

LOW-DOSE BPA PAPER IN TOXICOLOGICAL SCIENCES FATALLY FLAWED AND SHOULD BE RETRACTED: PLAGUED BY MASSIVE ERRORS & POOR DESIGN. CONTAMINATED CONTROLS ALSO RAISE ISSUES ABOUT FEDERAL BREEDING COLONY INTEGRITY

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Bisphenol A (BPA) has become nearly ubiquitous in our environment and can be found in many different products, including the plastic in water bottles and baby bottles, thermal paper for printers, and even in dental sealants and medical devices including intravenous fluid and chemotherapy bags and tubing^(26,27,28,29,30,31).

Thousands of studies have been published that examine the levels of BPA and other endocrine disruptors present in the environment along with the mechanisms and pathways those chemicals may take to affect or harm living things. There is, however, considerable controversy concerning negative health effects of so-called “low-dose” exposures.

One recent study, published in the journal *Toxicological Sciences*, stated as its goals the desire to help shed light on the conflicting body of evidence. Unfortunately, that study, "Toxicity Evaluation of Bisphenol A Administered by Gavage to SPRAGUE-DAWLEY Rats from Gestation Day 6 through Postnatal Day 90"⁽¹⁾ is fatally flawed.

The study⁽¹⁾ by Delclos *et al.*, concluded that low-dose BPA is not harmful. However, that conclusion cannot be scientifically justified by the experiment as conducted. This study suffers from numerous and massive errors including; by the eventual admission of the authors⁽¹⁾; the violation of one of the scientific method's paramount experimental rules: the control subjects were contaminated by the chemical substance being evaluated.

Additionally, while the study authors claim to have no idea of the BPA contamination source⁽¹⁾, an examination of the paper and a current understanding of the science indicate numerous avenues of contamination that are obvious, well-established, and should have been considered in any careful experiment design and conduct.

This letter will explore a number of those sources and their consequences in more detail below, but it merits consideration at this stage that one possible contamination source could jeopardize results of experiments underway in the very substantial, multi-agency program known as the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA).

Test Animal Contamination Concern For CLARITY-BPA

Delclos *et al.* state that the test animals in their experiment were obtained from the NTP breeding colony⁽¹⁾. If so, then their contaminated controls may also hold significance for the scores of investigators participating CLARITY-BPA.

“CLARITY-BPA is a collaborative effort between NTP and NIEHS with support from FDA to conduct a perinatal 2-year guide- line chronic rodent toxicity study on BPA⁽⁹⁵⁾.”

The program is designed to investigate “scientific uncertainties about BPA’s health effects to better inform regulatory decision-making. ...The CLARITY-BPA consortium is made up of NCTR staff, NIEHS and NTP staff, and 12 extramural grantees⁽⁹⁵⁾.”

The core guideline-compliant study is being conducted at FDA's National Center for Toxicological Research (NCTR) CLARITY-BPA investigators, both with government at universities, must use rats from the NTP breeding colony⁽⁹⁵⁾.”

The unknown source of the BPA contamination of controls raises the issue of whether the animals in the NTP breeding colony are regularly monitored for BPA and other endocrine disruptors.

Ongoing and regular population sampling by NTP would alert breeders to potential contamination. Prior to release, batch samplings would assure the receiving investigators of the purity of their test animals.

It is unknown whether the NTP breeding colony has monitoring protocols in place that adequately monitor test animals and assure investigators that the test subjects are uncontaminated and appropriate for experimentation.

Avoidable Errors Accumulate

In addition to the contamination error, the study fails on an accumulation of other serious errors:

- Potential contamination by other endocrine-disrupting compounds
- Unknown Combination Effects Of Stressors
- Use of oral gavage which introduces non-chemical stressors
- Use of oral gavage which fails to recreate appropriate BPA Absorption
- Inappropriate Rat Strain
- Poor choice of outcome measures
- Failure to use current science in BPA and Endocrine Disruptor Compound (EDC) research
- Erroneous claim that BPA is “weak” estrogen
- Failure to identify well-known exogenous source(s) of BPA contamination
- Failure to identify well-known exogenous source(s) and estrogenic activity of equipment and supplies
- Failure to measure BPA levels in any of the animals except in the test cohort at the end of the trial
- Failure to measure other exogenous estrogenic compounds in test animals
- Use of oral gavage
- Wrongly examining toxicity when timing is a bigger issue
- Failure to include disclosure of conflicts

