BISPHENOL A
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NOTE: This document provides references from the published scientific literature concerning bisphenol A, focusing on "low dose" *in vivo* effects, molecular mechanisms based primarily on *in vitro* studies, sources of exposure and pharmacokinetics. None of these reference lists should be considered to be comprehensive.
This is posted on the web at**:** http://endocrinedisruptors.missouri.edu/vomsaal/vomsaal.html

CURRENT NUMBER OF PUBLISHED STUDIES REPORTING SIGNIFICANT ADVERSE EFFECTS OR NO EVIEDNCE OF HARM DUE TO ADMINISTRATION OF LOW DOSES OF BISPHENOL A (BELOW 50 MG/KD/DAY) TO EXPERIMENTAL ANIMALS

151 STUDIES ON LOW DOSE EFFECTS OF BISPHENOL A IN ANIMALS

129 published studies reporting significant, and in many cases clearly adverse, effects
23 published studies reporting no evidence of harm

There are 2 major factors that predict the finding of no evidence of harm in these studies:

1. **Strain of rat used was a predictor of finding harm or no harm:**
   10 / 23 studies reporting no harm used the CD-SD (Crl:CD) rat
   – all 10 (100%) of the CD-SD rat studies reported no harm
   (3 / 12 negative studies funded by chemical corporations used the CD-SD rat)
   (7 / 11 negative studies funded by governments used the CD-SD rat)

The Charles River Sprague-Dawley (CD-SD) rat colony was subjected to selection over a 40-year period by Charles River for very large litters and large body size, and CD-SD rats are no longer similar in phenotype to the Sprague-Dawley rats that were purchased by Charles River in 1950. CD-SD rats require high doses of potent estrogenic drug ethinylestradiol to show responses. The CD-SD strain of rat is insensitive to any estrogenic chemical, not just bisphenol A.

2. **Source of funding:**
   12 / 23 studies reporting no harm were funded by chemical corporations

SOURCE OF FUNDING	STUDY OUTCOME HARM	NO HARM	
Government	**129 (92%)**	**11 (8%)**	**140**
Chemical Corporations	**0 (0%)**	**12 (100%)**	**12**
	129	**23**	**152**

This is a highly biased distribution of outcomes based on source of funding.

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I. BISPHENOL A: LOAEL AND REFERENCE DOSE

CASRN (80-05-7)
1 nM = 228 ppt (molecular weight = 228)
LOAEL = 50 mg/kg/day, Based on studies in the 1980s that used only very high doses, the lowest dose that was examined (50 mg/kg/day) caused adverse effects, and this is termed the LOAEL Acceptable Daily Intake level (also called the Reference Dose) = 50 µg/kg/day. The amount that is predicted based on models to be safe for humans is calculated by dividing the LOAEL by 1000 (see IRIS, US-EPA). Until the 1990's the assumption that 50 µg/kg/day bisphenol A was safe was not challenged by directly examining this dose to see if it actually caused effects.

IRIS (1988). Bisphenol A. (CASRN 80-05-7), US-EPA Integrated Risk Information System Substance file. http://www.epa.gov/iris/subst/0356.htm. Accessed, 2002.

II. US-NATIONAL TOXICOLOGY PROGRAM WITHIN THE NIH: PEER REVIEW OF THE "LOW DOSE" LITERATURE, INCLUDING BISPHENOL A, OCTOBER, 2000

NTP, N. T. P. (2001). Endocrine Disruptors Low Dose Peer Review, Raleigh, NC, http://ntp-server.niehs.nih.gov/htdocs/liason/LowDoseWebPage.html

III. ARTICLES DISCUSSING IMPLICATIONS FOR RISK ASSESSMENT OF FINDINGS:

1. AT DOSES BELOW THE PRIOR LOAEL (50 mg/kg/day)
2. OF NON-MONOTONIC (INVERTED-U) DOSE-RESPONSE FUNCTIONS
3. OF NO THRESHOLD FOR ENDOCRINE DISRUPTING CHEMICALS

Sheehan, D. M., Willingham, E., Gaylor, D., Bergeron, J. M. and Crews, D. (1999). No threshold dose for estradiol-induced sex reversal of turtle embryos: how little is too much? Environ. Health Perspect. 107:155-159.

