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Why Public Health Agencies Cannot Depend upon ‘Good Laboratory Practices’ as a Criterion for Selecting Data: The Case of Bisphenol A.

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Running title: 'GLP' is not a Guarantee of Reliable Science.

Key words: bisphenol A, endocrine disruptors, FDA, Food and Drug Administration, GLP, Good laboratory practices, low-dose, non-monotonic, positive control,

Abbreviations:

BPA: bisphenol A

DES: diethylstilbestrol

EFSA: European Food Safety Authority

FDA: Food and Drug Administration

GLP: Good Laboratory Practices

NIEHS: National Institute of Environmental Health Science

NIH: National Institute of Health

NOEL: No Observed Effect Level

NTP: National Toxicology Program

pM: picomolar

ppb: parts per billion

ppt: parts per trillion

RTI: Research Triangle Institute

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## Abstract

**Background:** In their safety evaluations of bisphenol A (BPA), the U.S. Food and Drug Administration (U.S. FDA) and a counterpart in Europe, European Food Safety Authority (EFSA), have given special prominence to two industry-funded studies that adhered to standards defined by ‘Good Laboratory Practices’ (GLP). These same agencies have given much less weight in risk assessments to a large number of independently replicated non-GLP studies conducted with government funding by the leading experts in various fields of science from around the world.

**Objectives:** We review differences between industry-funded GLP studies of BPA conducted by commercial laboratories for regulatory purposes and non-GLP studies conducted in academic and government laboratories to identify hazards and molecular mechanisms mediating adverse effects. The methods and results in the GLP studies that have emerged as pivotal in the U.S.FDA’s draft decision declaring BPA “safe” are examined in relation to findings from studies that were competitive for National Institutes of Health (NIH) funding, peer-reviewed for publication in leading journals, subject to independent replication, but rejected by the U.S. FDA for regulatory purposes.

**Discussion:** While the U.S. FDA and EFSA have deemed two industry-funded GLP studies of BPA to be superior to hundreds of studies funded by the US-NIH and NIH counterparts in other countries, the GLP studies on which the agencies based their decisions have serious conceptual and methodological flaws. In addition, the U.S. FDA and EFSA have mistakenly assumed that ‘Good Laboratory Practices’ yields valid and reliable scientific findings (i.e., “good science”). Their rationale for favoring GLP over hundreds of publically-funded studies ignores the central factor in determining the reliability and validity of scientific findings, namely independent replication, and

use of the most appropriate and sensitive state-of-the-art assays, neither of which is an expectation of industry-funded GLP research.

**Conclusions:** Public health decisions should be based upon studies using appropriate protocols with appropriate controls and the most sensitive assays, not GLP. Relevant NIH-funded research using state-of-the-art techniques should play a prominent role in safety evaluations of chemicals.

## **Introduction**

Regulatory agencies in the United States and the European Union have justified the decision to declare the estrogenic chemical bisphenol A (BPA) “safe” at current levels of human exposure based on a few studies conducted using “Good Laboratory Practices” (GLP). In contrast, these agencies have rejected for consideration in their risk assessment of BPA hundreds of laboratory animal and mechanistic cell culture studies conducted by academic and government scientists reporting harm at very low doses of BPA. A primary factor in rejecting these studies was because they were not conducted using GLP. We suggest here that decisions based on this logic are misguided and will result in continued risk to public health from exposure to BPA as well as other manmade chemicals.

“Good Laboratory Practices” are federal rules for conducting research on the health effects or safety testing of drugs or chemicals submitted by private research companies for regulatory purposes. The rules outline basic guidelines for conducting scientific research, including the care and feeding of laboratory animals, standards for facility maintenance, calibration and care of equipment, personnel requirements, inspections, study protocols and collection and storage of raw data (Goldman 1988). These regulations were developed in response to widespread misconduct by private research

companies; this misconduct was possible because their data usually do not go through the rigorous, multi-stage scientific review that is normal for academic data funded by federal agencies and published in the peer-reviewed literature. The lack of these safeguards from academic science had enabled fraud. The U.S. Food and Drug Administration (U.S. FDA) first issued rules for GLP in 1978 after a two-year federal investigation into sloppy laboratory practices of a number of private research companies (Lublin 1978; Markowitz and Rosner 2002). What began as serious concerns about poor quality research expanded into a criminal investigation of Industrial Bio-Test (IBT), one of the largest private laboratories at the time and a subsidiary of Nalco Chemical Company. In response to the federal investigation, the U.S. Environmental Protection Agency (U.S. EPA) demanded that 235 chemical companies re-examine the over 4,000 tests conducted by the laboratory. In 1983, three men from IBT were found guilty of deliberately doctoring data and sentence to prison. The fraudulent practices of IBT brought into question 15% of the pesticides approved for use in the U.S. That same year, the U.S. EPA issued similar GLP rules for regulatory testing.