Contaminated controls

A negative or naïve control is defined as a group of experimental units that have not had any experimental treatment applied, nor possess any quality that would have an effect on the results of the study. In the study under the microscope⁽¹⁾, in order to be a true negative control, all animals would need to be devoid of any BPA or other endocrine disruptors in their body that interact with or act similar to BPA. Unfortunately, this was not done, thereby rendering all further experimental results invalid⁽¹¹⁷⁾. The paper itself stated that:

“BPA-glucuronide was detected in the serum of vehicle and naïve control animals at levels similar to those detected in animals dosed with 2.5 ug BPA/kg bw/day. The presence of BPA-glucuronide, which is formed by metabolism of aglycone BPA in the body, in the

serum of control animals implies unintentional exposure of the animals to BPA rather than post-sampling contamination of the serum samples. The source of this exposure has not been definitively identified. Thus, the 2.5 µg BPA/bw/day dose group is not distinguishable from the two negative control groups.”⁽¹⁾

This finding automatically violates one of the most important functions of a control, in that no longer can the researchers determine if the low-dose BPA has an effect on the specific endpoints described in the paper^(2,38), since all rats, even the supposed negative controls were contaminated with BPA prior to experimentation.

If there are no differences in the low-dose BPA group and the control group, it is impossible to make the conclusion that low-doses of BPA do not have any effect on the rats when there is no real naïve control to compare to in the first place^(2,38,39,40). Additionally, without the comparison of a positive control (i.e. a control treated with BPA, in this case, that is known to promote adverse effects), it is even more difficult to determine if the negative result is real, or if it is a false negative^(3,35,39).

Unknown Combination Effects of Stressors

For the purpose of this and other tests of endocrine disruptors, it would not only be necessary to assure that the controls were not contaminated by the substance being investigated, but also for the presence of other endocrine disruptors as well.

Numerous recent studies have raised the confounding problems resulting from unknown antagonistic, agonistic, synergistic interactions which may possibly mask effects from a single compound under study^(96,97,98,99).

The National Institute of Environmental Health Sciences (NIEHS) has recognized this issue. In its 2012-2017 Strategic plan, the NIEHS stated that resolving the effects of interactions resulting from combinations of both chemical and non-chemical stressors was a high priority⁽¹⁰⁰⁾.

The NIEHS inclusion of non-chemical stressors speaks as well to gavage-induced stress which results in hormone releases that may alter the effects of a studied compound such as BPA.

Use of oral gavage which introduces non-chemical stressors

In addition to effects relating to internal concentrations of BPA or other compounds active at low doses, gavage can induce the production of stress hormones and alter the internal homeostasis of test animals^(101,102,103,104). A very recent BPA-related study that found “potential for gavage to influence gene expression in the developing brain”⁽¹⁰⁵⁾.

Altering the endocrinal homeostasis of test animals through experiment procedures with unknown or equivocal results may initiate undesirable phenomena with the potential to impact an experiment’s overall conclusions. This could be significant in cases where relevant concentrations of hormones and studied compounds are active in nano- or pico- concentrations.

Use of oral gavage which fails to recreate appropriate BPA Absorption

The Delclos *et al.* study used oral gavage dosing of pregnant rats during the experiment⁽¹⁾. Recent studies in BPA research have found that the oral gavage method may be an inappropriate technique

for BPA and other EDC experiments, as this method does not accurately represent the actual ingestion and absorption process by rodents and humans^(16,17,18,83,85).

Specifically, “most previous studies have relied on the gavage method, where BPA is distributed evenly in the gut. This is not how food actually enters our bodies. We chew it, move it around in our mouths, and it interacts with numerous surfaces--our tongue, cheeks, etc--before it enters the stomach.”⁽¹⁶⁾. In other words, using the gavage method would suggest that there shouldn't be any unmetabolized BPA in the blood after exposure, however many studies find that, in fact, there is^(16,17,83,84).