Sheehan, D. M. (2000). Activity of environmentally relevant low doses of endocrine disruptors and the bisphenol A controversy: Initial results confirmed. Proc Soc Exp Biol Med 224:57-60.

This article by Sheehan discusses the importance to science and regulators of the publication by Gupta reporting that she replicated our findings with bisphenol A in a series of studies, validating prior published findings in the eyes of unbiased scientists.

Sheehan, D.M. (2005). No-threshold dose-response curves for nongenotoxic chemicals: Findings and applications for risk assessment. Environ Res. Online, Nov. 2005 www.sciencedirect.com

We tested the hypothesis that no threshold exists when estradiol acts through the same mechanism as an active endogenous estrogen. A Michaelis-Menten (MM) equation accounting for response saturation, background effects, and endogenous estrogen level fit a turtle sex-reversal data set with no threshold and estimated the endogenous dose. Additionally, 31 diverse literature dose-response data sets were analyzed by adding a term for nonhormonal background; good fits were obtained but endogenous dose estimations were not significant due to low resolving power. No thresholds were observed. Data sets were plotted using a normalized MM equation; all 178 data points were accommodated on a single graph. Response rates from approximately 1% to >95% were well fit. The findings contradict the threshold assumption and low-dose safety. Calculating risk and

assuming additivity of effects from multiple chemicals acting through the same mechanism rather than assuming a safe dose for nonthresholded curves is appropriate.

vom Saal, F.S. and Sheehan, D.M. Challenging risk assessment. Forum for Appllied Research and Public Policy, 13(3):11-18, 1998.

vom Saal, F.S. and Welshons, W.V. NIH panel confirms that endocrine disrupting chemicals cause effects at very low doses. Risk Policy Report 7(11):47-50, November 30, 2000. Inside Washington Publishers. Source: Risk Policy Report via InsideEPA.com

vom Saal, F. S. V.; Richter, C. A.; Ruhlen, R. R.; Nagel, S. C.; Timms, B. G., and Welshons, W. V. The importance of appropriate controls, animal feed, and animal models in interpreting results from low-dose studies of bisphenol A. Birth Defects Research Part A-Clinical & Molecular Teratology. 2005; 73(3):140-145. ISSN: 1542-0752.

Interpreting results of studies that report only negative effects is problematic. A number of published studies to determine whether chemicals with estrogenic activity can cause effects at low doses have not taken into account the possibility that the commercial animal feed being used can mask effects of even potent estrogenic drugs such as diethylstilbestrol (DES). In addition, the sensitivity of the strain of animal being used for the specific category of chemical being tested has not always been described. For environmental chemicals, such as the estrogenic polycarbonate plastic monomer bisphenol A, DES is an appropriate positive control for estrogenic effects, and using an appropriate low dose of DES can eliminate the possibility of false-negative conclusions of safety when the above or other variables contribute to the negative outcome. Only when simultaneous positive effects of low doses of a positive control chemical such as DES and negative effects of environmentally relevant low doses of the test chemical are demonstrated within the same experiment are conclusions of no effect of the test chemical warranted, and this has not been reported for bisphenol A in any study. Instead, more than 90 refereed journal publications have reported effects due to exposure to low doses of bisphenol A in a wide variety of animals (for references see: http://rcp.missouri.edu/endocrinedisruptors/vomsaal/vomsaal.html). However, due to lack of attention to the importance of appropriate positive controls, a small number of studies reporting negative effects of bisphenol A have created a false sense of controversy regarding low-dose effects of bisphenol A.

vom Saal, F.S. and Hughes, C. (2005). 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.