Both the U.S. FDA (2008a) and European Food Safety Authority (ESFA 2006) have recently published documents demonstrating that their decision to continue to declare BPA safe at current exposure levels was based primarily on the results of a few industry-funded studies that followed GLP guidelines. These decisions stand in stark contrast to the decisions reached concerning the potential risks to human health reached by a panel of 38 experts at a U.S. NIH-sponsored conference, who published The Chapel Hill Consensus Statement (vom Saal et al. 2007) as well as five review articles (Crain et al. 2007; Keri et al. 2007; Richter et al. 2007a; Vandenberg et al. 2007a; Wetherill et al. 2007). These peer-reviewed articles covered approximately 700 articles concerning BPA and represented a comprehensive review of the literature as of the end of 2006. In addition, the U.S. FDA draft decision contradicted the conclusions reached by the National

Toxicology Program (NTP), which had spent two years investigating this question (NTP 2008). An important role of the NTP is to advise the U.S. FDA about the science relating to toxic chemicals in food, but in an unusual move the U.S. FDA chose to release its draft report prior to the release of the final report on BPA by the NTP and without indicating who at the U.S. FDA was involved in preparing the draft report (U.S. FDA 2008b). At a September 16, 2008 hearing regarding the draft report on BPA, the U.S. FDA announced that their goal was to have a subcommittee of the U.S. FDA Science Board complete a review of the draft decision by the end of October, 2008 (this would presumably also involve review by the subcommittee members of the approximately 1000 articles relating to BPA).

We will discuss below that the methods employed in chemical industry sponsored GLP studies are incapable of detecting low-dose endocrine disrupting effects of BPA and other hormonally active chemicals. Detecting endocrine disrupting effects at low doses of chemicals such as BPA requires sophisticated and modern assays and analyses that have been developed in advanced, usually federally-funded laboratories over the past decade. This is especially apparent when one examines what is now known about functional effects of BPA on a wide range of endpoints (Richter et al. 2007a; Welshons et al. 2006; Wetherill et al. 2007). These endpoints include those mediated by recently discovered estrogen response pathways initiated in human and animal cell membranes (referred as "non-classical or alternative estrogen response mechanisms"), which multiple laboratories have shown to be equally sensitive to BPA and estradiol in terms of activating effects in human and animal cells at low picomolar through low nanomolar concentrations (Alonso-Magdalena et al. 2008; Wetherill et al. 2007; Wozniak et al. 2005; Zsarnovszky et al. 2005).

The effects of BPA documented in these studies include a diverse array for which there are no data from GLP studies because the endpoints have not been examined: altered metabolism related to metabolic syndrome (Alonso-Magdalena et al. 2005; Alonso-Magdalena et al. 2006; Alonso-

Magdalena et al. 2008; Ropero et al. 2008); altered adiponectin secretion (Hugo et al. 2008), which is a condition predicting heart disease and type 2 diabetes (Lang et al. 2008); altered epigenetic programming leading to precancerous lesions of the prostate (Ho et al. 2006); differential growth patterns in the developing prostate (Timms et al. 2005); abnormal growth, gene expression and precancerous lesions of the mammary glands (Soto et al. 2008); and adverse effects on the female reproductive system, including uterine fibroids, para-ovarian cysts and chromosomal abnormalities in oocytes (Newbold et al. 2007; Susiarjo et al. 2007). There is also a large literature on neuroanatomical, neurochemical and behavioral abnormalities caused by low doses of BPA (Leranth et al. 2008; Richter et al. 2007a), which also are not capable of being detected by current GLP studies conducted for regulatory purposes because of their out-of-date assays.

The approaches used by the academic and government scientists to study the potential health hazards of BPA contrast sharply with those still used by the chemical industry that are relied on by regulatory agencies in the US and Europe, including the two studies identified by both the U.S. FDA and EFSA as central to the decision to declare BPA safe at current human exposure levels (Tyl et al. 2002; Tyl et al. 2008a). By using outdated and insensitive assays that were supposed to have been replaced by a new battery of screens and tests by 2000 (as mandated by the US Congress in 1996 in the Food Quality Protection Act (1996), but which has, as yet, still not occurred), these studies conducted using GLP fail to find any adverse effects.

