

COMMENTS ON THE REPORT OF THE EXPERT PANEL ON BISPHENOL A

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P 78, L 29-34

Nagel et al. (205, 206) noted that 17 β -estradiol is extensively protein-bound in vivo and bisphenol A is minimally protein-bound. They suggested that estrogenicity can be more accurately predicted by considering the free fraction of a chemical in serum. **[The Expert Panel notes that Figure 2 does not suggest that bisphenol A is more potent than 17 β -estradiol in vivo than in vitro. The Expert Panel also notes that Nagel et al. appeared to be referring primarily to prediction of developmental effects in the prostate rather than the estrogenic endpoints discussed in this section.]**

RESPONSE

The note in bold represents a fundamental misunderstanding of the role of plasma binding proteins in the pharmacokinetics of steroids and chemicals such as BPA. Due to limited solubility in aqueous medium, steroids and BPA are bound with low affinity and high capacity to albumin, and in human blood (used in the Nagel et al. 1997 study), estradiol and BPA bind to sex hormone binding globulin, a glycoprotein to which these compounds bind with higher affinity relative to albumin. This is true at any time in life. It is accepted that the biologically active fraction of total circulating steroid (as well as BPA) is the fraction that is not bound to plasma proteins; protein-bound steroids or BPA cannot diffuse through the cell membrane (discussed in Ref 206). While the specific focus of the Nagel study was on the potential effects of a higher percent free BPA relative to estradiol on development, the impact of an elevation in free (bioavailable) BPA due to reduced binding in plasma is not restricted to development. The comment in bold is incorrect in that in vitro studies, with few exceptions, are not conducted with medium containing the concentration of plasma proteins that would be present in vivo. Therefore, the appropriate conclusion from the Nagel study is that in an in vitro study in serum-free medium or medium with only a small fraction of the plasma proteins that would be present in vivo, similar amounts of BPA and estradiol will enter the cell, and the greater inhibitory action of plasma proteins on estradiol uptake will not be recognized. For example, if 10-fold more BPA than estradiol enters cells from blood in vivo and BPA is 10 times less potent than estradiol when tested in serum free medium, then, in vivo, BPA will actually have equal potency with estradiol, while in vitro, BPA will appear less potent than estradiol. Any clinician knows that it is the free concentration of steroid hormone that is clinically relevant, but this appears to have not been taken into account here in relation to the fact that in vitro assays typically assume equal access to the receptor from outside of the cell when measuring potency. The point here is not that BPA and estradiol have similar affinities for ER alpha or beta, but that plasma proteins modulate cell uptake and access to the receptor, and thus modulate the cellular response (potency) of the compounds.

P 79, L 30-33.

The assertions of some investigators notwithstanding, the Expert Panel notes that oral bisphenol A does not consistently produce estrogenic responses and, when seen, estrogenic effects after oral treatment occur at high dose levels.]

RESPONSE

What is the panel referring to here? In general, the uterotrophic response is less sensitive than other responses to BPA, regardless of route of exposure. This was also discussed in detail in two review articles that were not cited in the report.

vom Saal, F.S. and Hughes, C. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. *Environ. Health Perspect.* 113:926-933, 2005.

vom Saal, F.S. and Welshons, W.V. Large effects from small exposures: II. The importance of positive controls in low-dose research on bisphenol A. *Environmental Research* 100:50-76, 2006.

In particular, the statement made here: “the Expert Panel notes that oral bisphenol A does not consistently produce estrogenic responses” was discussed in the reviews by vom Saal and Hughes and by vom Saal and Welshons. There are serious flaws in the design, analysis and conclusions of articles that this panel is uncritically accepting as evidence of the absence of low-dose effects of BPA. The two reviews listed above that were not included in this report provide important information refuting the conclusion by the panel that: “oral bisphenol A does not consistently produce estrogenic responses”. Since the decision was made to exclude this information from the report, it will be presented here in a number of different sections that follow.

P 90, L 3-7

“The classical ERs are cytosolic receptors that, when bound, translocate to the nucleus where they produce their activity through alterations in genomic transcription. In contrast, a membrane-bound ER has been described in murine pancreatic islet cells (249-252). This membrane-bound receptor regulates calcium channels and modulates insulin and glucagon release. Bisphenol A has been shown to activate this receptor in vitro at a concentration of 1 nM, which is similar to the active concentration of diethylstilbestrol (249, 251).”

First of all, the person(s) who wrote this is a few decades out of date with regard to knowledge of estrogen receptors, and it would be an embarrassment to the NTP to leave in the report on BPA that ERs are cytosolic receptors. This fact also suggests that the panel members need to make sure that this report contains accurate information. Throughout the report there are numerous factual errors that I found. However, I did not attempt to proof read for accuracy the entire report. Clearly, this needs to be done by someone who knows something about basic endocrinology. BPA is a sex hormone mimicking chemical, the rules that govern the study of systemic toxicants do not apply to BPA, which has been the subject of a number of recent reviews that were not cited in this report.