In a study using a dog model, researchers compared gavage methods of BPA delivery versus sublingual exposure methods, and found that BPA absorption into the body was significantly higher using sublingual methods than gavage techniques⁽¹⁷⁾. Specifically, the levels of BPA in the dogs' bodies were significantly higher in those dogs' receiving the sublingual BPA treatment than the levels of BPA in those dogs' receiving BPA through gavage. The study concluded that BPA is more efficiently and rapidly absorbed through the mucosa of the mouth, resulting in much higher BPA levels in the body than using gavage methods⁽¹⁷⁾.

This means that a study using gavage results in BPA levels in the bodies of their test subjects that are significantly lower than they would be in a more “real world” scenario such as sublingual absorption and therefore the conclusions from which would be inaccurate. Similar studies in the mice model have found similar results, in that exposure of BPA through consumption of a diet treated with BPA results in a more natural route of exposure than through oral gavage^(18,86).

Inappropriate Rat Strain

It has been found that there are clear species and strain-specific differences in sensitivity to various estrogenic compounds and EDCs^(4,5,41,42,44,45), so using an animal model that has been shown to respond to the compound(s) in question is crucial for accurate interpretations of results. Specifically, depending upon the strain of rat utilized, results for similar experiments have been found to be markedly different.

Different strains of rats appear to have varying sensitivities to compounds with various estrogenic activities, for example with Charles River Sprague-Dawley rats, studies have found that they are very insensitive to BPA and other estrogenic compounds, thus using them in low-dose studies is inappropriate and not representative of how the compounds act in more sensitive strains or species^(4,5,6,43,44,45). In the current FDA study under review, they chose to use Sprague-Dawley rats as their animal model⁽¹⁾.

According to the results of one meta-analysis of all BPA literature through the end of 2004, 90% of government-funded peer-reviewed research articles found adverse effects of BPA exposure⁽⁵⁾. A side note: the same meta-analysis discovered that of the industry-funded research articles on BPA, 0% found any adverse effects of BPA exposure, which raises more red flags⁽⁵⁾.

Taking only the government-funded peer-reviewed research articles in consideration, when those studies that used Charles River Sprague-Dawley rats were removed from the meta-analysis, the number of studies finding adverse effects of BPA exposure increased from 90% to 96%⁽⁵⁾. This jump indicates that the use of the appropriate species be used in BPA experiments, as some strains (including Charles River Sprague-Dawley rats) are not sensitive enough to accurately reflect what could be occurring in the human model⁽⁶⁾.

Poor choice of outcome measures

Another flaw with the FDA study is that they chose to focus primarily on the toxic effects of BPA exposure on a set of anatomical outcome measures that has been shown to be unacceptable and unrepresentative of BPA or other EDC exposure over the years^(6,7). A major part of the problem is that studies by federal agencies are required to follow OECD Guidelines (OECDG), which often necessitates the use of multi-generational tests in order to stand up in a court of law if needed⁽⁶⁾.

The problem with OECDG multi-generational testing regimes is that they use out-of-date testing methods and outcome measures which do not fully capture the effects of BPA exposure, and completely ignore the more contemporary methods and outcome measures like gene expression, DNA methylation, cellular pathways, or epigenetics^(6,7).

Specifically, the FDA study ignored current science that indicates that DNA methylation and other epigenetic effects along with interference with cellular signaling pathways are responsible for more relevant biological phenomena that affect obesity^(47,48,49), cancer^(50,51,52), Alzheimer's Disease^(53,54,55) and other maladies promoted by BPA and other EDCs^(56,57,58,59,60).

Including the FDA study current under review, these types of studies focus mainly on anatomical measurements, gross pathological observations, and other macroscopic outcome measures⁽¹⁾. Since the 1990s, there have been vast improvements in BPA and EDC research methods, with the understanding that out-of-date OECDG methods established as early as the 1950s are not sensitive enough to detect effects of low-dose exposure of BPA and other EDCs. However, federal regulatory agencies still insist on following out-of-date protocols that were created prior to the improvements in our understanding of BPA and EDC exposure on more sensitive measures (DNA methylation, cellular signaling pathways, etc.)^(7,61,62).