### **Reliability and Validity**

Reliability and validity are separate issues, although in the experimental research described here, validity and reliability basically refer to research that is credible. Golafshani (2003) noted in a review that “reliability” refers to the extent to which results are consistent over time and are an accurate representation of the total population under study. Of central importance is that the results

of a study must be reproduced under a similar methodology in order to be considered to be reliable. “Validity” refers to whether the research measures that which it was intended to measure, and valid findings are considered to be “true”. In other words, reliability is determined by whether the results are replicable while validity is assessed by whether the methods used result in finding the truth as a result of the investigator actually measuring what the study intended to measure.

### **Use of ‘Good Laboratory Practices’ in Regulatory Decision-Making**

Despite strong evidence of aberrations in animals exposed during fetal and neonatal life caused by low doses of BPA in studies conducted by the world’s leading academic and government experts in the field of endocrine disruption, endocrinology, neurobiology, reproductive biology, genetics, metabolism, etc., a relatively small number of studies reporting no adverse effects at low doses of BPA have continued to be promoted by the chemical industry and used by regulatory agencies (e.g., Ashby et al. 1999; Cagen et al. 1999; Tyl et al. 2002; Tyl et al. 2008a). According to the U.S. FDA, these are accepted because they used GLP (U.S. EPA 2008), with the implication that studies not employing GLP are not reliable or valid (U.S. FDA 2008a).

**GLP does not guarantee reliability or validity of scientific results.** Unfortunately, while GLP creates the semblance of reliable and valid science, it actually offers no such guarantee. It specifies nothing about the quality of the research design, the skills of the technicians, the sensitivity of the assays or whether the methods employed are current or out-of-date (all of the above are central issues in the review of a grant proposal by a NIH panel). GLP simply means that the laboratory technicians/scientists performing experiments follow highly detailed U.S. EPA requirements [or in the European Union, Organization for Economic Co-operation and Development (OECD) requirements] for record keeping, including details of the conduct of the experiment, and archiving relevant biological and chemical materials (U.S. EPA 2008).

These record-keeping procedures in GLP were instituted because of widespread misconduct being committed by commercial testing laboratories (see discussion above). These fraudulent results were possible because contract lab studies used in the regulatory process are rarely subject to the checks and balances that peer-reviewed, replicated scientific findings undergoes. Without that acid test of reliability (replication by other independent scientists), other procedures were needed. Hence GLP was implemented, despite its severe limitations.

**NIH-funded research subject to more stringent reviews than GLP.** While few NIH-funded investigators adhere to GLP-mandated record-keeping, the procedures of GLP are actually surpassed by the procedures required for NIH-funded science that is published in peer-reviewed journals. NIH-funded studies pass through three phases of peer-review that are far more challenging than GLP requirements: First, the principal scientists must have demonstrated competence to conduct the research, and experimental methods, assays, laboratory environment, etc. must involve use of state-of-the-art techniques to be competitive for NIH funding. Second, results are published in peer-reviewed journals, with detailed evaluations by independent experts examining all aspects of the study. And third, the findings are challenged by independent efforts to replicate; for example, the initial findings concerning the stimulating effects of estrogenic chemicals on the mouse prostate (Nagel et al. 1997; vom Saal et al. 1997) were independently replicated and extended by Gupta (2000), which led to an editorial identifying “initial results confirmed” (Sheehan 2000).

Typically, within a laboratory, interesting findings are also followed by subsequent publications extending the prior findings; examples are the findings of BPA effects on  $\beta$  cells in the mouse pancreas (Alonso-Magdalena et al. 2005; Alonso-Magdalena et al. 2006; Alonso-Magdalena et al. 2008) and the effects of estrogenic chemicals and drugs on the developing mouse prostate that followed earlier findings (described above) from this same group (Timms et al. 2005; Richter et al. 2007b). In particular, independent replication by competent, respected scientists is the main

criterion of acceptance of the findings as having been demonstrated to be reliable and having been validated by virtue of coming to the same conclusion using a variety of sophisticated techniques in multiple publications.

An important criticism of the approach taken by the US FDA in its assessment of the now approximately 1000 articles on BPA is that it appears to have made no attempt to “connect the dots” between replicated studies; but, instead, the US FDA appears to have assessed each study without regard to whether it had been confirmed by other studies.