Welshons, W.V., Thayer, K.S., Taylor, J., Judy, B. and vom Saal, F.S. Large effects from small exposures: I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environ. Health Perspect.* 111: 994-1006, 2003.

vom Saal, F.S. and Hughes, C. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. *Environ. Health Perspect.* 113:926-933, 2005.

vom Saal, F.S. and Welshons, W.V. Large effects from small exposures: II. The importance of positive controls in low-dose research on bisphenol A. *Environmental Research* 100:50-76, 2006.

Welshons, W.V. and vom Saal, F.S. Large effects from small exposures: III. Mechanisms mediating responses to the low doses of the plastic monomer bisphenol A. *Endocrinol.* 147:S56-S69, 2006.

A number of important papers reporting effects of BPA that are mediated via receptors associated with the cell membrane and that initiate calcium influx and rapid changes in cells were not cited nor were they discussed in this section of the report. This is a serious omission of critical information. It is important to note that these studies show effects at doses far below 1 nM (228 ppt) BPA, and that effects via these receptors reveal a similar potency of BPA and estradiol. Thus, while the affinity of BPA for ER alpha or beta is lower than that of estradiol, this difference does not occur for the receptors that mediated these rapid cellular responses. Importantly, these rapid signaling pathways interact with the traditional nuclear hormone receptor pathways.

Farach-Carson, M.C. and P.J. Davis, Steroid hormone interactions with target cells: cross talk between membrane and nuclear pathways. *J Pharmacol Exp Ther*, 2003. 307(3): p. 839-45.

Information about very low dose effects of BPA via rapid response systems not covered in the initial draft of the report follows:

The rapid effects of BPA on intracellular calcium ion concentration have been measured and compared to that of estradiol in breast cancer cell models. A rapid influx of calcium was observed in response to BPA that was significant at the lowest dose tested: 0.1 nM. This response was not blocked by anti-estrogens, demonstrating ER-transactivation independence of these rapid signaling effects of BPA at and below nanomolar concentrations.

Walsh, D.E., P. Dockery, and C.M. Doolan, Estrogen receptor independent rapid non-genomic effects of environmental estrogens on $[Ca^{2+}]_i$ in human breast cancer cells. *Mol Cell Endocrinol*, 2005. 230(1-2): p. 23-30.

A concentrations of BPA as low as 1 pM caused an influx of calcium within 1 minute of exposure in pituitary cells.. In addition, these low concentrations of BPA could induce rapid release of prolactin from pituitary cells via effects that are mediated by the elevation of intracellular calcium. The effects of BPA are similar to those observed for estradiol.

Watson, C.S., C.H. Campbell, and B. Gametchu, Membrane oestrogen receptors on rat pituitary tumour cells: immuno-identification and responses to oestradiol and xenoestrogens. *Exp Physiol*, 1999. 84(6): p. 1013-22.

Watson, C.S., N.N. Bulayeva, A.L. Wozniak, and C.C. Finnerty, Signaling from the membrane via membrane estrogen receptor-alpha: estrogens, xenoestrogens, and phytoestrogens. *Steroids*, 2005. 70(5-7): p. 364-71.

Wozniak, A.L., N.N. Bulayeva, and C.S. Watson, Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca^{2+} fluxes and prolactin release in GH3/B6 pituitary tumor cells. *Environ Health Perspect*, 2005. 113(4): p. 431-9.

Rapid modulation of ERK-signaling in response to BPA has been observed in neurons and immune cells.

Canesi, L., L.C. Lorusso, C. Ciacci, M. Betti, M. Zampini, and G. Gallo, Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. *Gen Comp Endocrinol*, 2004. 138(1): p. 58-69.

In developing neurons of the rat cerebellum, rapid low dose BPA-mediated effects on ERK-signaling have been observed and characterized in vivo and in vitro in isolated primary cultures of cerebellar granule cell neurons. BPA stimulated an inverted U-shaped dose response with equal efficacy and potency as estradiol in the low dose range (1 pM – 1 nM). At concentrations of BPA and estrogen in the micromolar range, an increases in ERK-phosphorylation were again observed. Developmental and pharmacological studies of the rapid actions of estrogen and BPA on cerebellar signaling have revealed a mechanism that results in cell specific activation of ERK1/2-phosphorylation that involved G-proteins, PKA and the Src-family tyrosine kinase. Of considerable interest is the finding that when cerebellar cells were exposed to a mixture of estrogen and BPA at different concentrations, a dose dependant inhibition of these stimulatory actions was caused by BPA with an apparent IC₅₀ in the pM range.

Zsarnovszky, A., H.H. Le, H.S. Wang, and S.M. Belcher, Ontogeny of rapid estrogen-mediated extracellular signal-regulated kinase signaling in the rat cerebellar cortex: potent nongenomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. *Endocrinology*, 2005. 146(12): p. 5388-96.

A related in vivo finding is that in the rat brain. BPA (40 •g/kg) can also antagonizes the action of estradiol in the adult rat hippocampus by blocking the stimulatory effect of estradiol on synaptogenesis.

MacLusky, N.J., T. Hajszan, and C. Leranth, The environmental estrogen bisphenol a inhibits estradiol-induced hippocampal synaptogenesis. *Environ Health Perspect*, 2005. 113(6): p. 675-9.

