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Topic 3.15

Endocrine disruption occurring at doses lower than those predicted by classical chemical toxicity evaluations: The case of bisphenol A*

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Abstract: The meaning of the term "low dose" is discussed in relation to endocrine toxicity data for chemicals. Consideration is also given to experimental conditions likely to impinge on the interpretation and extrapolation of such low-dose effects, and the importance of gathering appropriate control data is emphasized. In the specific case of bisphenol A (BPA), it is concluded that despite the extensive endocrine disruptor (ED) database available for this chemical, it is still not possible to locate a single study that passes the most rudimentary scientific requirements—that the observations are capable of independent confirmation. Two possible explanations for this are considered. First, that BPA possesses subtle low-dose ED toxicities that only become evident under certain undefined experimental conditions. Until these conditions are defined and understood, it will be a matter of chance what individual investigators observe experimentally for BPA or any other chemical. Second, that the general failure of investigators to define and understand natural variability among control parameters monitored in ED studies allows artefactual positive results to be encountered for chemicals, especially in limited and nonreproduced studies. Whichever of these conclusions is correct, the positive low-dose data currently available for BPA cannot be extrapolated to humans with any confidence.

INTRODUCTION

Evaluation of the possibility that one or more environmental factors may be adversely affecting the reproductive capacity of humans and wildlife has been severely hampered by inadequate epidemiological evidence for induced effects, and by the absence of agreed and developed test methods and experimental data. Consequently, disparate experimental data have been published in the absence of a unified approach, and the field has been assailed by controversy, sinking, in some instances, to overt invective. The most controversial of issues, and the subject of this review, has been whether certain endocrine disruptors (EDs) are able to induce adverse effects at dose levels below those expected based upon the results of classical reproductive and developmental toxicity evaluations. Allied to this question is the suggestion that these "low-dose" toxicities follow an inverted-U shaped dose response, thus explaining the absence of effects at the "higher" doses used in the classical toxicity evaluations. The controversy that followed the initial report of such effects has centered on whether the effects reported can be independently reproduced, and more specifically, whether they are of general biological significance, or are limited to highly specific and undefined conditions of the experimental model used to establish them.

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In attempting here to summarize the low-dose debate, two quotations from a recent book by Stephen Jay Gould [1] enable the discussion to be placed in a larger scientific context:

> "Many supposed debates in science arise from confusion engendered by differing uses of words, and not from deep conceptual muddles about the nature of things."

> "Replication 'with difference' builds the best case for a generality—for how can we prove a coordinating hypothesis unless we can apply it to multiple cases?"

Specific answers are not provided to the many questions posed in this article, but the rehearsing of these questions should aid their resolution. Primary attention is focused here on bisphenol A (BPA) as this chemical has the largest available low-dose ED database.

DISCUSSION

This review describes the underlying scientific issue awaiting resolution before a clear and scientifically justified position can be taken on low-dose endocrine toxicities. The issues raised are discussed in the context of a series of figures and tables.

Meaning of the terms "low-dose effects" and "inverted-U" dose response

The schematic shown in Fig. 1 demonstrates the paucity of human data on EDs. In contrast, there are well-established carcinogen databases for both humans and rodents, enabling firm correlations of activities to be established. The absence of a corresponding human database for EDs forces reliance on data derived from rodent studies. Most rodent ED data are derived from classical toxicology evaluations using dose levels related to the maximum tolerated dose (MTD) for the chemical. These studies are then used to define a no adverse effect level (NOAEL) for the chemical. There are three situations where one can consider the possibility of effects occurring below this NOAEL level. First, there is the possibility that a study with larger group sizes, and a higher resolving power, might have led to the observation of effects. This possibility applies to all toxicological evaluations. A more interesting possibility is that monitoring precursor events associated with the adverse effect in question might lower the NOAEL. This concern is also not unique to EDs. For example, it has yet to be considered whether the induction

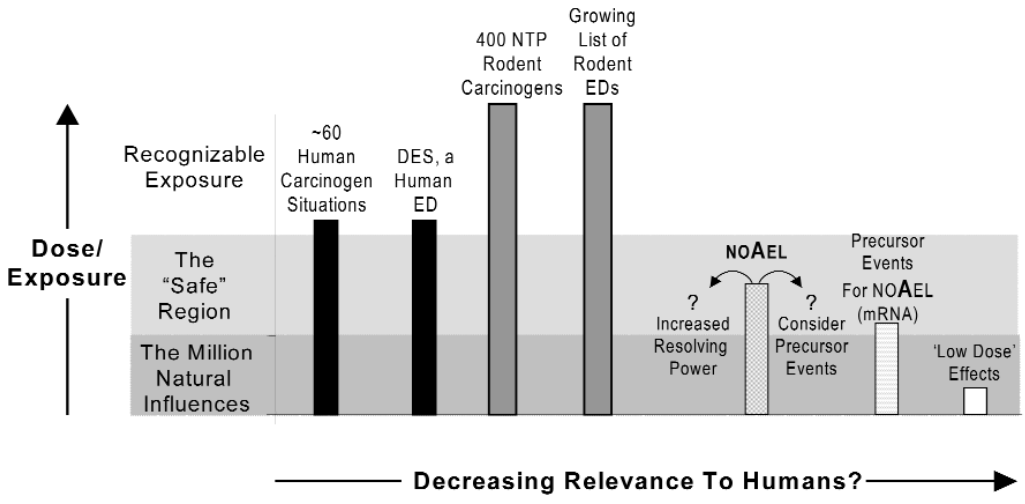

Fig. 1 Basis for considering an effect to be observed at a "low dose".