Thus, collectively, there are many phases that are used to verify the reliability and validity of NIH-funded published research that have been completely ignored by the US FDA, while industry-funded GLP research is rarely, if ever, subject to these central requirements and yet is accepted by regulatory agencies as reliable and valid.

**FDA’s misguided ‘gold standard’.** In this light, the U.S. FDA’s reliance upon GLP as the “gold standard” is scientifically misguided. Furthermore, U.S. FDA administrators are ignoring published critiques of the GLP studies it considers reliable and valid, such as the Tyl et al. (2002) study and two coordinated studies conducted at the same time by Ashby et al. (1999) and Cagen et al. (1999). Each was an industry-funded study conducted using GLP. Each was harshly criticized in peer-reviewed publications by academic scientists and government panels (CERHR 2007; NTP 2001; vom Saal and Hughes 2005; vom Saal and Welshons 2006). Yet the U.S. FDA and EFSA panels still assert that these studies represent the “gold standard” in toxicological research.

Specifically, Cagen et al. (1999) and Ashby et al. (1999) were recently rejected by the NTP CERHR panel on BPA as unusable for consideration in its evaluation of the health hazards posed by BPA (CERHR 2007). Both the Ashby et al. (1999) and Cagen et al. (1999) studies reported finding no effect of their positive control (the estrogenic drug diethylstilbestrol, DES) on any outcome,

although these failures were not acknowledged by the authors in either published article. In experimental science, the failure of a positive control to show an effect indicates the experiment failed, which is the conclusion reached by the CERHR, NTP panel (CERHR 2007).

The Tyl et al. 2002 study, which the U.S. FDA still accepts as a major study for determination of the safety of BPA (U.S. FDA 2008a, b), was criticized by a NTP panel that met in 2000 to examine the “low dose” issue (NTP 2001), as well as in subsequent publications (vom Saal and Hughes 2005; vom Saal and Welshons 2006), for using an insensitive rat (the CD-SD rat) that requires extremely high doses ( $\geq 50 \mu\text{g}/\text{kg}/\text{day}$ ) of the potent estrogenic drug ethinylestradiol to show effects such as those examined in the study by Tyl et al. (2002); this dose of ethinylestradiol is over 100-times higher than the approximately  $0.3 \mu\text{g}/\text{kg}/\text{day}$  used by women in oral contraceptives. The fact that Tyl et al. (2002) adhered to GLP did not protect them from using insensitive animals. This led the NTP panel that met in 2000 to state: “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.” (NTP Report 2001, p VIII).

Thus when reviewed by other scientists, three prior major GLP studies of BPA have been found to be so flawed as to be useless for guiding regulatory agencies in decision-making. A new GLP study has now been published by Tyl et al. (2008a). Close examination of this study also reveals fatal flaws. These flaws render it useless for regulatory purposes, even though it conforms to GLP.

### **Examples of Flaws Ignored by the U.S. FDA and EFSA in a recent GLP Study of BPA.**

In summary, the flaws in Tyl et al. (2008a) are:

- The high dose required for the positive control (estradiol) to cause an effect means the system used by Tyl et al. (2008a), at least in her laboratory, is relatively insensitive to

exogenous estrogens and thus inappropriate for studying low-dose effects of estrogenic compounds such as BPA. The lack of response to low doses of estradiol or BPA in the Tyl laboratory is puzzling in that the strain of mice used in these experiments (the CD-1 mouse) has been reported in over 20 other peer reviewed publications to show adverse effects in response to very low doses of BPA (vom Saal 2008), as well as many other studies showing low-dose effects in response to the natural hormone estradiol, the estrogenic drugs ethinylestradiol and DES, and to and other estrogenic chemicals.

- Tyl et al. (2008a) used insensitive, out-of-date protocols and assays that are incapable of finding many of the adverse effects reported by more sophisticated studies conducted by independent, NIH-funded scientists as well as scientists funded by government agencies in other countries.
- In the specific case of testing for changes in prostate weight, Tyl et al. (2008a) report an abnormally high prostate weight for control animals that exceeds by over 70% the prostate weights reported by other studies for animals of the same strain and similar age (for example, Gupta 2000; Ruhlen et al. 2008). This suggests that the dissection procedures for the prostate in the Tyl laboratory included other non-prostatic tissues in the weight measurements, rendering them unusable for studying weight changes in the prostate in response to BPA or estradiol; neither chemical showed any effect on the selected endpoints, which directly contradicts other findings concerning opposite effects of low and high doses of estrogen on the prostate (vom Saal et al. 1997; Putz et al. 2001; Timms et al. 2005).