Failure to use current science in BPA and EDC research

Even though OECDG-based testing regimes do not accurately reflect the harmful public health hazards of BPA or other EDC exposures, federal regulatory outcomes and decisions are still based on those studies using OECDG methods and not based on the many hundreds of academic studies that use more modern techniques and have overwhelmingly found harmful effects of BPA and other EDCs on various human health aspects^(6,7,26,46,61,62,63,64).

Both the United States FDA and European Food Safety Authority have in recent years published information showing that their conclusions regarding the safety of BPA has been based solely on the results of a small number of studies following OECD guidelines, while ignoring the expert advice and research of over 700 peer-reviewed academic articles as of 2006^(6,7). Since that time, there have been many hundreds more articles demonstrating harmful public health effects of BPA and other EDCs, while the FDA and other federal regulatory agencies continue to ignore this mountainous evidence and respond with their own poorly-designed experiments and out-of-touch science^(6,7,26,46,61,62,63,64).

Erroneous claim that BPA is “weak” estrogen

It was originally thought that BPA was a relatively weak estrogenic compound, evidence is mounting that BPA is just as strong an estrogenic compound than natural hormones like estradiol^(5,32,36). In other words, what was thought of as being a relatively benign, BPA can actually cause significant

problems at the levels currently present in the environment including but not limited to cancer, low testosterone and low sperm motility, miscarriages, and birth defects^(23,33,34,35,37).

In the investigation by Delclos *et al.*, the authors stated that “BPA-induced effects partially overlapped those induced by EE₂ estradiol, consistent with the known weak estrogenic activity of BPA”⁽¹⁾. While this may have been the understanding very early on in BPA-related research^(65,66,67), a significant amount of research has been done since then that refutes this “weak” claim. In fact, many studies are now finding that BPA can elicit estrogen-like activity that has been shown to be as strong as, if not stronger, than EE₂, the mechanisms by which are different than “traditional” EE₂ pathways^(8,9,68,69,70).

Specifically, more recent studies have found that BPA promotes very fast responses, even at low doses, via “non-classical estrogen pathways” and the activation of many different transduction signaling pathways in different cell types which when combined results in greater effects related to gene expression triggered by estrogenic action^(8,9,10,71,72).

Failure to identify well-known exogenous source(s) of BPA contamination

Feed: One potential source of BPA contamination in the rats in this and many other EDC research studies is the specific diet or feed that is provided to the animals. The FDA paper itself acknowledged this piece of information and stated that “since many of the effects of BPA are thought to occur through interference with estrogen signaling pathways, a soy- and alfalfa-free diet was used and dietary levels of the phytoestrogens genistein and daidzein were monitored, in addition to BPA.”

In fact, most of commercial rodent feed contains the phytoestrogens genistein and daidzein, which show a strong affinity for estrogen receptor beta and have been shown to be, at times, more potent than the effects of environmental estrogens such as DDT and BPA^(11,12,73,74). Studies have confirmed that it is the feed itself that can activate estrogen receptors as a result of estrogenic compounds in the feed, and that it is not due to the metabolism of the food in the gut^(21,75,76).

While they were careful to choose an appropriate diet for minimizing phytoestrogen content in the rodent diets during the experiment, they failed to take into consideration the diet the rats received prior to starting the experiment. In the FDA paper, it was stated that “while in the breeding colony, [the breeders] were maintained with their dams on NIH-41 irradiated feed pellets” from Harlan Laboratories⁽¹⁾.