A recent study examined the effects of low-level BPA (1 pg/ml to 1 µg/ml) on the differentiation of serum-free mouse embryo (SFME) cells and astrocyte progenitor cells (Yamaguchi et al 2006), and the expression of glial fibrillary acidic protein (GFAP) expression as a marker of differentiation was measured. They found that GFAP expression was significantly increased in SFME cells in the presence of 1-100 pg/ml BPA, and these increases were due to activation of signal transducer and activator of transcription 3 (STAT3) and Smad1 by the low-level BPA.

Yamaguchi, H., J. Zhu, T. Yu, K. Sasaki, H. Umetsu, Y. Kidachi, and K. Ryoyama, Low-level bisphenol A increases production of glial fibrillary acidic protein in differentiating astrocyte progenitor cells through excessive STAT3 and Smad1 activation. *Toxicology*, 2006. 226(2-3): p. 131-42.

In isolated murine midbrain astrocytes or in astrocyte/neurons co-culture BPA caused increased GFAP immunoreactivity and increased intracellular Ca⁺⁺ in response to dopamine at concentrations as low as 0.1 pM and 1 pM. An inverted U-shaped low dose was observed between 0.1 pM – 10 pM, with a second phase of effects observed at concentrations of 10 nM to 1 •M. Theses effects were not

observed in response to estradiol at any concentration, suggesting unique actions of BPA (discussed further below).

Miyatake, M., K. Miyagawa, K. Mizuo, M. Narita, and T. Suzuki, *Dynamic changes in dopaminergic neurotransmission induced by a low concentration of bisphenol-A in neurones and astrocytes*. *J Neuroendocrinol*, 2006. **18**(6): p. 434-44.

CARCINOGENICITY

P 109, L 40

While the paper by Huff (Ref 267) was included in the reference list, the panel did not mention any of his arguments concerning his reanalysis of the NTP BPA cancer data and his conclusion that BPA was related to a number of cancers using current methods. At the very least, the panel should acknowledge that there is controversy concerning this subject. Also, the paper by Ho et al. (Ref 336) identifying that neonatal exposure to 10 micrograms / kg /day BPA resulted in prostate interepithelial neoplasia in adulthood in rats was not mentioned in this section. The paper by Vandenberg et al (2006) on the effects of BPA on mammary gland pre-cancerous lesions should also be included here.

IN VIVO STUDIES

P 93, L 30.

The panel should be aware that a new published paper from the lab of Patricia Hunt showing that a very low dose of BPA administered to a pregnant female mouse disrupts chromosomes in the oocytes of the developing female fetus provides additional support for her initial 2003 paper. This shows that the effects of developmental exposure to BPA lead to transgenerational effects that will not be observed until the F2 generation

Susiarjo, M., Hassold, T.J., Freeman, E., Hunt, P.A. Bisphenol A exposure in utero disrupts early oogenesis in the mouse. *PLoS Genet* 3(1) 2007: e5. doi:10.1371/journal.

A major deficiency in the review prepared by the panel is the lack of attention to the strain of rat used in specific studies. For example, there are examples where the panel identified the rat used as being Sprague-Dawley instead of the very different rat that is referred to as the Charles River-Sprague Dawley Crj (CD-SD) rat. As pointed out in vom Saal and Hughes (2005 – reference added above), according to Charles River (CRL 2004), a breeding colony of rats were purchased by Charles River from Sprague-Dawley in 1950. This colony was continuously subjected to selective breeding for rapid postnatal growth and large litter size, and then in both 1991 and 1997 new colonies from selected animals were established.

Every study conducted with the CD-SD rat has concluded that BPA causes no effects at low doses. The CD-SD rat requires very high doses of the potent orally active estrogenic drug, ethinylestradiol, to show effects. This is an inappropriate model animal to use to detect low dose effects of BPA. The rule is to use the most sensitive model animal, not one that is the least sensitive of any model animal available.

Table 1. Biased outcome in low-dose *in vivo* bisphenol A research conducted with just rats and mice due to: 1. source of funding and 2. use of a rat strain (CD-SD) that is insensitive to any exogenous estrogen as of November, 2006.

| SOURCE OF FUNDING | ALL STUDIES REPORTED EFFECT | | CD-SD RAT STUDIES REPORTED EFFECT | | ALL STUDIES EXCEPT CD-SD RATS | |
|-----------------------|-----------------------------|--------------|-----------------------------------|--------------|-------------------------------|----------|
| | HARM | NO HARM | HARM | NO HARM | HARM | NO HARM |
| Government | 138 (92%) | 14 (8%) | 0 (0%) | 10 (100%) | 138 (97.2%) | 4 (2.8%) |
| Chemical Corporations | 0 (0%) | 11 (100%) | 0 (0%) | 3 (100%) | 0 (0%) | 8 (100%) |

STUDIES REPORTING NO *IN VIVO* EFFECTS OF LOW DOSES OF BISPHENOL THAT USED AN INSENSITIVE RAT TO ANY ESTROGEN, THE CHARLES RIVER SPRAGUE-DAWLEY (CD-SD) RAT

Elswick, B.A., Welsch, F., and Janszen, D.B. (2000). Effect of different sampling designs on outcome of endocrine disruptor studies. *Reprod. Toxicol.* 14:359-367.

