

LETTER TO THE EDITOR

Flawed Experimental Design Reveals the Need for Guidelines Requiring Appropriate Positive Controls in Endocrine Disruption Research

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A study published in *Toxicological Sciences* (Ryan *et al.*, 2009) illustrates the importance of examining appropriate doses of both the positive control and the test chemical in research on endocrine-disrupting chemicals. For the three low doses of bisphenol A (BPA) that were fed to rats during pregnancy and lactation, there were no effects on female offspring (there were also no effects on male offspring from the same experiment; Howdeshell *et al.*, 2008). A review of the results of the positive control doses makes it clear that the experiment cannot adequately assess the consequences of low-dose exposure to BPA because the animal model is insensitive to low doses of the positive control estrogen. Therefore, conclusions being drawn from this experiment about low-dose responses to any estrogen are invalid, including that of “no harm” from the low doses of BPA that were tested. However, the experiment is important because it highlights the need to apply basic principles of study design, long known and accepted in studies

of hormones and hormonally active drugs, to toxicological studies of chemicals with hormonal activity.

Specifically, the study by Ryan *et al.* (2009) using long evans (LE) rats was designed to test low doses of BPA that had previously been reported to cause effects in mice. However, the authors did not establish the sensitivity of the LE rat to the positive control ethinylestradiol (EE) for the outcomes being examined prior to determining what doses of BPA to test in their study. The lowest maternal dose of EE reported to cause effects in LE rat offspring by these authors was between 5 and 50 µg/kg/day, depending on the response being examined. However, the lowest effect dose for EE in the LE rat (5 µg/kg/day) was 2500-fold higher than the maternal dose required to stimulate effects on offspring in mice (Thayer *et al.*, 2001). The clinically effective dose of EE in oral contraceptives is < 0.5 µg/kg/day. Ryan *et al.* (2009) thus report no effects of EE in their animal model at doses sufficient to cause temporary sterility in 99.7% of women who

properly use oral contraceptives (Thayer *et al.*, 2001). One potential contributor to the low sensitivity to estrogen in this experiment is the use of polycarbonate cages made from BPA.

Numerous reports show that for the types of outcomes examined in LE rat offspring, maternal doses of BPA between 100- and 1000-fold greater than the effective EE dose would be required (Richter *et al.*, 2007; Timms *et al.*, 2005). Thus, the minimum dose of BPA predicted to produce an effect on rodent reproductive organs observed at 5 µg/kg/day EE would be 500 µg/kg/day BPA. However, the only doses of BPA tested in LE rats were 2, 20, and 200 µg/kg/day. The appropriate conclusion to be drawn from this experiment is that due to the relative insensitivity of the outcomes examined in LE rats to the positive control estrogen, effects previously observed in numerous studies in response to low doses of BPA in other more sensitive model animals were not observed.

By failing to establish the sensitivity of the animal model to the class of chemical being tested, the authors violated U.S. National Toxicology Program (NTP) recommendations for low-dose studies of endocrine disrupting chemicals. The NTP (2001) recommends that: "Because of clear species and strain differences in sensitivity, animal model selection should be based on responsiveness to endocrine-active agents of concern (i.e., responsive to positive controls), not on convenience and familiarity" (p. VII). This comment was prompted by a BPA study published in Toxicological Sciences that used an insensitive rat and did not include a positive control but concluded, as did Ryan *et al.* (2009), that BPA caused no harm at low doses (Tyl *et al.*, 2002). It is unacceptable in any research with experimental animals to not include both a negative control and "appropriate" positive control doses if the conclusion reached is no harm due to low-dose exposure to BPA or any other endocrine disrupting chemical.

In summary, publishing studies that conclude no harm in response to low doses of endocrine disrupting chemicals, when the studies did not include a positive control (Tyl *et al.*, 2002), included inappropriate doses of positive controls (Ryan *et al.*, 2009; Tyl *et al.*, 2008), or included positive controls that showed no effect (Cagen *et al.*, 1999), is inappropriate in peer-reviewed journals (Myers *et al.*, 2009a,b; vom Saal and Welshons, 2006). Such studies violate basic principles of study design. To avoid allowing flawed research to enter the peer-reviewed literature, we recommend adoption of the following criteria:

(1) Determine the sensitivity of systems being examined in a model animal by establishing the dose-response relationship for an appropriate positive control prior to designing an experiment to test chemicals for activity via similar mechanisms; Ryan *et al.* (2009) would have examined higher doses of BPA if they had followed this approach.

(2) Include appropriate doses of "concurrent" positive controls, such as EE, for estrogenic test chemicals.

The latter is required to determine the relative potency and efficacy of a test chemical such as BPA in the model system at

the time the chemical is tested since there are a myriad of factors that can alter outcomes from one experiment to another. In particular, a response to only very high concurrent positive control doses (e.g., Ryan *et al.*, 2009; Tyl *et al.*, 2008) is not relevant for predicting receptor-mediated low-dose activity of hormonally active chemicals (Myers *et al.* 2009a,b). We urge adoption of these essential control standards for publication of research on endocrine-disrupting chemicals.

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