Bisphenol A (BPA) is the monomer used to manufacture polycarbonate plastic, the resin lining of cans, and other products, with global capacity in excess of 6.4 billion lb/year. Because the ester bonds in these BPA-based polymers are subject to hydrolysis, leaching of BPA has led to widespread human exposure. A recent report prepared by the Harvard Center for Risk Analysis and funded by the American Plastics Council concluded that evidence for low-dose effects of BPA is weak on the basis of a review of only 19 studies; the report was issued after a delay of 2.5 years. A current comprehensive review of the literature reveals that the opposite is true. As of December 2004, there were 115 published in vivo studies concerning low-dose effects of BPA, and 94 of these report significant effects. In 31 publications with vertebrate and invertebrate animals, significant effects occurred below the predicted "safe" or reference dose of 50 microg/kg/day BPA. An estrogenic mode of action of BPA is confirmed by in vitro experiments, which describe disruption of cell function at 10(superscript)- 12(/superscript) M or 0.23 ppt. Nonetheless, chemical manufacturers continue to discount these published findings because no industry-funded studies have reported significant effects

of low doses of BPA, although > 90% of government-funded studies have reported significant effects. Some industry-funded studies have ignored the results of positive controls, and many studies reporting no significant effects used a strain of rat that is inappropriate for the study of estrogenic responses. We propose that a new risk assessment for BPA is needed based on a) the extensive new literature reporting adverse effects in animals at doses below the current reference dose; b) the high rate of leaching of BPA from food and beverage containers, leading to widespread human exposure; c) reports that the median BPA level in human blood and tissues, including in human fetal blood, is higher than the level that causes adverse effects in mice; and d) recent epidemiologic evidence that BPA is related to disease in women.

Vom Saal, F.S. and Welshons, W.V. (2005). Large effects from small exposures. II. The importance of positive controls in low-dose research on bisphenol A. Environ. Res. Online: Nov, 2005 www.sciencedirect.com.

Over six-billion pounds per year of the monomer bisphenol A (BPA) are used to manufacture polycarbonate plastic products, resins lining cans, dental sealants, and polyvinyl chloride plastic products. There are 109 published studies as of July 2005 that report significant effects of low doses of BPA in experimental animals, with many adverse effects occurring at blood levels in animals within and below average blood levels in humans; 40 studies report effects below the current reference dose of 50mug/kg/day that is still assumed to be safe by the US-FDA and US-EPA in complete disregard of the published findings. The extensive list of significant findings from government-funded studies is compared to the 11 published studies that were funded by the chemical industry, 100% of which conclude that BPA causes no significant effects. We discuss the importance of appropriate controls in toxicological research and that positive controls are required to determine whether conclusions from experiments that report no significant effects are valid or false.

vom Saal, F.S., Nagel, S.C., Timms, B.G. and Welshons, W.V. (2005). Implications for human health of the extensive bisphenol A literature showing adverse effects at low doses: a response to attempts to mislead the public. Toxicology 212:244-52, author reply 253-4.

Welshons, W.V., Thayer, K.S., Taylor, J., Judy, B. and vom Saal, F.S. (2003). Large effects from small exposures: I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. Environ. Health Perspect. 111(8): 994-1006.

IV. REPORT IN 1936 THAT BISPHENOL A HAD FULL ESTROGENIC ACTIVITY IN A STUDY OF ESTROGENIC CHEMICALS, AND THEN IN 1938, REPORT ON THE SYNTHESIS OF A SIMILAR MOLEDULE, DES

(The 1936 publication was 17 years prior to synthesis of polycarbonate from bisphenol A in 1952)

Dodds, E.C. and Lawson, W. Synthetic oestrogenic agents without the phenanthrene nucleus. Nature 137:996, 1936.

Approximately 70 mg/kg/day bisphenol A was injected into ovariectomized adult rats, and cornification of the vaginal epithelium, similar to that caused by estradiol, was observed.