We elaborate on these points below:

**Aberrant insensitivity of CD-1 mouse to estrogens.** In Tyl et al. (2008a), estradiol was used as a positive control: it was fed to female mice prior to and during pregnancy and lactation at 80 - 220

$\mu\text{g/kg/day}$ , and then after weaning estradiol was fed to offspring at doses of 80 – 100  $\mu\text{g/kg/day}$ .

Estradiol was used as a positive control because BPA is a manmade endocrine disrupting estrogenic chemical.

Many published findings reporting effects of very low doses of positive control estrogens and BPA in CD-1 mice demonstrate that in the test system reported in the Tyl et al. (2008a) study, the CD-1 mouse was somehow rendered insensitive. The fact that a dose of 100-200  $\mu\text{g/kg/day}$  of estradiol was necessary to show an effect of the positive control predicts that Tyl et al. (2008a) should not detect effects of BPA below 10-100 milligrams/kg/day, far above the low-dose range relevant to human exposures that was supposedly of interest.

For nuclear estrogen receptor mediated effects via regulation of gene activity (nuclear estrogen receptors are transcription factors whose activity is regulated by binding to estrogen), prior studies have typically shown a 1000-fold lower activity for BPA relative to estradiol or potent estrogenic drugs, including DES and ethinylestradiol. For example, Richter et al. (2007b) reported an increase in androgen receptor gene activity to estradiol at 1 pM (0.28 pg/ml) in fetal CD-1 mouse prostatic mesenchyme cells in primary culture, and the same response was found for BPA at 1000 pM (228 pg/ml); the *in vitro* response to estradiol was predicted by the response of the prostate to increasing free serum estradiol from 0.2 to 0.2 pg/ml in male mouse fetuses via estradiol administration to the mother (vom Saal et al. 1997). Other research showed that a significant effect on development of the male reproductive system in CF-1 mice occurred at a 0.002  $\mu\text{g/kg/day}$  maternal dose of ethinylestradiol (Thayer et al. 2001), similar to effects observed with 2 – 20  $\mu\text{g/kg/day}$  BPA (vom Saal et al. 1998). Research in Taisen Iguchi's laboratory showed accelerated puberty in CD-1 (ICR) mice at a DES dose of 0.02  $\mu\text{g/kg/day}$  (the positive control), and the same response to BPA occurred at 20  $\mu\text{g/kg/day}$  (Honma et al. 2002), again, revealing a 1000-fold difference between the positive control estrogen and BPA.

There are numerous other examples of findings where a higher dose of BPA was required to cause the same effect as the positive control estrogen (estradiol, ethinylestradiol or DES) in studies where the effects are mediated by the classical nuclear estrogen receptors, in contrast to the more recently discovered rapid signaling estrogen response system where BPA and these positive control estrogens have equal potency, as described above. In summary, CD-1 mice have been used by a large number of academic and government investigators and have been reported in peer-reviewed publications to be sensitive to positive control estrogens within the range of human sensitivity based on *in vivo* and *in vitro* studies via the classical estrogen receptor alpha-mediated response mechanism. The CD-1 mouse is the animal model that has been used by the US National Institute of Environmental Health Sciences (NIEHS) for decades, since it is considered the best animal model for predicting the effects of developmental exposure to estrogen in humans (Newbold 1995; Newbold et al. 2007).

The failure of a traditional toxicological studies conducted by Tyl et al. (2008a, b) to detect the wide range of adverse effects of even relatively high doses of BPA or of low doses of estradiol that have been reported in numerous studies by academic and government scientists provides evidence that the GLP protocols established long ago by regulatory agencies to determine the toxicity of chemicals are inappropriate for detecting the endocrine disrupting activity of chemicals with hormonal activity such as BPA. Indeed, this was the premise of the Congressional mandate in the Food Quality Protection Act (1996) for the EPA to establish a new set of assays for endocrine disrupting chemicals, although this process has been systematically delayed and is over 8 years behind the Congressionally mandated date of 2000 to have these new assays validated.

Citing Tyl et al. (2008a) the EFSA report on BPA stated: “The positive control substance, 17 $\beta$ -oestradiol, resulted in reproductive and developmental toxicity” (EFSA 2006). This report failed to acknowledge that only a very high dose of the positive control was sufficient to elicit effects, and

that this meant that the experiments conducted in the Tyl laboratory were for some reason very insensitive to any estrogen and thus inappropriate for use in a study to examine low-dose estrogenic effects of BPA.