Ema, M., S. Fujii, M. Furukawa, M. Kiguchi and A. Ikka Tand Harazono (2001). Rat two-generation reproductive toxicity study of bisphenol A. *Reprod. Toxicol.* 15:505-523.

Kamata, R.; Koda, T.; Morohoshi, K.; Umezu, T., and Morita, M. (2005). RNA constitution and estrogen-responsive gene expression in the ovariectomized rat uterus. *Analytical Biochemistry* 341(1):131-140.

Kato, H., Furuhashi T., Tanaka, M., Katsu, Y., Watanabe, H., Ohta, Y., Iguchi, T. (2006). Effects of bisphenol A given neonatally on reproductive functions of male rats. *Reprod. Toxicol.* 22:20-29. PMID: 16311018. The findings reported are consistent with other findings that the CD-SD (Crj:CD (IGS) strain of rat is insensitive to low doses of estrogen. The positive control dose of estradiol of 0.9 mg/kg/day is not a positive control for low dose effects, but, instead, would be an appropriate positive control for high-dose effects.

Kim, HS, Han, SY, Kim, TS, Kwack, SJ, Da Lee, R, Kim, IY, Seok, JH, Lee, BM, Yoo, SD and Park, KL (2002). No androgenic/anti-androgenic effects of bisphenol-A in Hershberger assay using immature castrated rats. *Toxicol. Lett* 135: 111-123.

Kobayashi, K., M. Miyagawa, R. S. Wang, S. Sekiguchi, M. Suda and T. Honma (2002). Effects of in utero and lactational exposure to bisphenol A on somatic growth and anogenital distance in F1 rat offspring. *Ind. Health* 40:375-81.

Kobayashi, K. ; Miyagawa, M.; Wang, R. S.; Suda, M.; Sekiguchi, S., and Honma, T. (2005). Effects of in utero and lactational exposure to bisphenol A on thyroid status in F-1 rat offspring. *Industrial Health.* 43(4):685-690.

Kwon, S., D. B. Stedman, B. A. Elswick, R. C. Cattley and F. Welsch (2000). Pubertal development and reproductive functions of CrI:CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. *Toxicol Sci* 55:399-406.

Masutomi, N., M. Shibutani, H. Takagi, C. Uneyama, K. Y. Lee and M. Hirose (2004). Alteration of pituitary hormone-immunoreactive cell populations in rat offspring after maternal dietary exposure to endocrine-active chemicals. *Arch. Toxicol.* 78:232-40.

Nagao, T., Y. Saito, K. Usumi, M. Kuwagata and K. Imai (1999). Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. *Reprod Toxicol* 13:303-11. The reproductive function in CD-SD rats treated subcutaneously (s.c.) with 300 mg/kg bisphenol A or 2 mg/kg estradiol benzoate (this is a very high dose to use as a positive control) from postnatal Day 1 to 5 was examined after puberty as well as histopathologic changes in reproductive organs.

Takagi, H., M. Shibutani, N. Masutomi, C. Uneyama, N. Takahashi, K. Mitsumori and M. Hirose (2004). Lack of maternal dietary exposure effects of bisphenol A and nonylphenol during the critical period for brain sexual differentiation on the reproductive/endocrine systems in later life. 78:97-105.

Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, Veselica MM, Fail PA, Chang TY, Seely JC, Joiner RL, Butala JH, Dimond SS, Cagen SZ, Shiotsuka RN, Stropp GD, and Waechter JM (2002), Three-Generation Reproductive Toxicity Study of Dietary Bisphenol A in CD Sprague-Dawley Rats. *Toxicol. Sci.* 68: 121-146.

NOTE: This study also used a type of animal feed (Purina 5002) that contains contaminants that can mask the effects of DES (discussed in more detail below), and also used the CD-SD strain of rat that only responds to very high doses of the estrogenic drug ethinylestradiol.

However, on Page 206, L 47, the panel made the following statement concerning this study:

Utility (Adequacy) for CERHR Evaluation Process: This exceptional study is very useful for the evaluation process, and will carry significant weight in the evaluation of structural, histogenic, and fertility endpoints. Given the information provided here and in vom Saal and Hughes (2005 ref provided above), the panel should re-consider this judgement.

Yamasaki, K., M. Sawaki, S. Noda, N. Inmatanaka and M. Takatsuki (2002). "Subacute oral toxicity study of ethinylestradiol and bisphenol A, based on the draft protocol for the 'Enhanced OECD Test Guideline no. 407'." *Arch. Toxicol.* 76: 65-74.