Dodds, E. C., W. Lawson and R. L. Noble (1938). Biological effects of the synthetic oestrogenic substance 4: 4'-dihydroxy- a: B-dimethylstilbene. Lancet 234:1389-1391.

Sir Charles Dodds received the Nobel prize for discovering the extremely potent estrogenic drug diethylstilbestrol (DES) in 1938, which was more powerful than any of the estrogenic

chemicals, including bisphenol A, examined in this 1936 article. Bisphenol A was thus never used as a drug, and, instead, in 1957 bisphenol A was used as the monomer to make polycarbonate plastic and resins used to line cans.

V. DEFINITION OF "LOW DOSE"

Studies in which doses below the currently accepted LOAEL reported by the US-EPA (IRIS) of 50 mg/kg/day showed significant effects are included here in the list of low-dose positive effects, since they would impact the LOAEL used to calculate the acceptable daily intake value.

An alternative approach suggested by the NTP Low Dose Peer Review Panel (2001) was that doses 10-fold below the LOAEL (5 mg/kg/day) were recommended for inclusion in the new "low dose" range, based on the assumption that doses 10-fold below the LOAEL can be considered the no adverse effect level (NOAEL). This assumption has now shown to be false by the large number of studies cited below that show effects not only below 5 mg/kg/day, but below the predicted safe dose of 50 µg/kg/day.

VI. RELATIONSHIP BETWEEN BISPHENOL A AND DISEASE IN HUMANS

Sugiura-Ogasawara, M., Ozaki, Y., Sonta, S., Makino, T. and Suzumori, K. (2005). Exposure to bisphenol A is associated with recurrent miscarriage. Human Reproduction Online, June 9.

BACKGROUND: Little is known about the influence of high exposure to bisphenol A on recurrent miscarriage and immunoendocrine abnormalities. METHODS: Serum bisphenol A, antiphospholipid antibodies (aPLs), antinuclear antibodies (ANAs), natural killer cell (NK) activity, prolactin, progesterone, thyroid-stimulating hormone (TSH) and free T4 were examined in 45 patients with a history of three or more (3–11) consecutive first-trimester miscarriages and 32 healthy women with no history of live birth and infertility. Subsequent pregnancy outcome and embryonic karyotype of abortuses were examined prospectively. RESULTS: The mean 6 SD values for bisphenol A in patients were 2.59 6 5.23 ng/ml, significantly higher than the 0.77 6 0.38 ng/ml found for control women (P 5 0.024). High exposure to bisphenol A was associated with the presence of ANAs but not hypothyroidism, hyperprolactinaemia, luteal phase defects, NK cell activity or aPLs. A high level of bisphenol A in itself did not predict subsequent miscarriage. CONCLUSION: Exposure to bisphenol A is associated with recurrent miscarriage.

Takeuchi T, Tsutsumi O, Ikezuki Y, Takai Y, Taketani Y. 2004. Positive relationship between androgen and the endocrine disruptor, bisphenol A, in normal women and women with ovarian dysfunction. Endocr J 51:165-9.

This study was performed to investigate the serum levels of bisphenol A (BPA), an endocrine disruptor, in women with ovarian dysfunction and obesity. Fasting serum samples were obtained from 19 non-obese and 7 obese women with normal menstrual cycles: 7 patients with hyperprolactinemia, 21 patients with hypothalamic amenorrhea, and 13 non-obese and 6 obese patients with polycystic ovary syndrome (PCOS). BPA was measured by an enzyme-linked immunosorbent assay. BPA was detected in all human sera. Serum BPA concentrations were significantly higher in both non-obese and obese women with polycystic ovary syndrome (1.05 +/- 0.10 ng/ml, 1.17 +/- 0.16 ng/ml; $p<0.05$, respectively) and obese normal women (1.04 +/- 0.09 ng/ml, $p<0.05$) compared with those in non-obese normal women (0.71 +/- 0.09 ng/ml). There was no difference among women with hyperprolactinemia, women with hypothalamic amenorrhea, and non-

obese normal women. There were significant positive correlations between serum BPA and total testosterone ($r = 0.391$, $p<0.001$), free testosterone ($r = 0.504$, $p<0.001$), androstenedione ($r = 0.684$, $p<0.001$), and DHEAS ($r = 0.514$, $p<0.001$) concentrations in all subjects. These findings show that there is a strong relationship between serum BPA and androgen concentrations, speculatively due to the effect of androgen on the metabolism of BPA.