Based on the preliminary report released by the U.S. FDA regarding BPA (U.S. FDA 2008a), it appears that the U.S. FDA has followed the lead of the EFSA in its lack of understanding of the importance of the dose of the positive control estrogen required to cause adverse effects. The consequence is that the U.S. FDA has relied primarily on the Tyl et al. study, with the result that the U.S. FDA has assured Americans that BPA is “safe” at current human exposure levels.

Several factors might account for the insensitivity of the CD-1 mouse in the Tyl et al. studies conducted at the Research Triangle Institute (RTI), a testing facility that conducted these (as well as previous) studies that were funded by the American Chemistry Council. One possibility is that the diet used in these studies may have interfered with the results. The feed used by Tyl et al. (2008a) in this experiment (Purina 5002) has been shown by others to interfere with responses to exogenous estrogenic chemicals, blocking adverse effects documented on other diets. For example, a number of years ago Julius Thigpen and colleagues at NIEHS recommended against the use of Purina 5002 in studies of endocrine disrupting chemicals (Thigpen et al. 2003). While Tyl et al. (Tyl et al. 2008a) measured some specific phytoestrogens in the 5002 feed by chemical analysis, Heindel and vom Saal (2008) point out in a report on NIH-sponsored meetings on this subject that this is an insufficient control for total dietary estrogenic contaminants that can disrupt studies involving the effects of estrogenic chemicals.

A second possibility is that there are strain differences in sensitivity developed in the CD-1 mouse sold by the various Charles River Laboratories located in different regions. We consider this unlikely because most laboratories regularly replace their CD-1 mouse breeder stock from Charles

River Laboratories, and practices there make it unlikely that the sensitivity of this outbred stock to estrogens has changed dramatically over a very short period of time. Also, because the Research Triangle Institute where the Tyl studies were conducted is very near the laboratories of the NIEHS, it is likely that the CD-1 mice used by these two programs were purchased from the same breeding facility.

**Use of insensitive, out-of-date protocols and assays.** Another serious concern about the two recent Tyl et al. (2008 a, b) studies is the experimental approach used, thus raising questions about the validity of the studies. The study design used by Tyl et al. has been superseded by advances in both experimental design and analytical tools developed by NIH-funded scientists (and their counterparts in Europe and Asia) since the mid-1990s. The methods used by Tyl et al., primarily wet weight changes of tissues, gross histological changes, and developmental landmarks such as vaginal opening, were established procedures by the 1950s. Thus, a major limitation of the Tyl studies is the failure to measure more meaningful and sensitive endpoints in order to detect the effects of low dose BPA exposure, which are often not macroscopic in nature. Indeed, the director of the reproductive division of the National Health and Environmental Effects Research Laboratory at the U.S. EPA, stated in 2001 that the inconclusive results concerning effects of BPA on reprotoxicology, can only be solved by understanding the mechanisms (Triendl 2001). With current GLP standards it is not possible to study mechanisms, because they still rely on out-of-date assays.

As one example of a comparison between the approach by Tyl et al. (2008a) and independent government-funded academic scientists, extensive research has been conducted in the laboratory of Ana Soto and by other independent academic and government scientists describing effects of exposure of female mice and rats to very low doses of BPA during perinatal development on the mammary glands. While Tyl et al. (2008a) report no low dose effects of BPA on the mammary glands using conventional histological analysis, there have been consistent findings of adverse

effects of low doses of BPA from studies that utilized more sophisticated and sensitive analysis of whole mounted mammary glands to facilitate detection of microscopic lesions, coupled with immunostaining for regulatory proteins as well as techniques for determination of aberrant gene expression associated with progression to cancer. These peer-reviewed published studies have reported detecting changes during embryonic development of mammary glands as well as abnormalities detected during adolescence through adulthood that are indicative of mammary gland cancer as well as other developmental abnormalities (Colerangle and Roy 1997; Durando et al. 2007; Markey et al. 2001; Markey et al. 2005; Moral et al. 2008; Munoz-de-Toro et al. 2005; Murray et al. 2007; Nikaido et al. 2004; Vandenberg et al. 2007b; Vandenberg et al. 2006; Wadia et al. 2007; LaPensee et al. 2008).