Comparison of the response of the CD-SD rat to BPA and ethinylestradiol

The CD-SD strain of rat showed some responses to a 50- $\mu\text{g}/\text{kg}/\text{day}$ dose of ethinylestradiol administered for 28 days, and more responses at the very high dose of 200 $\mu\text{g}/\text{kg}/\text{day}$ (Yamasaki et al. 2002). Ethinylestradiol is the potent estrogenic drug used by women in birth control pills at a dose of 0.5 $\mu\text{g}/\text{kg}/\text{day}$ (based on a 60-kg woman). The CD-SD rat thus has a very low sensitivity to ethinylestradiol, since relative to women it requires 100 – 400-fold higher doses to produce effects. In contrast, the fetal male CF-1 mouse examined in the initial vom Saal laboratory studies with BPA responded to ethinylestradiol with significant changes in adult sperm production and prostate size at a maternal oral dose of 0.002 $\mu\text{g}/\text{kg}/\text{day}$ (Thayer et al. 2001). The CF-1 male mouse fetus is thus between 25,000 - 100,000-times more sensitive to ethinylestradiol relative to the CD-SD rat. Yamasaki et al. (Yamasaki et al. 2002) also reported that a 600-mg/kg/day dose of BPA was required to see effects in CD-SD rats. This 600-mg/kg/day dose is over 200,000-times higher than the BPA doses used in studies showing low-dose effects of BPA conducted in the vom Saal laboratory, and as indicated above, it is also dramatically higher than doses of BPA required to cause effects in over 130 other low-dose BPA studies conducted with other types of rats and various mouse strains; there are also many low-dose BPA studies showing effects with other experimental vertebrate and invertebrate

animals.

Thayer KA, Ruhlen RL, Howdeshell KL, Buchanan D, Cooke PS, Welshons WV, et al. 2001. Altered reproductive organs in male mice exposed prenatally to sub-clinical doses of 17 α -ethinyl estradiol. *Human Reprod* 16: 988-996.

Another major deficiency in the panel report is that in addition to not paying attention to the sensitivity of the animal model to exogenous estrogens, no concern is expressed based on whether an appropriate positive control, such as ethinylesradiol or DES, was included. In fact, most of the studies reporting finding no low dose effects of BPA did not include a positive control, leaving one with no idea as to why the experiment failed. For example, could the experiment have been contaminated by other estrogens? At least in some cases, the answer is quite likely yes, as shown below.

Two industry-funded studies (Ashby et al. 1999; Cagen et al. 1999) were designed with DES included as a positive control, which was reported (Toloken 1998) by industry spokesmen at a public news briefing about the Cagen et al. study. A critique (ENDS 1998) pointed out that the positive control, DES, failed to show a difference from the negative controls in each of these studies (Ashby et al. 1999; Cagen et al. 1999). However, the authors did not indicate in either of the published articles (Ashby et al. 1999; Cagen et al. 1999) that DES had been included in the study as the positive control. Subsequent studies funded by chemical corporations, all of which have reported the absence of significant effects for low doses of BPA, avoided this problem by simply not including a positive control in the experiment.

An NTP (2001) panel consisting of 36 internationally recognized experts commented on the issue regarding: “a study in which the positive control does not produce the expected positive response. The prudent course of action in such cases may be to declare the study inadequate and repeat it, regardless of the experimental outcome in the test groups” (NTP 2001; p 5-10). The NTP panel went on to note that: “For those studies that included DES exposure groups, those that showed an effect with BPA showed a similar low-dose effect with DES (e.g. prostate and uterus enlargement in mice), while those that showed no effect with BPA also found no effect with DES.” As articulated by the NTP panel, only by including in an experiment a positive control estrogenic chemical, such as DES or ethinylestradiol, can the reason for the failure to find low-dose effects of BPA be determined to be due to either inactivity of the uchemical, insensitivity of the model animal, or some other variable, such as the type of feed used.

The inclusion of a positive control in an experiment and the failure to find a difference between the positive control and negative control is not a “weakness” as described in the current panel report, it is a fatal flaw, and the study should be rejected. Designing a study with a positive control and when the positive control fails to show an effect, not identifying the actual design as including a positive control in the published report is fraud.

On page 132, L 21-22 the absence of a significant difference between the negative and positive control (DES) in the study by Cagen et al. is identified as a “weakness” rather than a fatal flaw.

NTP. 2001. Final Report of the Endocrine Disruptors Low Dose Peer Review Panel. Raleigh, NC. <http://ntp.niehs.nih.gov/index.cfm?objectid=06F5CE98-E82F-8182-7FA81C02D3690D47>. [accessed 17 February 2005].

Ashby J, Tinwell H, Haseman J. 1999. Lack of effects for low dose levels of bisphenol A (BPA) and diethylstilbestrol (DES) on the prostate gland of CF1 mice exposed in utero. *Reg Tox Pharm* 30: 156-166.

Cagen SZ, Waechter JM, Dimond SS, Breslin WJ, Butala JH, Jekat FW, et al. 1999. Normal reproductive organ development in CF-1 mice following prenatal exposure to Bisphenol A. *Tox Sci* 11: 15-29.