VII. 129 *IN VIVO* STUDIES PUBLISHED IN PEER-REVIEWED JOURNALS REPORTING THAT BISPHENOL A CAUSES SIGNIFICANT EFFECTS IN ANIMALS WITH DOSES BELOW THE PUBLISHED LOAEL OF 50 mg/kg/day. ALSO INCLUDED ARE SIGNIFICANT EFFECTS AT DOSES IN THE PART PER BILLION (PPB) RANGE FOR AQUATIC ANIMALS

Adriani, W., Della Seta, D., Dessi-Fulgheri, D., Farabollini, F., Laviola, G. (2003). Altered profiles of spontaneous novelty seeking, impulsive behavior, and response to D-amphetamine in rats perinatally exposed to bisphenol A. Environ. Health Perspect. 111:395-401.

A dose of 40 µg/kg/day was fed to pregnant and lactating Sprague-Dawley rats. Bisphenol A-exposed female offspring (but not male offspring) showed neophobia (less time spent in a novel environment) than controls. In contrast, males exposed to bisphenol A showed a feminization of their response to a delay in receiving food. Finally, the normal increase in activity in response to an amphetamine injection (1 mg/kg) is reduced in males by bisphenol A.

Aikawa, H., S. Koyama, M. Matsuda, K. Nakahashi, Y. Akazome and T. Mori (2004). Relief effect of vitamin A on the decreased motility of sperm and the increased incidence of malformed sperm in mice exposed neonatally to bisphenol A. Cell Tissue Res. 315:119-24.

Injection (sc) of bisphenol A (50 µg/animal, about 15-20 mg/kg/day) for the first 5 days after birth resulted in a decrease in the percentage of moving sperm, and an increase in the incidence of malformed sperm, in the epididymides of mice at 10 weeks of age, although no marked changes were found in the testicular histology between BPA-treated and vehicle-treated control mice. The deteriorating effects of BPA were ameliorated by the concurrent administration of 100 IU of retinol acetate (RA). Neonatal treatment with BPA (0.5 µg/animal, about 150-200 µg/kg/day) for 5 days resulted in an increase in the incidence of malformed sperm, whereas the BPA effect became more severe in mice nursed by mothers fed a vitamin A-deficient (VAD) diet only a few days before and after parturition. On the other hand, neonatal treatment with 20 µg estradiol for the first 5 days after birth resulted in an increase in the number of estrogen receptor alpha (ERalpha)-positive cells in the epithelium of the vas deferens, whereas only a few epithelial cells showed weak ERalpha-positive signals in the vehicle-treated control mice at 18 days after birth. This increase, however, was suppressed by the concurrent administration of RA. Five daily neonatal treatments with 50 microg BPA led to no significant increase in the number of ERalpha-positive cells. These findings clearly showed that in mice, exposure to a relatively large dose of BPA causes damage to the motility and morphology of sperm, but the BPA effect is, to some extent, inhibited by a supplement of VA, and enhanced under a VAD condition.

Akingbemi, B.T., Soitas, C.M., Koulova, A.I., Kleinfelter, G.R. nd Hardy, M.P. (2004). Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig Cells. Eodocrinol. 145: 592-603.