of subtle pathological changes in the rodent liver, such as the appearance of enzyme-altered foci, at subcarcinogenic dose levels, should contribute to an assessment of the NOAEL for the liver carcinogen in question. Consideration of such questions hinge on the definition of an adverse effect, as opposed to an observed effect. The advent of genomic techniques will increase the need to answer such questions, as there one is faced with changes to the mRNA levels associated with a precursor to an adverse effect. General consideration by toxicologists of low-dose ED effects for chemicals was triggered by observations reported [2]. In that case, effects on the mouse prostate gland were reported at dose levels orders of magnitude lower (20 μg/kg) than would have been suggested by the standard toxicology data for this chemical (5 mg/kg; discussed later). Although these data [2] are often referred to as representing an inverted-U dose response there were, in fact, only two doses evaluated. These data, therefore, raise the prospect of a surge of ED activity for BPA within the region lying below its classical NOAEL and within the area of natural biological variability (for example, circadian rhythms, transitory changes after eating).

The concept of low-dose "inverted-U", and "U-shaped" dose–response relationships is not novel (reviewed in [3]), but to date there are few, if any, confirmed and agreed examples within the toxicology literature. Perhaps the most compelling example of a U-shaped dose response is provided by the data of Almstrup et al. [4]. These authors showed that certain phytoestrogens are able to inhibit the testosterone initiated proliferation of MCF7 cells at low doses, by inhibiting the conversion of testosterone to estradiol via aromatase enzymes, while stimulating proliferation at higher dose levels due to the intrinsic estrogenic activity of the phytoestrogens. The issue of relevance to this discussion is whether the effects reported for BPA are of general significance, because if they are, a new approach to establishing NOAEL levels for chemicals will be required. An assessment of the total available low-dose database for BPA is given later herein.

Integration of all relevant data

Consideration of low-dose chemical toxicities form only one possible component of current attempts to discern which environmental influences are associated with the adverse effects that are driving the science of endocrine disruption—effects such as the current incidences of human prostate, testicular, and breast cancer. To study chemical contaminants in isolation will delay resolution of these key underlying questions. The hypothesis that milk consumption may be associated with human prostate cancer (Fig. 2) [5] and the observation of reduced human sperm motility following the Kobe earthquake [6] provide two examples of the many complex issues underlying the questions being addressed in the field of ED.

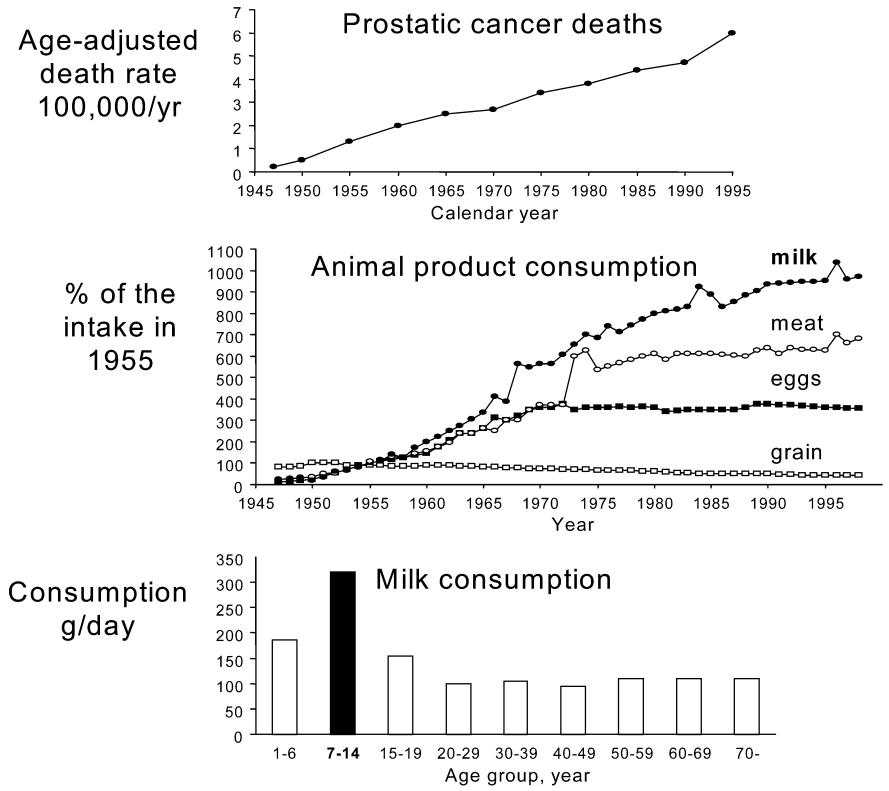

Fig. 2 Possible correlations between increases in prostate cancer in Japan and increases in milk consumption over the same period [5].