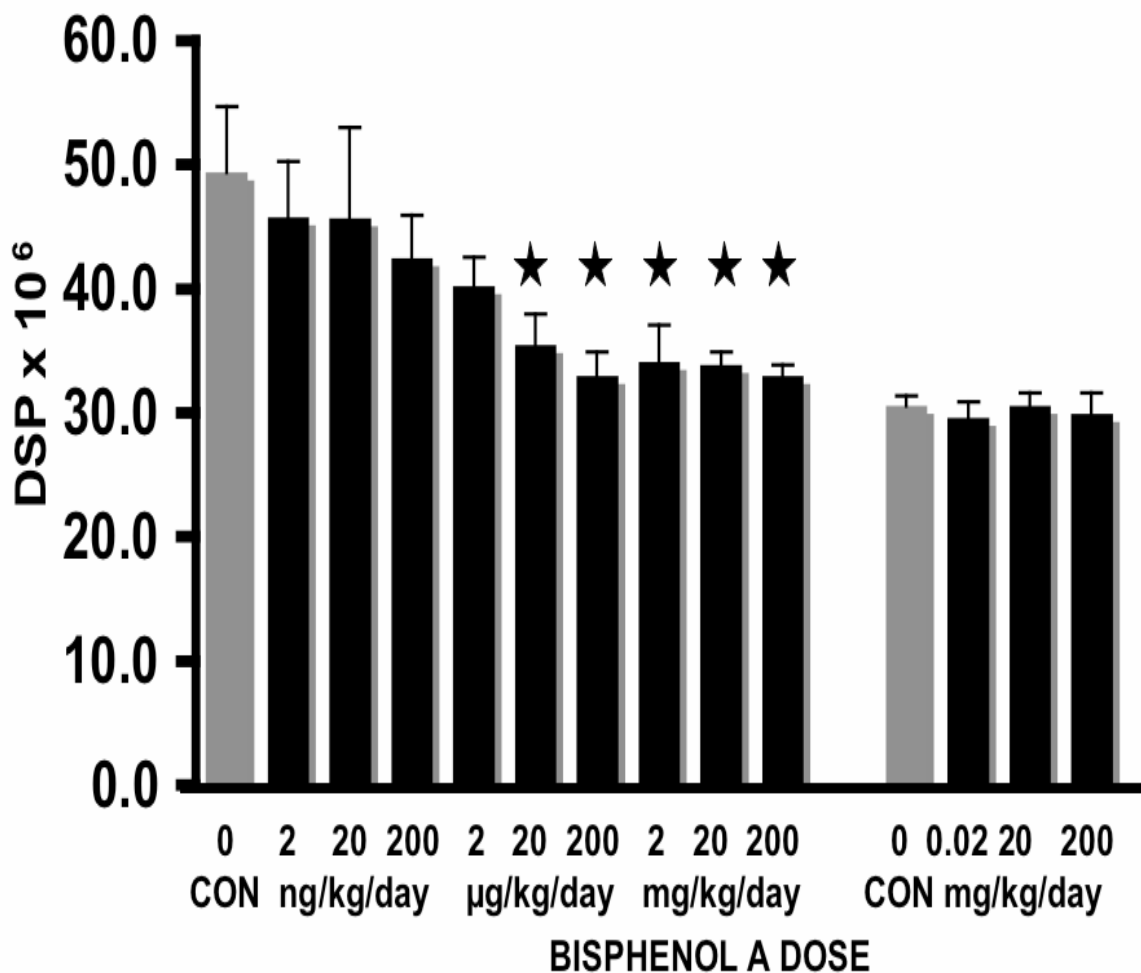
ENDS. 1998. Industry oestrogen study "fundamentally flawed". *The ENDS Report* 285: 4-5.
Toloken S. 1998. SPI study disputes endocrine disruptor findings. *Plastic News*. 16 October.

An example of the problem caused when a study does not include a positive control and finds no significant differences between the negative control and test chemical on any outcome was discussed in detail in the paper by vom Saal and Welshons (2006 reference provided above). The following figure comparing the findings from Sakaue et al and Ashby et al (presumably the Ashby study was an exact replication of the Sakaue study using the same type of rat and same feed) was included in the vom Saal and Welshons publication.

Ashby, J., H. Tinwell, P. A. Lefevre, R. Joiner and J. Haseman, 2003. The effect on sperm production in adult Sprague-Dawley rats exposed by gavage to bisphenol A between postnatal days 91-97. *Toxicol. Sci.* 74(1), 129-38.

Sakaue, M., S. Ohsako, R. Ishimura, S. Kurosawa, M. Kurohmaru, Y. Hayashi, Y. Aoki, J. Yonemoto and C. Tohyama, 2001. Bisphenol A affects spermatogenesis in the adult rat even at a low dose. *J. Occupat. Health* 43, 185-190.

COMPARISON OF TWO STUDIES OF BISPHENOL A EFFECTS ON DAILY SPERM PRODUCTION IN ADULT MALE RATS



Sakaue et al., 2001
J. Occup. Health

Ashby et al. 2003
Toxicol. Sci.