Male Long-Evans rats were fed a 2.4 µg/kg/day dose of bisphenol A from postnatal day (PND) 21-35. Bisphenol A suppressed serum LH (by 60%)and testosterone (by 35%) and estradiol (by 30%); the latter finding was consistent with the inhibition of the enzyme aromatase mRNA in Leydig cells. An interesting aspect of this finding is that all of these effects were observed at very low dose of 2.4 µg/kg/day but not at higher doses. This is similar to the finding of Wetherill et al (2002) and vom Saal et al. (1997) in human prostate cancer cells that effects observed at low doses of bisphenol A can disappear at higher doses. Treatment of Leydig cells (the cells in the testes that secrete testosterone) in culture with a 2.3 pg/ml (0.01 nM) dose of bisphenol A resulted in a 25% decrease in testosterone synthesis associated with a decrease in the androgen-synthesizing enzymes 17α-hydroxylase and C17-20 lyase. Aromatase activity was also decreased. Treatment of pregnant and lactating rats (Gestation day 12 – PND 21) with 2.4 µg/kg/day bisphenol A resulted in a 38% decrease in testosterone in testicular interstitial fluid collected in later adult male offspring, showing permanent inhibitory effects of developmental exposure to a very low dose of bisphenol A on testosterone synthesis. Other long-term effects of developmental bisphenol A exposure were a decrease in seminal vesicle weight as well as an increase in body weight (all measure on postnatal day 90), replicating the finding of Howdeshell et al.1999 in mice. The findings thus demonstrate an effect of a very low dose of bisphenol A fed by gavage to Long-Evans rats (Charles River) during postnatal life from gestation day 12 – postnatal day 21 (weaning). These long-term consequences again show the sensitivity of developing animals to exposure to very low doses of bisphenol A.

Al-Hiyasat, A. S., H. Darmani and A. M. Elbetieha (2002). Effects of bisphenol A on adult male mouse fertility. Eur. J. Oral Sci. 110:163-167.

A study of fertility and sperm production in male Swiss mice reported effects of low doses of bisphenol A. In this study, bisphenol A was fed by gavage to male mice for 30 days at doses of 5, 25 and 100 µg/kg/day (note: the doses were incorrectly reported in the paper as ng/kg/day instead of µg/kg/day). At the end of the 30-day treatment period, the males were placed with two females for 10 days, after which the reproductive organs of the males were examined. All doses of bisphenol A resulted in a decrease in body weight, and all organ weights were thus corrected for body weight. The 5 and 25 µg/kg/day doses of bisphenol A significantly reduced testis and seminal vesicle weights relative to controls administered just vehicle each day. The 25 and 100 doses of bisphenol A resulted in a significant decrease in daily sperm production as well as a decrease in the number of sperm per mg epididymis, which was associated with a significantly lower proportion of pregnancies in the females housed for 10 days with the males. These findings suggest a very high level of sensitivity of these mice to bisphenol A. In addition, the findings show that at least up to 10 days after the termination of treatment, effects of bisphenol A are still detected in the male reproductive system.

Al-Hiyasat AS, Darmani H, Elbetieha AM. 2004. Leached components from dental composites and their effects on fertility of female mice. Eur J Oral Sci 112:267-272.

This study investigated the effects of leached components from a resin-based dental composite (Z-100) and bisphenol A (BPA) on female mouse fertility. Leached components or BPA (5, 25 and 100 micro g kg(-1)) were administered intragastrically daily to the test and distilled water to the control groups for 28 d. Female mice were then mated with sexually mature untreated males and their fertility was assessed. The results revealed a significant reduction in the number of pregnancies - 54.5% vs. 100% (control) - in mice treated with the leached components from the dental composite, which also showed an increase of 142% in relative ovary weights. Exposure to 25 and 100 micro g kg(-1) BPA resulted in significant increases in the total number of resorptions out of the total number of implantations and significant increases in relative uterine weights. Relative ovarian weights were significantly increased at the highest dose. High performance liquid chromatography analysis showed that tri-(ethylene glycol)-dimethacrylate (TEG-DMA)

was the major leached component (total: 5945 micro g ml(-1)) from the composite, followed by bisphenol A glycerolate dimethacrylate (BIS-GMA) (total: 2097 micro g ml(-1)) and BPA (total: 78 micro g ml(-1)). The results suggest that leached components frog/m the dental composite used and commercially purchased BPA have adverse effects on the fertility and reproductive system of female mice.