Consideration of the conditions under which experimental data are generated

Although the route of administration adopted in an ED study is rarely discussed by investigators, it has the power to affect seminally interpretation and extrapolation of the derived data. This can be illustrated by the data shown in Figs. 3 and 4. The data in Fig. 3 illustrate the highly significant observation that equally effective doses of diethylstilbestrol (DES), and the phytoestrogen genistein (GEN) in the immature mouse uterotrophic assay are also associated with equally effective carcinogenic responses in the mouse uterus [7]. The mouse data were extrapolated to infants consuming soy-based infant formula by the authors [7]. However, in terms of risk assessment, it is critical to note that the data shown in Fig. 3 were all generated using the subcutaneous route of administration, while infants are exposed to soy formula by the oral route. The uterotrophic activities of GEN in immature rodent uterotrophic assays shown in Fig. 4 illustrate the critical importance of the route of exposure selected; in particular, the relative insensitivity of the oral route. Thus, data generated for GEN using the subcutaneous injection route is of little value when estimating potential hazards posed by oral exposure to this agent. The general point intended here is that it is inappropriate to sum together data for a chemical generated using a variety of routes of exposure—but such is a common practice in ED risk assessment, as discussed later in relation to the low-dose ED effects reported for BPA.

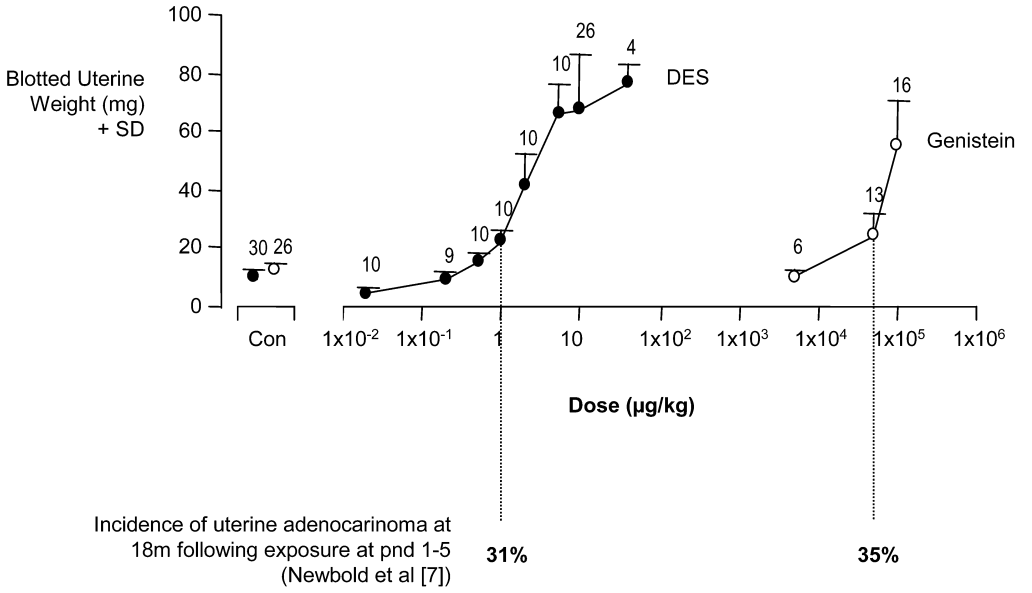

Fig. 3 Dose levels that yield equal levels of activity for DES and GEN in the immature mouse uterotrophic assay ([58] and Ashby et al., unpublished, 2002) are also associated with the induction of equal incidences of uterine adenoma in the mature mouse when exposed on postnatal days 1–5 [7].