Similar to the findings for the mammary gland, Ogura et al. (2007) reported that if analyzed by conventional histological methods (staining with hematoxylin and eosin), prenatal exposure to low doses of BPA or DES showed no effects on prostate development, while if the sections were analyzed using antibodies that identified basal cells and basal cell squamous metaplasia, then significant effects were revealed. Squamous metaplasia of basal cells is indicative of abnormal proliferation and function of the prostate stem cell population that is thought to transform into neoplastic cells, and Ho et al. (2006) reported that neonatal exposure to very low doses of BPA caused 100 percent of male rats to develop high grade prostatic intraepithelial neoplastic (PIN) lesions later in life. All of these studies were rejected by the U.S. FDA as not adequate for making regulatory decisions about the safety of BPA. Instead, the U.S. FDA relied upon Tyl et al. (2008a), even though the study used techniques that Ogura et al. (2007) showed lacked the sensitivity of 21st century experimental approaches.

Tyl et al. (2008a) did not examine any neurobehavioral endpoints, yet findings regarding changes in brain structure, brain chemistry and behavior represent the largest portion of the published low-dose

BPA literature. The NTP report (NTP 2008) and the NIEHS conference consensus report (vom Saal et al. 2007) both indicated concern about neurobehavioral effects of low doses of BPA, and the absence of studies that included neurobehavioral endpoints is a glaring omission in the Tyl et al. (2008a, b) publications.

**Flawed prostate dissection.** Data presented in Tyl et al. (2008a) raise questions about the adequacy of techniques used in their studies of BPA. Specifically, Tyl et al. (2008a) report the prostate in 3.5-month-old control male CD-1 mice weigh over 70 mg [see Table 3 in Tyl et al. (2008a) for data on F1 retained males].. This average control weight contrasts sharply with those reported from other laboratories. Specifically, the weight of the prostate in 2 - 3-month-old CD-1 mice using the dissection technique in both the vom Saal and Gupta laboratories and at NIEHS (Newbold, personal communication) is about 40 mg (Gupta 2000; Ruhlen et al. 2008). Other laboratories report that prenatal exposure to very low doses of BPA and positive control estrogens increased prostate size, prostatic androgen receptors and prostate androgen receptor gene activity (Gupta 2000; Richter et al. 2007b; Timms et al. 2005; vom Saal et al. 1997), but the increased prostate size of experimental animals exposed to BPA in these laboratories weigh less than the prostates in Tyl et al's (2008a) control animals. This raises serious questions about Tyl et al's procedures and/or animals. The weight of prostate reported by Tyl et al. (2008a) suggests that the technique they used for dissecting the prostate resulted in non-prostatic tissue being weighed along with prostate. The seminal vesicle, coagulating glands and dorso-lateral prostate all merge together where the ejaculatory ducts enter the urethra, and there are also fat deposits on the prostate. This poses a challenge for those without proper training in distinguishing these different tissues during dissection in mice.

Alternatively, it is known that as male rodents age, they are prone to develop prostatitis. While this inflammatory disease leads to an increase in prostate size and could thus account for the very large prostate weights reported by Tyl, anyone familiar with the appearance of prostatitis would detect

this abnormality upon histological examination, which Tyl et al (2008a) supposedly conducted.

Also, prostatitis is rare in young-adult mice or rats (Cowin et al. 2008), and the size of the prostates in the Tyl et al (2008a) study were similar to those for middle-aged and old male mice.

The findings regarding effects of BPA on the prostate presented by Tyl et al. (2008a) are thus suspect, and cannot be used as evidence that other earlier studies (Gupta 2000; Timms et al. 2005; vom Saal et al. 1997) are not replicable. Given these problems in prostate weight measurements, it is not surprising that even very high doses of BPA or estradiol in Tyl et al. (2008a) had no effect on the prostate, in sharp contrast to other studies that showed stimulation of the prostate at low doses of estrogen and inhibition at high doses (Putz et al. 2001; Timms et al. 2005).

In addition to the problem associated with the very high weight of the prostate in the Tyl et al. (2008a) study, in a separate measurement Tyl et al. combined the anterior prostate (coagulating gland) and seminal vesicle and presented these two organs as one combined outcome measure. This is wrong and misleading. The coagulating glands emerge as the anterior ducts of the prostate from the dorso-cranial region of the urogenital sinus, while the seminal vesicles bud from the proximal region of the Wolffian ducts. Prior publications have reported that elevated estrogen is associated with an increase in prostate size associated with an increase in prostate androgen receptors, while a decrease in seminal vesicle size occurs associated with a reduction in 5-alpha-reductase, an enzyme that converts testosterone to the more potent androgen 5-alpha-dihydrotestosterone (Nonneman et al. 1992). Low doses of BPA were shown to decrease the size of organs that differentiate from the embryonic Wolffian ducts (epididymides and seminal vesicles) while increasing the size of regions of the prostate that develop from the urogenital sinus (vom Saal et al. 1998). Combining these different organs together (it is technically not difficult to separate them) was thus inappropriate in that they develop from different embryonic tissues that show markedly different responses to estrogenic chemicals during development. In fact, Ogura et al. (2007) reported that the anterior

prostate (coagulating glands) showed the greatest expression of ER-alpha, and also showed the most pronounced indication of basal cell squamous metaplasia, relative to other regions of the prostate.

