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

John Ashby
Syngenta Central Toxicology Laboratory, Macclesfield, Cheshire, UK

The rodent 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 increasing 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
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 B6C3F1 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 estrogenicity 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) |
|----------------|-----------------|-------------|
| A (mg/kg) | | |
| Uterine blotted wt | — | — | — | — | — | — | — | — | — | — |
| Epithelial LI | — | — | — | — | — | — | — | — | — | — |
| Glandular LI | — | — | — | — | — | — | — | — | — | — |
| Stromal LI | — | — | — | — | — | — | — | — | — | — |
| Epithelial height | — | — | — | — | — | — | — | — | — | — |
| Endometrial height | — | — | — | — | — | — | — | — | — | — |
| B (mg/kg) | — | — | — | — | — | — | — | — | — | — |
| Uterine blotted wt | — | — | — | — | — | — | — | — | — | — |
| Epithelial LI | — | — | — | — | — | — | — | — | — | — |
| Glandular LI | — | — | — | — | — | — | — | — | — | — |
| Stromal LI | — | — | — | — | — | — | — | — | — | — |
| Epithelial height | — | — | — | — | — | — | — | — | — | — |
| Endometrial height | — | — | — | — | — | — | — | — | — | — |
| C (mg/kg) | — | — | — | — | — | — | — | — | — | — |
| 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).

*Statistically significant changes.

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

| Parameter | 0.1 | 0.5 | BPA (mg/kg) |
|-----------|-----|-----|-------------|
| 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 practicability 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 uterotropic 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 end point assays. 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).

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