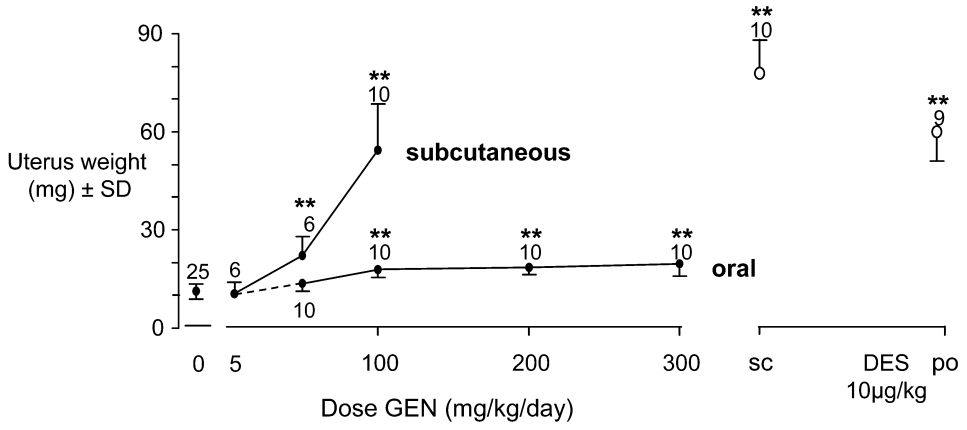

Fig. 4 Relative activity of GEN and DES in the immature mouse uterotrophic assay using either oral or subcutaneous routes of exposure [59].

Precursors of adverse effects

It is probable that precursor events to a toxic response will occur at dose levels below the NOAEL for the adverse toxic effect under study. This is illustrated by the data shown in Fig. 5. In the studies referred to therein, Gould et al. [8] confirmed the absence of uterotrophic activity for BPA in the immature rat at dose levels previously reported as inactive [9]. However, other effects were observed by Gould et al. in the treated uteri, despite the absence of uterine growth. Whether or not these changes are associated with the "beginnings" of an uterotrophic response, or with parallel phenomena, remains to be determined. Such questions will increasingly be posed by the trend to generate genomic data at dose levels below the NOAEL dose for adverse effects in the tissue under study. Such precursor/parallel ef-

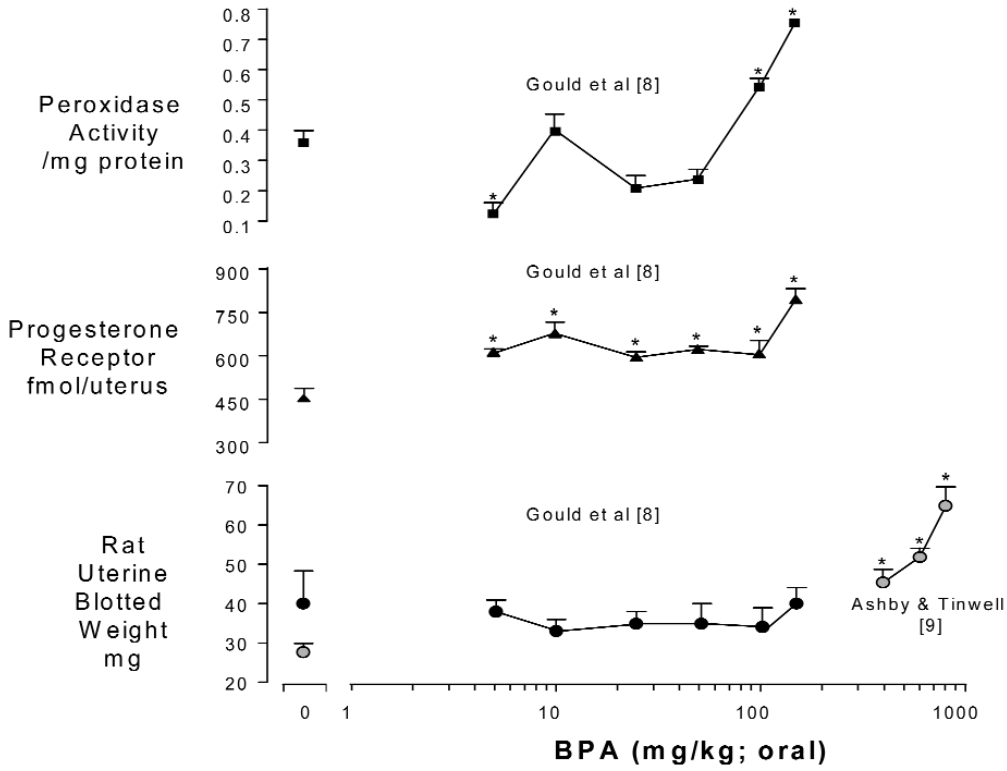

Fig. 5 Reported activities of BPA in the immature rat uterus.

fects are probably unconnected with the low-dose effects discussed for BPA in the next section, and the clear separation of these two low-dose effects will aid the interpretation of each of them in terms of human risk assessment.

"Low-dose" effects reported for BPA

The first report of low-dose effects induced by a chemical in the mouse prostate gland was for DES given orally [10]. Those data are shown in Fig. 6, together with mouse uterotrophic assay data for DES reported earlier ([11]; subcutaneous injection of DES and see Fig. 4). These increases in prostate weight were small and were not exceptional given that they were generated following exposure in utero, and that positive effects at similar dose levels were known for DES in the immature mouse uterotrophic assay (Fig. 4) [11]. Much more unexpected was the observation that BPA was as effective as DES at increasing mouse prostate gland weight (Fig. 7) [2] despite its essential inactivity in the mouse uterotrophic assay (oral or subcutaneous administration of BPA) [12]. Comparison of the effects shown in Figs. 6 and 7 reveals that BPA, a chemical generally considered to be a weak estrogen, is as active in the mouse prostate as is the reference and potent estrogen DES. It is this stark contrast of activities that has led to the many subsequent studies and discussions on BPA.