Coming directly from the Harlan Laboratories website, it does not appear that the NIH-41 diet is phytoestrogen-free. Specifically “the diets 2014, 2016, 2019, and 2020X while not completely “free” are best described as minimal phytoestrogen diets. Quarterly testing of these diets for isoflavones (phytoestrogens from soybean) typically reveals levels ranging from less than detectable to 20 ppm. For some perspective, most traditional rodent diets contain from 200 to 500 ppm isoflavones. Phytoestrogen minimal diets such as 2014, 2016, 2019, and 2020X are generally acceptable for studies where phytoestrogens are of concern.”⁽¹³⁾

In other words, NIH-41 from Harlan Laboratories does not appear to be one of their phytoestrogen minimal diets, indicating that prior to experimentation, the rats at the facility used by the FDA study were, in fact, exposed to a range of phytoestrogens on a daily basis, thus indicating a possible source for the pre-experimental contamination of the rats. After getting in touch directly with Harlan Laboratories about NIH-41, they confirmed that this particular feed contains soy-based products

(5% soybean meal as well as 2% soy oil), thus confirming the potential for exogenous phytoestrogen exposure prior to testing^(25,77).

In regards to the experimental food, while it was reported to be free of the phytoestrogens genistein and daidzein, it was also reported by the FDA researchers to contain “BPA levels below the average analytical blanks.”⁽¹⁾ Even though they controlled for certain phytoestrogens in the animal feed, BPA itself was found in the diet and thus a possible contributor to the BPA contamination of the rats during experimentation^(3,25,77).

Finally, since recent research has indicated that the half-life of BPA may be longer than initially thought^(6,14,78), the low levels of BPA in the feed could be more of a problem than the FDA researchers assumed.

Failure to identify well-known exogenous source(s) of BPA and estrogenic activity of equipment and supplies

Use of polysulfone cages: In addition to the feed, there are many other exogenous sources of estrogens that the rats were exposed to both prior to and during experimentation by the FDA researchers.

Specifically, laboratory animals are in other words inundated with possible sources of BPA and other EDC exposure, including from bedding, cages, water bottles, water, and disinfectant cleaners^(6,11,79,80,81). According to the FDA researchers, their rats were housed in polysulfone cages. While they indicated that equipment used was “low BPA” and that levels were monitored regularly, it wasn’t clear what those values actually were.

Polysulfone pellets are commonly used in many types of equipment for both research and medicine. In fact, one study focusing on kidney hemodialyzers found that polysulfone pellets, which are major components of the hemodialyzer machines, leach a significant amount of BPA into the environment^(15,82).

When combined to create the final machine, the polysulfone does not appear to leach as much BPA as it did when in the pellet form; however, it is possible that after long term exposure, patients requiring prolonged use of this polysulfone-based equipment could be adversely affected^(15,82).

Finally, there was a failure on the part of the FDA researchers to test the estrogenic activity of all these aforementioned possible exogenous sources of estrogens. It is clear they were not in touch with the current state of EDC research, as they would have been more careful testing the estrogen content of everything the rats came in contact with prior to and during experimentation, since many studies have found vast variability in the sources of possible EDC contamination that must be minimized as much as possible in BPA and other EDC experiments.

Failure to measure BPA levels in any of the animals except in the test cohort at the very end of the trial

By not measuring BPA levels of any of the animals from the very beginning, and only measuring BPA levels at the very end of the trial, effectively no consideration was given to actual levels of BPA in the animals. Without having a starting point to compare to, it's near impossible to draw any conclusions based on what was seen at the end⁽³⁸⁾. If the researchers had tested the animals at the

very beginning, they would have found that all animals were contaminated with BPA, even the controls, and at that point some amendments to the protocol could be made.

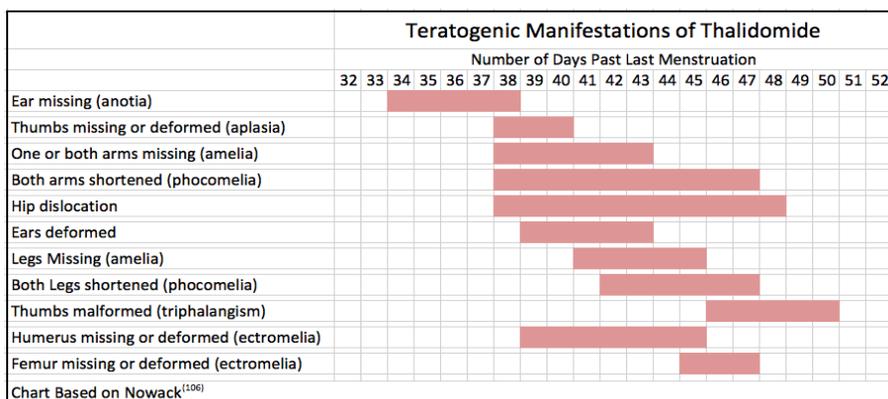
In addition, failure to measure BPA levels in test animals makes it impossible to measure (as opposed to estimate) serum concentrations and their possible correlation to effects or to whether the method used for administering the BPA (oral gavage) may have been flawed.