**Conclusion.** In that the Tyl et al (2008a) control data are not consistent with the prior published literature for prostate weight of young-adult CD-1 male mice, and methods used were inappropriate for revealing an extensive body of adverse effects detected using more sophisticated approaches, we deem the findings by Tyl et al. to be invalid. There are already hundreds of studies showing adverse effects of BPA in animals, with many conducted at current human levels of exposure to BPA, and it is thus unlikely that academic scientist would bother to replicate the outdated approaches used in the Tyl et al. (2008a, b) studies. This lack of replication is typical of GLP studies, which tend to involve unnecessarily large numbers of animals (over 8000 rats were killed in the Tyl et al. 2002 study), and reliability appears to be accepted as assured due to the excessive numbers of animals that are used. While killing excessive numbers of animals is accepted as “good science” by the U.S. FDA, the use of arbitrarily large numbers of animals per group (>20 animals per treatment group is common) actually violates guidelines in the NIH Guide for the Care and Use of Animals in Research that governs research conducted by academic and government scientists. For research with animals to be approved by any university Animal Care and Use Committee, group sizes must be based on power analysis conducted using historic data. Based on this criterion in the NIH Guide, all of the Tyl et al. studies were significantly over powered and thus in direct violation of federal guidelines for conducting animal research, a fact about which U.S. FDA regulators seem unaware.

Each of the four main industry-funded GLP studies of BPA (the two Tyl et al. studies plus Cagen et al. (1999) and Ashby et al. (1999)) is flawed and not appropriate for use in health standard setting. Clearly, meeting GLP standards is not a guarantee of reliable or valid science. The fact that the U.S. regulatory community is willing to accept these industry-funded, antiquated and flawed studies as proof of the safety of BPA, while rejecting as invalid for regulatory purposes the findings from a

very large number of academic and government investigators using 21st century scientific approaches, is of great concern. The basis for these decisions by U.S. and E.U. regulatory agencies should be thoroughly investigated, particularly since the NTP concluded that exposure of human infants to BPA was in the range that had been shown to cause harm in experimental animals (NTP 2008), and both the Canadian Ministry of Health and Ministry of the Environment recently concluded that BPA was a toxic chemical (Environment Canada 2008).

Problems inherent with reliance on GLP as a standard for choosing data are compounded by the process used by federal agencies to determine membership on science advisory panels. Leading experts qualified by specific experience on the chemical/endpoints under consideration are often specifically excluded from membership. For example, the U.S. FDA's BPA review panel was identified as an 'expert panel,' when in fact the panel was composed largely of scientists lacking any experience in research with BPA. This process, which appears to consider almost any scientist knowledgeable about a chemical to create bias, makes it vastly more difficult for the panel to integrate scientific data from the relevant literature, especially, as with BPA, there are almost 1000 relevant studies and the review panel is provided with very little time to become knowledgeable about the details. It means that the depth of knowledge present on this and similarly constituted government regulatory agency panels is unlikely to be sufficient to subject draft assessments to the scrutiny that peer-review by experts normally entails. Combined with reliance on GLP data, this process has a high potential to yield flawed assessments that jeopardize public health.

We are not suggesting that GLP should be abandoned as a requirement for industry-funded studies. We object, however, to regulatory agencies implying that GLP indicates that industry-funded GLP research is somehow superior to NIH-funded studies that are not conducted using GLP. This argument demonstrates a lack of understanding of the profound difference between the use of replication as a mechanism to assess reliability and the methods used to assess validity for peer-

reviewed published academic studies, while GLP was instituted with the expectation that this type of verification would not occur.

Public health decisions should be based upon studies using appropriate protocols and the most sensitive assay. They should not be based upon criteria that include or exclude data depending upon whether the studies are GLP or not. Simply meeting GLP requirements is insufficient to guarantee scientific reliability and validity.

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