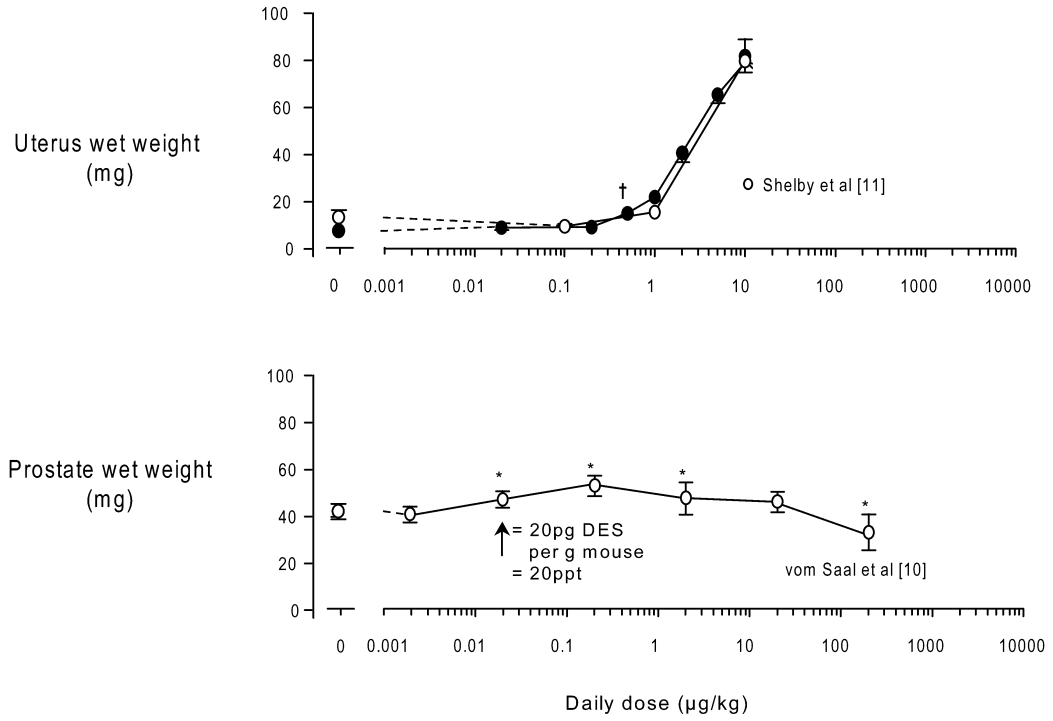

Fig. 6 Activity of DES in the immature mouse uterotrophic assay and comparison with changes in prostate weight induced following exposure in utero. *Reported as statistically significant.

