

## TOXICOLOGICAL HIGHLIGHT

# Is It Time to End Concerns over the Estrogenic Effects of Bisphenol A?

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For more than a decade, there has been a heated controversy over whether or not the environmental chemical, bisphenol A, exerts adverse estrogenic effects in animal studies, and by extrapolation, in humans. In the present issue of *Toxicological Sciences*, Ryan *et al.* (2009) publish a detailed study that throws cold water on this controversy by showing complete absence of effect of a range of bisphenol A exposures perinatally on reproductive development, function, and behavior in female rats. Will this help to resolve the controversy? Before considering details of the study, and their implications, it is appropriate to consider the backdrop to these studies and the fundamental scientific issues on which they impact. If, like me, you are uninvolved in bisphenol A research, you may be puzzled by the seemingly never-ending controversy, as the scientific process usually sorts out what is right, given time. So why has this not happened? Therefore, without wishing to detract in any way from the impact and importance of the study by Ryan *et al.* (2009), I have tried below to evaluate the wider (scientific) aspects of this topic and to emphasize how the present study contributes to resolving some of the controversial issues. Let me begin with a dose of scientific philosophy.

Anyone involved in biomedical research knows that scientific “facts” that remain untouched by the ravages of further research are a rare commodity. Usually, most facts change their form with time (evolve), and their meaning, and probably their importance, also will change. Disappointing as this may be for our scientific egos, it represents something far more important—scientific progress. It is inevitable that scientific progress, in the form of new facts, will trample over our bright ideas, hypotheses, and even over our results. This apparently destructive process is in fact constructive, and as scientists, we have to embrace this evolution and to accept that the sacrifice of our initial beliefs (and our precious results and

our interpretations) is part and parcel of advancing understanding. I spout this scientific philosophy as it seems to me that research on bisphenol A has been trying to go through this process but has become literally bogged down in the mire of controversy, much of which stems from the earliest findings and seems to have little to do with the current state of the science. The study by Ryan *et al.* (2009) can be considered as another attempt to release bisphenol A research from the grip of the mire back into the normal pathway of scientific evolution.

The results from Ryan *et al.* (2009) are unequivocal and robust and are based on a valid and rational scientific foundation. They tell us that, *in vivo* in female rats, bisphenol A is an extremely weak estrogen—so weak that even at levels of exposure 4000-fold higher than the maximum exposure of humans in the general population there are no discernible adverse effects, whereas the potent estrogen ethinyl estradiol (EE; the positive control) caused major adverse effects at doses used in earlier contraceptive pills and that were associated with increased risk of thromboembolism in women. In this particular study, the end points of interest were female reproductive development and function and several aspects of adult sex hormone-dependent behavior, but the results join with others from the same and other groups that show a similar absence of reproductive effects in male rats and mice when exposed orally to bisphenol A (Ema *et al.*, 2001; Howdeshell *et al.*, 2008; Tinwell *et al.*, 2002; Tyl *et al.*, 2002). These findings come from studies involving large numbers of animals and were conducted by recognized world expert groups. Yet, to judge from correspondence in the wider (nonscientific) media (blogs and environmental websites) on the publication by Ryan *et al.* (2009), this research is flawed and incorrect, for example, “because it uses an estrogen-insensitive rat strain,” which is patently not the case (see below). The latter argument has been used previously to “explain away” the lack of repeatability of many of the studies on bisphenol A (Myers *et al.*, 2009), although it seems unlikely that the rather minor differences in

strain sensitivity can account for how a compound as weakly estrogenic as bisphenol A can induce estrogen receptor (ER)-mediated effects *in vivo* as claimed (discussed further below).

One comment has suggested that because the study by Ryan *et al.* (2009) had earlier been submitted to another journal and then withdrawn (to undertake further studies suggested by reviewers) was an indication that it was fundamentally flawed. Clearly, such a comment cannot come from anyone who knows and respects the scientific peer review process. Rejections and requests for more data are an integral part of this process, and they ensure, as best as possible, that any deficiencies in design, execution, or interpretation get spotted and ironed out; if they cannot be ironed out, then the manuscript will not get published. Nobody claims that this is a perfect process, but in the wider scheme of things, it works extremely well. If flawed or suboptimal/too preliminary studies do get published, they will not stand the test of repeatability, which is why this second step in the scientific process is so important. Indeed, it is this very test that bisphenol A research has repeatedly failed. One scientifically logical explanation for this failure is that the more detailed larger studies, in which no effects of bisphenol A were found, have used a route of exposure to bisphenol A (oral route) relevant to humans (Kang *et al.*, 2006), rather than injections and implants in many of the earlier studies which will have resulted in markedly higher internal exposure—yet, the initial studies are still advocated by some as correct and the more detailed “repeat” studies as being wrong for a variety of “design” reasons (Myers *et al.*, 2009; Richter *et al.*, 2007). These arguments run counter to the normal scientific evolution process, as outlined above and in many respects defy common sense.