What is clear from the data presented in the figure comparing the Ashby and Sakaue findings is that in attempting to replicate the BPA finding from the Chiharu Tohyama laboratory at the Japanese NIH (Sakaue et al. 2001) with the same strain used in the study conducted in the Tohyama laboratory as well as the same type of feed, the mean daily sperm production value for the negative controls in the Ashby et al. (2003) study was identical to the maximum inhibitory effect reported by Tohyama laboratory after exposure to BPA. However, in the abstract, Ashby et al. (2003) stated: “No explanation for our failure to replicate the effects reported by Sakaue et al. is evident.” If a positive control, such as DES, had been included in the Ashby et al. (2003) experiment, it would have allowed this group to determine whether daily sperm production in the negative controls in the Ashby laboratory was maximally suppressed by some contaminating factor, and the animals were thus insensitive to the suppressive effect of any estrogen. Instead, by not including a positive control,

Ashby et al. (2003) concluded that low doses of BPA caused no significant effects, consistent with 4 other published reports from his laboratory about BPA, and in marked contrast to numerous other published studies reporting in vivo effects of similar low doses of BPA on testicular sperm production in mice and rats, as well as many other outcomes described in the other studies conducted in the Ashby laboratory at Zeneca.

FEED AS A SOURCE OF VARIABILITY IN OUTCOME IN RESEARCH ON BPA AND OTHER ESTROGENIC CHEMICALS

The NIH recently held two workshops to address the issue of contaminants in feed and batch-to-batch variability in feed, and a manuscript has been written and submitted regarding the contaminants found in feeds used in laboratory research. Importantly, both soy-based and casein-based feeds show significant contamination with estrogenic compounds as well as batch-to-batch variability in estrogenic contaminants (not just variable levels of soy phytoestrogens).

In more detail, a critical issue in experiments concerning effects of low doses of estrogenic chemicals such as BPA is that a common rodent feed used in toxicological studies (Purina 5002) has been reported by investigators at NIH to be highly variable in its estrogenic activity. These investigators reported that some batches of this feed were able to interfere with the ability to detect puberty-accelerating effects of DES in female CD-1 mice, due to the feed maximally advancing the age at puberty in control females (Thigpen et al. 2003). The use of this particular feed in the Cagen (Cagen et al. 1999) and Tyl (Tyl et al. 2002) studies raises the possibility that endocrine disrupting components in this feed played a role in the failure of these studies to show low-dose effects of BPA; the Cagen (Cagen et al. 1999) study also failed to find significant effects of the positive control DES, while the Tyl (Tyl et al. 2002) study did not include a positive control.

The panel report did not recognize the Tyl et al study as having any weaknesses and obviously did not take into account the absence of the need to include a positive control (to determine whether the animals were contaminated by estrogenic contaminants in the Purina 5002 feed that was used), and the insensitivity of the CD-SD rat used in this study.

Thigpen JE, Haseman JK, Saunders HE, Setchell KDR, Grant MG, Forsythe DB. 2003. Dietary phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. *Comp Med* 53: 477-485.

OTHER COMMENTS

P 175, L 8.

I have to wonder how carefully this report was prepared if the lack of clarity about the strain of mouse used and the method of assigning males to groups could be included as a criticism. The beginning of the “Animals” section of this publication (see p 243) states: “CF-1 mice were purchased from Charles River Laboratories...”. Regarding the selection of males, it was stated that they were “randomly selected” and that the males were those used to report the prostate data in the Nagel et al study discussed below, where we clearly identified that only one randomly selected male per litter was used.

P 175, L 43

The Nagel et al. study is also criticized as lacking information about the animal that was used and for inappropriate experimental methods. Again, on p 71 it is stated that the CF-1 mouse (*Mus musculus domesticus*) was purchased from Charles River Laboratories....". On page 72 we stated that "one male per litter was randomly selected". Inclusion of these criticisms in the report weakens the impact of the findings. This is unacceptable. Clearly, someone should have more carefully reviewed the methods before making these criticisms. I hope that prior to publication a more thorough review of criticisms of papers is conducted. If two of my articles contain inaccurate criticisms, I have to assume that there are many more errors in this report. Are these errors random or directed at one segment of this contested literature?

P 177, L 14 and P 178, L 28.

The statement that the panel is unaware why the 0.1 microgram per kg per day dose of DES should be expected to cause an effect (this was the positive control chemical and dose used by Cagen et al. and Ashby et al.) is very disturbing. This suggests that the panel is unaware of the published DES literature. There are numerous published studies showing effects of DES at this dose, as well as at lower doses, with a number of these papers coming from studies conducted by scientists in the NTP (numerous papers by John McLachlan and Retha Newbold going back 20 years).

The lack of a positive finding for the positive control chemical means that the experiments conducted by Ashby et al. and Cagen et al. failed and should be rejected! Anyone trained in experimental research should know this. Thus, it appears that in an attempt to justify including the Ashby et al and Cagen et al findings in this report, the panel attempts to make it appear that the positive control dose was not appropriate. It would clearly be inappropriate to ignore the published studies showing DES at the dose used by Ashby and by Cagen should have produced positive effects on the outcomes that they examined.