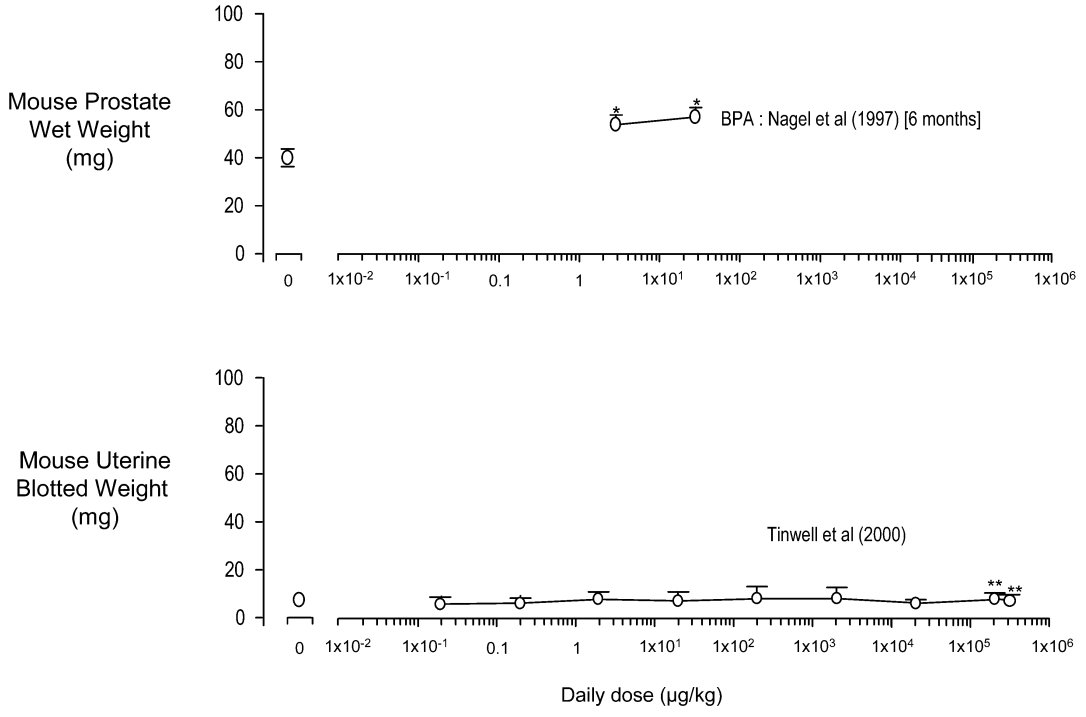

Fig. 7 Activity of BPA in immature mouse uterotrophic assay and comparison with changes in prostate weight induced following exposure in utero. *Reported as statistically significant.

Since those original studies on BPA many investigators have studied the ED effects of BPA in both mice (Table 1) and rats (Table 2). The rat multigeneration assay data for BPA [13,14] are generally considered by regulators to have defined its NOAEL at 5 mg/kg—against which value the low-dose effects reported for BPA can be assessed (unshaded areas of Tables 1 and 2).

Table 1 Reported activities of BPA in reproductive/developmental studies conducted in the mouse. Abstracts have been omitted. The shaded area of the table represents activities observed at or above the generally accepted NOAEL value for BPA in the rat [13,14].

Mouse strain	**Exposure period**	Route of admin.	Dose range	Min. positive dose	**Effects reported**	Ref.
CF_1	GD11–17	Oral	2 & 20 μg/kg	2 μg/kg	Increase in prostate & preputial gland wt. Decreased DSP & epididymal wt	[2,15]
CF_1	GD11–17	Oral	2.4 μg/kg	2.4 μg/kg	Increase in body wt; advance in age of 1st oestrus	[32]
CD-1	GD14–18 (in utero) ± GD14–18 (adult exposure during pregnancy)	Oral	10 μg/kg	10 μg/kg	Maternal behavior affected in mice exposed either in utero or during adulthood	[33]
ICR	GD11–17	s.c.	2 & 20 μg/kg	20 μg/kg	Advance in age at vaginal opening and at 1st estrus	[34]
CD-1	GD9–20	Implant	25 & 250 μg/kg	25 μg/kg	Increased mammary gland maturation	[35]
CD-1	GD16–18	Oral	50 μg/kg	50 μg/kg	Increased AGD & prostate wt; decreased epididymal wt	[36]
C57BL/6N	PND35–63 & PND35–91	DW	139 μg–12.7 mg/kg	139 μg/kg	Decreased testosterone; testicular morphology affected	[37]
ICR	GD11–17 PND1–5	s.c.	10 & 100 mg/kg	10 mg/kg 100 mg/kg	Decreased corpora lutea Increase vaginal stratification & polyovular follicles	[38]
CD-1	GD6–15	Oral	500–1250 mg/kg	500 mg/kg	Maternal & fetal toxicity	[39]
CD-1	Continuous breeding	Diet	300–1300 mg/kg	600 mg/kg	Reduction in testes wt and sperm motility	[40]
CF_1	GD11–17	Oral	0.2–200 μg/kg	–	No effects	[41]
CF_1	GD11–17	Oral	2 & 20 μg/kg	–	No effects	[24]
C57BL/6N	GD11–17; PND21–43; PND70–77	Oral	2–200 μg/kg	–	No effects	[42]

The top seven entries in Table 1 provide evidence of sub-NOAEL effects for BPA in the mouse. None of these data provide an independent confirmation of any of the other studies. Two of these studies are difficult to integrate with the others because they employed either subcutaneous injection or implantation of the BPA. The middle entries in Table 1 represent activities for BPA within its acknowledged active dose range. The final entries in Table 1 represent the results of three independent and unsuccessful attempts to confirm the original observations made for BPA [2,15]. One is therefore faced with a set of disparate observations made for low doses of BPA, and three unsuccessful attempts to confirm one of those observations. The strain of mouse used does not appear to be the critical determinant of activity for BPA (Table 1), nor does the diet employed (discussed further in [16]). Thus, the "generality of effect" mentioned earlier has yet to be established for low-dose effects of BPA in the mouse.

Table 2 Reported activities of BPA in reproductive/developmental studies conducted in the rat. Abstracts have been omitted except for the replicated studies by Welsch [51,52]. The shaded area of the table represents activities observed at or above the generally accepted NOAEL value for BPA in the rat [13,14].