Wrongly examining toxicity when timing is a bigger issue

One problem that had plagued early BPA and EDC research was examining these compounds in terms of their toxicity, while advancements in this type of research has demonstrated that the timing of the exposure is markedly more critical than simple toxicity alone. Cytotoxicity functions at a coarser level in which cell death and malfunction can be easily observed. Endocrine disruptors function at the level of hormone receptors^(105, 107-110) and also exhibit measurable epigenetic effects⁽¹¹¹⁻¹¹³⁾, and affect gene expression^(114,115).

In other words, a certain level of BPA exposure to an adult may not have much of an effect, while if that same level of BPA is exposed to a developing embryo at a very specific point in its development, a cascading series of events can be "programmed" into the embryo that could develop into significant health problems later in life^(4,22,23,24,87,88).

There is also evidence in the literature that the adult endocrine system is able to maintain a sort of homeostasis, or regulation of a constant balanced environment, thereby may not be as sensitive to the effects of EDC exposure as say if it were going through puberty, a state of marked hormonal flux, or embryonic development, both which have been shown to be significantly more sensitive to BPA and other EDC exposure at those specific times^(4,24,89,90,91,92).



Choosing an outdated model such as "toxicity" is inappropriate when timing is what is most crucial in determining adverse effects of BPA and other EDC exposure.

Significantly, the drug Thalidomide passed traditional toxicity testing and yet caused one of the most prominent pharmaceutical disasters in history⁽¹¹⁶⁾.

One important lesson from the Thalidomide disaster concerns the timing of exposures since BOA and other EDCs can affect hormones, especially in developing As can be seen in the following chart, the drug's effects resulted in very different deleterious effects depending upon the time of exposure.

The lesson to be learned is that the evaluation of endocrine disrupting compounds needs to go beyond traditional toxicity testing. It needs to be tested at a wide variety of relevant concentrations and be thoroughly evaluated at many different timing intervals of exposure using the most sensitive tests possible

Failure to disclose conflicts of interest

Conflicts of interest, while seemingly short and often times an after-thought for some researchers, is a critical part of the publication process. Even if there are no conflicts of interest, it is in the researcher's best interest to provide this information. The existence of conflicts of interest may negatively influence the results of the study in question, essentially invalidating the conclusions presented^(19,93,94).

By disclosing any conflicts, or indicating "none" if that happens to be the case, important information is presented to the reader so that they may be able to cast their own judgment on the legitimacy and objectiveness of the researcher's results and conclusions^(20,93,94). A failure to disclose conflicts of interest casts a skeptical eye on the validity of the entire study even when the experiment is conducted properly.

Conclusions

Given the number of failures, it is hard to see how this paper can be corrected, especially with a tainted data set. The only alternative is for it to be re-done with the failings corrected and a new, valid set of data.

The only other conclusion that can be drawn about a study with so many crippling, obvious, and avoidable flaws is that the regulatory agencies of the U.S. government need to raise their standards to match those of the National Institutes of Health.

NIH has three stringent levels of scrutiny involving peer review: Before grants are awarded, the competency of the investigators is assessed as are, experiment design, appropriate outcomes and use of the most current science and lab techniques⁽⁷⁾. This is followed by post-investigation peer-review, publication in peer-reviewed journals and efforts to follow up the study with replication and updated results. Conflicts of interest are required to be declared.

Declaration of Conflicts of Interest

Neither Lewis Perdue nor Rebecca L. Yeaman have any conflicts of interest to disclose. They claim to be able to speak freely without risking project funding because they have no grants or compensation of any form from any source, either private or public.

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