Another reason why bisphenol A research has been bogged down is the thorny issue of “low-dose” effects and inverted U-shaped dose-response curves (also termed “hormesis”). These claim to show that effects of bisphenol A occur at both very low and much higher concentrations and may disappear or diminish at intermediate concentrations; this has been advocated as one contributor to the nonrepeatability of some studies. As already mentioned, others have suggested that a more important contributor to the lack of repeatability of certain bisphenol A studies has been the use of “insensitive” strains of mice or rats for studies that showed no reproductive effects when compared with CD1 mice (Myers *et al.*, 2009; Richter *et al.*, 2007). The studies by Ryan *et al.* (2009) address both of these issues. Their studies cover a 100-fold range of bisphenol A doses, with the lowest dose used (2  $\mu\text{g}/\text{kg}/\text{day}$ ) estimated to be  $\sim 40$ -fold higher than the median calculated intake of bisphenol A in the U.S. population (Lakind and Naiman, 2008). Ryan *et al.* (2009) show no evidence for effects of bisphenol A on the end points measured at either high or low doses, and sufficient animals were used for these studies to discern such effects if present. Furthermore, their studies used an even wider range of doses (ranging from 0.05 to 50  $\mu\text{g}/\text{kg}/\text{day}$ ) for the control estrogen EE and found no significant

evidence for a U-shaped dose-response curve but found significant adverse effects at doses of 5–50  $\mu\text{g}/\text{kg}/\text{day}$  depending on the parameter measured. In terms of estrogen sensitivity, Ryan *et al.* (2009) show that both Long-Evans (used for most of their studies) and Sprague-Dawley rats show comparable sensitivity to EE as do CD1 mice and, more importantly, that this is similar to EE sensitivity in humans. Therefore, based on the results of Ryan *et al.* (2009) and other similarly detailed studies that examined effects of bisphenol A on different end points (Ema *et al.*, 2001; Howdeshell *et al.*, 2008; Tinwell *et al.*, 2002; Tyl *et al.*, 2002), the only scientifically logical conclusion is that bisphenol A, at doses considerably in excess of human exposure levels, does not reliably affect parameters of development and function in male or female rats/mice that are estrogen sensitive.

U-shaped dose-response curves could occur if a ligand, such as bisphenol A, activated two separate pathways with differing threshold sensitivities, but which impinge on a similar downstream pathway. To characterize this, careful and detailed studies are needed. An intrinsic element of this work-up is to recognize that different pathways will be involved at “low” and “high” doses, and this is where confusion has reigned with bisphenol A. Those who are strong believers in the estrogenic effects of bisphenol A have continued to argue that estrogenic effects occur at low doses, whereas the larger more detailed studies say this is not the case, including Ryan *et al.* (2009), as outlined above. More importantly, it simply is not feasible that low- and high-dose effects, if they occur, are both mediated via classical ERs, as it does not fit with what we know about how ERs work (and no cogent, alternative explanation has been offered). On the other hand, it does not rule out the possibility that estrogen-sensitive processes can be affected by bisphenol A via an alternative, non-ER-mediated pathway (e.g., Hugo *et al.*, 2008). However, if this is the case, then either extremely high doses of bisphenol A are required to activate these pathways or they do not impinge on the “classic” rodent estrogen end points such as uterine weight increase or gender-specific behavior (Ryan *et al.*, 2009). It is still feasible that bisphenol A may affect cellular/mechanistic targets that are not obviously estrogen sensitive (Bouskine *et al.*, 2009) or are estrogen sensitive but not ER mediated (Hugo *et al.*, 2008), but such studies so far remain restricted to *in vitro* approaches, and no evidence has been presented for their involvement in the controversial results referred to above.