Rat strain	**Exposure period**	Route of admin.	Dose range	Min. positive dose	**Effects reported**	Ref.
SD	GD6–21	Oral	20 µg–50 mg/kg	20 µg/kg	Multiple male and female parameters affect	[20]
SD	PND91–PND96	Oral	2 ng–200 mg/kg	20 µg/kg	Males only studied. Reduced daily sperm production at PND126	[21]
SD	GD1–PND21 GD14– PND6	Oral	40 µg/kg 400 µg/kg	40 µg/kg	Male and female play behavior affected	[18]
SD	Pregestation-PND21	Oral	40 µg/kg	40 µg/kg	Sexual activity impaired in males; sexual motivation and receptivity affected in females	[19]
SD	GD6–PND22	Oral	0.1 & 1.2 mg/kg	1.2 mg/kg	Altered patterns of estrous cyclicity	[43]
Wistar	Gestation & lactation	DW	~1.5 mg/kg	~1.5 mg/kg	Sexual differentiation of locus coeruleus and behavior affected; no effects on reproductive organs or sex hormones.	[44]
Wistar	GD8–birth	Implant	25 µg & 250 µg/kg	25 µg/kg	Differentiation pattern of periductal stromal cells of the ventral prostate affected	[17]
Wistar	PND2–12	s.c.	37 mg/kg	37 mg/kg	Reduction of epithelial cell height in efferent ducts only at PND18	[45]
Wistar	PND2–12	s.c.	37 mg/kg	37 mg/kg	Males only studied. Significant increase in testis wt & marked effects on pubertal spermatogenesis at PND18. Effects not evident at PND25. Increased testis wt at PND90–100	[46]
Wistar	PND2–12	s.c.	37 mg/kg	37 mg/kg	Increased plasma testosterone; increased germ cell volume/Sertoli cell at PND18	[47]
Wistar	PND22–32	s.c.	50 mg/kg	50 mg/kg	Males only studied. Increase in lateral prostate wt at PND120	[48]
F344	PND28–72	Diet	235–950 mg/kg	235 mg/kg	Significant decrease in seminalvesicle, dorso-lateral prostate, preputial glands, hypophysis and body wt; testicular toxicity at PND72	[49]
SD	2 generations	Oral	0.2 mg–200 mg/kg	–	Multiple parameters studied. AGD reduced in F1 generation, but considered not to be toxicologically significant by investigators.	[14]
SD	GD2–PND21	Oral	1 µg–10 mg/kg	–	Males studied. Significant increase in ventral prostate wt at PND177 but considered by investigators to be due to sampling design. Studied repeated by Welsch et al. 2001.	[50]
SD	GD2–PND21	Oral	1 µg–10 mg/kg	–	Females studies. No effects on developmental landmarks, estrous cyclicity, organ wts, fertility and fecundity observed (PND2–~10 months).	[51]
SD	GD2–PND21	Oral	1 µg–15 mg/kg	–	No effects observed	[52]

(*continues on next page*)

Table 2 (*Continued*).

Rat strain	**Exposure period**	Route of admin.	Dose range	Min. positive dose	**Effects reported**	Ref.
SD	3 generations	Diet	1 mg–500 mg/kg	–	Multiple parameters studied. Total live pups/litter and ovarian wt reduced, but considered by investigators to be due to toxicity. No effects at or below 5 mg/kg/day.	[13]
Wistar	Pregestation–PND22	Oral	4 μg–4 mg/kg	–	Males only studied. No effects at PND90 on tissue wts or developmental landmarks.	[53]
SD & AP	GD6–21	Oral	20 μg–50 mg/kg	–	No effects in male or female offspring	[22]
SD	PND91–PND96	Oral	20 μg–200 mg/kg	–	No effects on adult male sperm parameters	[23]
SD	PND1–5	s.c.	3 mg/kg	–	No effects in both sexes at PND21 and PND98	[54]
SD	GD11–PND20	Oral	3.2–32 mg/kg	–	No effects on both sexes (pubertal development in females only; male and female reproductive tissues PND180)	[55]
Wistar	PND2, 4, 6, 8, 10	s.c.	~10 mg/kg (0.5 mg/rat)	–	No effects of testicular wt; seminiferous tubule diameter; pituitary FSHb expression; inhibin expression in testes	[56]
AP	PND22–36; PND36–55	Oral	100–200 mg/kg	–	Males only studied. No effects on time of prepuce separation or wts of seminal vesicles, ventral prostate, testes or epididymides.	[57]
CD	GD6–15	Oral	160–640 mg/kg	–	No effects on fetus	[39]

The rat is the normal species used in chemical toxicity evaluation, and consequently, many ED studies on BPA in this species have been reported (Table 2). As is evident by the shaded area in Table 2, most of these studies gave negative results, including what are normally considered definitive rat two-generation [14] and three-generation [13] studies. Interest, therefore, centers on the four studies listed at the top of Table 2. These showed effects at dose levels well below the suggested NOAEL value of 5 mg/kg BPA and employed oral administration of the test chemical (one study is difficult to interpret due to use of an implant of BPA [17]). Two of the four studies [18,19] measured endpoints that are rarely encountered in toxicology (play behavior and sexual motivation, respectively) and are not assessed here. Thus, there are two "standard" ED toxicity evaluations of BPA in the rat [20,21]. Extensive studies aimed at confirming the first of these reports yielded uniformly negative results in two strains of rat, including the SD rats employed in the original publication [22].