PUBLISHED PAPERS SHOWING EFFECTS AT THE LOW DOSE OF DES USED BY ASHBY AND BY CAGEN CAUSED EFFECTS; THIS LIST ALSO INCLUDES PAPERS THAT REPORTED LOW-DOSE EFFECTS OF BPA

Atanassova, N., C. McKinnell, K. J. Turner, M. Walker, J. S. Fisher, M. Morley, M. R. Millar, N. P. Groome and R. M. Sharpe (2000). Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and the relationship to adult testis size and fertility: evidence for stimulatory effects of low estrogen levels. *Endocrinology* 141:3898-3907.

Gupta, C. (2000). Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. *Proc. Soc. Exp. Biol. Med.* 224:61-68.

Honma, S., Suzuki, A., Buchanan, D. L., Katsu, Y., Watanabe, H. and Iguchi, T. (2002). Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. *Reprod. Toxicol.* 16:117-122.

Nikaido, Y., K. Yoshizawa, N. Danbara, M. Tsujita-Kyutoku, T. Yuri, N. Uehara and A. Tsubura (2004). Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring. *Reprod Toxicol* 18:803-811.

Timms, B. G., K. L. Howdeshell, L. Barton, S. Bradley, C. A. Richter and F. S. vom Saal (2005). Estrogenic chemicals in plastic and oral contraceptives disrupt development of the mouse prostate and urethra. *Proc. Natl. Acad. Sci.* 102:7014-7019.

Thuillier, R., Wang, Y. and Culty, M. (2003). Prenatal Exposure to Estrogenic Compounds Alters the Expression Pattern of Platelet-Derived Growth Factor Receptors alpha and beta in Neonatal Rat Testis: Identification of Gonocytes as Targets of Estrogen Exposure. *Biol. Reprod.* 68:867-880.

vom Saal, F. S., B. G. Timms, M. M. Montano, P. Palanza, K. A. Thayer, S. C. Nagel, M. D. Dhar, V. K. Ganjam, S. Parmigiani and W. V. Welshons (1997). Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *Proc. Nat. Acad. Sci.* 94(5): 2056-2061. (BPA was not examined in this study)

Wang, Y., R. Thuillier and M. Culty (2004). Prenatal estrogen exposure differentially affects estrogen receptor-associated proteins in rat testis gonocytes. *Biol Reprod* 71:1652-64.

P 177, L 18. “Support not indicated”. Are the members of the panel really unaware that John Ashby, in whose lab 5 studies reporting no low-dose effects of BPA were conducted, worked for Zeneca, and these studies, along with the Cagen studies, were a coordinated set of studies funded by chemical industry trade organizations (CEFIC and SPI)? The reality that who you work for and who funds the study influences the results of research cannot be denied (tobacco, beverage industry, lead industry, bisphenol A, atozine, etc.). Regardless of the data (100% of industry-funded BPA studies report finding no effects of low doses of BPA), defenders of the corporations that manufacture BPA will fight to not allow this information to be discussed. A document that provides this information is posted at: <http://endocrinedisruptors.missouri.edu/vomsaal/vomsaal.html>

P 179, L 13. The statement: “[It was not clear if the data presented were covaried with body weight.]” We state in this paper the following “We found that prenatal treatment with bisphenol A significantly reduced the number of days between vaginal opening and first vaginal oestrus, which is highly correlated with the first postpubertal ovulation ... based on analysis of covariance adjusted for body weight at weaning”. This is another example of inaccurate criticism.

P 179, L 34-39.

The critique here is interesting with regard to the process referred to as puberty. This is a prolonged process that has various landmarks, such as vaginal opening, and culminates in females with the first ovulation, signaling the onset of fertility and end of the pubertal transition. The epidemiological literature examines events such as time of appearance of pubic hair and breast buds and the interval to the first menstruation as biologically meaningful information. My recommendation is that the person(s) who wrote this critique should consult other members of the panel who have experience studying puberty and eliminate this critique.

Another critique here was: “Although the authors identified a litter-based analysis, in Study figure 1, the n values exceed the number of dams, suggesting that some of the data were analyzed on a per pup basis.”

RESPONSE

There is a lack of understanding of the intrauterine position phenomenon. Litters are delivered by Cesarean section and the intrauterine position of fetuses is recorded; pups are reared by foster

mothers. The 0M, 1M and 2M animals are siblings, and animals from 21 litters per treatment (control and BPA) were examined. This was explained in the publication.

P181, L 30-31.

Any scientist who considers a new finding that no other study has ever examined to be a “weakness” is coming from a very different place than I am regarding the view that until shown to be reliable or not, data are data. BPA is a selective estrogen receptor modulator (SERM) that this report suggests has some unique effects. When we reported that BPA increased prostate size, the person who made this statement would have probably considered our finding to be a “weakness”. Now, that finding has been replicated and BPA treatment in the same dose range results in pre-cancerous prostate lesions in adulthood. The perception that this finding is a “weakness” is not appropriate, any more than the finding by MacLusky et al. (2005 reference provided above) that BPA causes the opposite effect relative to estradiol in the hippocampus is a “weakness”. The panel appears not to have connected this finding to that of Honma et al (2002) that adult AGD is increased by prenatal BPA exposure. This bizarre comment should be deleted from this report.

The purpose of a report such as this is to “connect the dots”. One can only hope that this will occur prior to the final report being issued.