One study had suggested that bisphenol A, administered perinatally to mice via an Alzet minipump, could affect the development or expression of aspects of sexual behavior in males and females in adulthood (Rubin *et al.*, 2006). An earlier study, using a more appropriate route of bisphenol A exposure (oral), found only minor effects on sexual behavior in males and augmentation of (estrogen independent) normal sexual behavior of females (Farabollini *et al.*, 2002). Several robust estrogen-mediated behavioral effects in females were investigated by Ryan *et al.* (2009), including aspects of sexual

(lordosis quotient) and more general (saccharin preference) behavior, both of which show marked male-female differences in untreated animals. Perinatal exposure to bisphenol A had no effect on these behaviors in female rats, whereas EE markedly altered them in the direction of males (“masculinization of behavior”). The same authors have previously found no effects of bisphenol A on sexual behavior in male rats (Howdeshell *et al.*, 2008), although it would not be expected that perinatal exposure to bisphenol A would disrupt sexual behavior in male rats due to any estrogenic effect. This is because in rodents, perinatal masculinization of the brain and of several key sexual behaviors, although androgen dependent, are in fact estrogen mediated (Meisel and Sachs 1994). Testosterone produced by the testis is converted via aromatase within discrete brain regions into estradiol, which then causes biological effects via ERs. Therefore, perinatal exposure to exogenous estrogens can induce male-specific sexual behavior in females, as shown presently for EE by Ryan *et al.* (2009). This is not the case in humans or nonhuman primates in which male-specific sexual behaviors appear to be unequivocally androgen mediated (via androgen receptors), and no role for estrogens in such processes has been described (Swaab 2007). Therefore, an additional reason to be unconcerned about the weak estrogenic effects of bisphenol A is that it cannot affect the programming or maintenance of sexual behavior in humans, at least via any estrogenic (ER mediated) process. This is important to mention because of a recent publication showing that occupational exposure of Chinese men to bisphenol A, resulting in > 50-fold higher levels of exposure than in the general population, was associated with increased sexual dysfunction (Li *et al.*, 2009). If this association reflects cause and effect, it raises further puzzles regarding mechanisms, as neither effects via ERs nor via androgen receptors can account for these findings (Sharpe 2009).

Ryan *et al.* (2009) and other similarly detailed studies in rodents more or less close the door on the possibility that bisphenol A is an environmental chemical to be concerned about because of its ER-mediated estrogenic activity. For sure, bisphenol A could contribute to the additive effects of the mixture of estrogenic chemicals to which we are all exposed (Kortenkamp 2007), but its incredibly low estrogenic potency *in vivo* via oral exposure (the predominant route of exposure for the general population) and the low levels of exposure of the general population (Calafat *et al.*, 2008) mean that its contribution to mixture estrogenic effects will be minute. I recognize that this statement will run counter to the strong convictions of some, but I base it on objective, scientific principles of evaluation. Bisphenol A research has put one of these principles firmly under the spotlight, namely, the almost complete inability for different laboratories to reproduce the same results, although much of this may be explained by use of different routes of exposure (and therefore different levels of target tissue exposure). If an earlier result cannot be reproduced in a huge study conducted in a scientifically rigorous manner,

as exemplified by Ryan *et al.* (2009), then the original result fails one of the golden rules that govern scientific research. When this happens repeatedly, as is the case with bisphenol A, then there can be no logical, scientifically based reason for continuing to espouse that the original results are the only ones that are correct, rather the converse.

Fundamental, repetitive work on bisphenol A has sucked in tens, probably hundreds, of millions of dollars from government bodies and industry which, at a time when research money is thin on the ground, looks increasingly like an investment with a nil return. All it has done is to show that there is a huge price to pay when initial studies are adhered to as being correct when the second phase of scientific peer review, namely, the inability of other laboratories to repeat the initial studies, says otherwise. If this short opinion piece does nothing else, I hope that it will remind us all of the central importance to be attached to the repeatability of experiments and how we should react when a study proves to be unrepeatable. As scientists, we all like our ideas and hypotheses to be proved correct; yet, there is equal merit in being proved wrong. The ideal hypothesis is one that can be shot at, and in most cases, it ends up full of holes (at best). This is the tried and trusted way via which scientific understanding moves onward, and ultimately, our own convictions and presumptions cannot stand in its way.

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