Sakaue et al. [21] reported that exposure of 13-week-old SD rats to low doses of BPA for 6 days, with termination at 18 weeks, significantly reduced daily sperm production (DSP). Four separate attempts to confirm those observations yielded uniformly negative results [23]. Results from those four repeat studies, together with the data originally reported [21], are shown schematically in Fig. 8. The repeat studies initially employed RM3 diet, but two subsequent studies using Purina 5002, and a final study using CE2 diet (as used by Sakaue et al.) were also conducted. Discussions with Sakaue and coworkers ensured that every attempt was made to replicate the experimental conditions employed by Sakaue et al. Two points in Fig. 8 are of particular interest. First, Sakaue et al. [21] reported positive results from a repeat study. This is an unusual practice that adds weight to their observations. All of the test and control data reported by Ashby et al. [23] have DSP values within the range of the test data re-

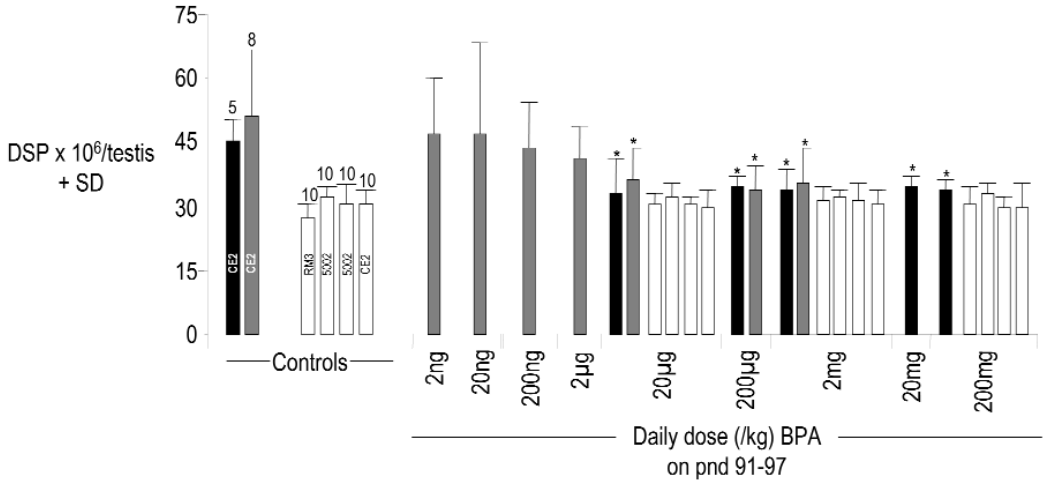

Fig. 8 Comparison of the daily sperm production (DSP) data published by Sakaue et al. [21] (black and gray columns) with those of Ashby et al. [23] (white columns). All data were generated in 13-week-old SD rats exposed orally to BPA for 6 days. Termination was at week 18. *Reported to be statistically significantly reduced by Sakaue et al. [21]. The control group sizes shown apply equally to all of the test.

ported by Sakaue et al. This suggests that an unaccountable difference in DSP control values between the two laboratories may be at the root of the divergence in test results for BPA.

Variation in ED parameter control values, both within and between laboratories, is emerging as one of the central issues in ED research. For example, similar arguments to those rehearsed above for the Sakaue repeat studies, have been made [16] to account for the failure [24] to confirm the original mouse prostate gland effects reported for BPA [2]. Variability in rat control testes weights has also been associated by Sharpe et al. [25] with the inability of several groups to confirm the reduction in rat testes weight induced by butyl benzyl phthalate [26]. In another case, similar problems with variability in control rat prostate gland weight were discussed [27] when attempting to confirm the data reported for nonylphenol (NP) [28]. A final example of this problem is the failure of Odum et al. [29,30] to confirm the reported activities [31] of NP and DES in the rat mammary gland. In that case, it was concluded that the use of a single control database over several independent studies in the initial report compromised the original observations. These several instances indicate that it would be profitable to understand, and then control, the several possible sources of control-variability for the major endpoints studied in ED. At present, this endeavor is being conducted ad hoc within a series of nonreplicated and often inconclusive chemical toxicity evaluations.

Despite the extensive ED database available for BPA it is still not possible to locate a single study that passes the most rudimentary scientific requirement—that the observations are capable of independent confirmation. Two explanations for this can be considered, as follows:

- BPA possesses subtle low-dose ED toxicities that only become evident under certain undefined experimental conditions. Until these conditions are defined and understood it will be a matter of chance what individual investigators observe experimentally for BPA or any other chemical.
- Failure to define and understand natural variability among control parameters monitored in ED studies allows artefactual positive results to be encountered for chemicals, especially in limited and unreproduced studies.

Which ever of these conclusions is correct, the positive low-dose data currently available for BPA cannot be extrapolated to humans with any confidence.

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