, Connor C, Sauer MJ. Evaluation of a recombinant yeast cell estrogen screening assay. Environ Health Perspect 105:734–742 (1997).
15. Papaconstantinou AD, Umbreit TH, Fisher BR, Goering PL, Lappas NT, Brown KM. Bisphenol A-induced increase in uterine weight and alterations in uterine morphology in ovariectomised B6C3F1 mice: role of the estrogen receptor. Toxicol Sci 56:332–339 (2000)
16. Mehmood Z, Smith AG, Tucker MJ, Chuzel F, Carmichael NG. The development of methods for assessing the in vivo oestrogen-like effects of xenobiotics in CD-1 mice. Food Chem Toxicol 38:493–501 (2000).
17. Tinwell H, Joiner R, Pate I, Ashby J. Uterotrophic activity of Bisphenol A (BPA) in the immature mouse. Regul Toxicol Pharm 32:118–126 (2000).
18. Ema M. Two-Generation Reproduction Study of Bisphenol A in Rats. Final Report. Study No SR-98101. Hokkaido, Japan:Chemical Compound Safety Research Institute, 2000.
19. Tyl R, Myers C, Marr M, Chang T, Seely J, Brine D, Veselica M, Fail P, Joiner R, Butala J, et al. [Abstract]. Three-generation reproductive toxicity study of bisphenol A (BPA) administered in the diet to CD(R) (Sprague-Dawley) rats. Toxicologist 60:297 (2001).
20. Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lublahn DB. Estrogenic responses in estrogen receptor-alpha deficient mice reveal a distinct estrogen signaling pathway. Proc Natl Acad Sci USA 94:12786–12791 (1997).
21. Ghosh D, Taylor JA, Green JA, Lubahn DB. Methoxychlor stimulates estrogen-responsive mRNA in mouse uterus through a non-estrogen receptor mechanism. Endocrinology 140:3526–3533 (1999).
22. Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, Cullinan GJ, Bendele R, Kauffman FG, Bensch WR, et al. Raloxifene prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J Clin Invest 93:63–69 (1994).
23. Ashby J, Odum J, Foster P. Activity of raloxifene in immature and ovariectomized rat uterotrophic assays. Regul Toxicol Pharmacol 25:226–231 (1997).
24. Ashby J, Tinwell H, Odum J, Kimber I, Brooks AN, Pate I, Boyle CC. Diet and the aetiology of temporal advances in human and rodent sexual development. J Appl Toxicol 20:343–347 (2000).
25. Newbold RR, Jefferson WN, Padilla-Banks E, Walker VR, Pena DS. Cell response endpoints enhance sensitivity of the immature mouse uterotrophic assay. Reprod Toxicol 15:245–252 (2001).
26. Corrigendum. Corrigendum to "Cell response endpoints enhance sensitivity of the immature mouse uterotropic assay" [Reprod Toxicol 15 (2001) 245–252]. Reprod Toxicol 15:465–466 (2001).
27. Korach KS, Levy LA, Sarver PJ. Estrogen receptor stereochemistry: receptor binding and hormonal responses. J Steroid Biochem 27:281–290 (1987).