Alo, R., R. M. Facciolo, M. Madeo, G. Giusi, A. Carelli and M. Canonaco (2005). Effects of the xenoestrogen bisphenol A in diencephalic regions of the teleost fish Coris julis occur preferentially via distinct somatostatin receptor subtypes. Brain Res Bull 65(3):267-73.

The xenoestrogen bisphenol A, a contaminant used in the manufacturing of polymers for many consumer products, has been shown to mimic estrogenic actions. This xenoestrogen regulates secretion and expression of pituitary lactotrophs plus morphological and structural features of estrogen target tissues in rodents. Recently, ecological hazards produced by bisphenol A have drawn interests towards the effects of this environmental chemical on neurobiological functions of aquatic vertebrates of which little is known. In this study, the effects of bisphenol A (dose = 80 µg/ml) on the distribution of the biologically more active somatostatin receptor subtypes in diencephalic regions of the teleost fish Coris julis were assessed using nonpeptide agonists (L-779, 976 and L-817, 818) that are highly selective for subtype(2) and subtype(5), respectively. Bisphenol A proved to be responsible for highly significant increased binding levels of subtype(2) in hypothalamic areas, while markedly decreased levels of subtype(5) were found in these diencephalic areas, as well as in the medial preglomerular nucleus. The extensive distribution of somatostatin receptor subtype(2) and subtype(5) in the teleost diencephalic areas suggests that, like in mammals, this receptor system may not only be involved in enhanced hypophysiotropic neurohormonal functions but might also promote neuroplasticity events.

Aloisi, A. M., D. Della Seta, I. Ceccarelli and F. Farabollini (2001). Bisphenol-A differently affects estrogen receptors-alpha in estrous-cycling and lactating female rats. Neurosci. Lett. 310(1): 49-52.

The effect of a 40 µg/kg/day dose of bisphenol A fed to SD female rats during pregnancy and lactation or adult females during estrous cycles for 42 days was examined. Bisphenol A was administered orally in oil. Effects on ER alpha staining cells in the hypothalamus of was examined. With both pregnant and lactational exposure or during estrous cycles, bisphenol A caused an increase in the number of ER staining cells in the medial preoptic area of the hypothalamus. A related finding is that Funabashi et al. (2001; 2003) showed that bisphenol A increased progesterone receptor mRNA in the preoptic area of rats.

Aloisi AM, Della Seta D, Rendo C, Ceccarelli I, Scaramuzzino A, Farabollini F. (2002). Exposure to the estrogenic pollutant bisphenol A affects pain behavior induced by subcutaneous formalin injection in male and female rats. Brain Res 937:1-7.

We investigated the effects of perinatally administered bisphenol A (BPA), an environmental contaminant with estrogenic activity, on formalin-induced nociceptive responses. Male and female offspring of mother rats treated with BPA (40 µg/kg/day) or oil were cross-fostered after birth to obtain three homogeneous groups: BPA-prenatal, receiving BPA via the placenta; BPA-postnatal, receiving BPA through suckling; OIL, control, from mothers receiving only peanut oil (vehicle). All groups underwent a pain test with s.c. formalin injection (50 mul, 10%) or were sham injected (pricking with a syringe needle) in the dorsal hind paw. They were immediately placed in an open field apparatus where pain responses (licking, flexing and paw-jerk) were recorded for 60 min. Corticosterone, testosterone and estradiol serum levels were determined in blood obtained at the end of the experiment. BPA-prenatal treatment induced an increase in licking duration in females and in flexing duration in both sexes in the first half of the test (0-30 min after formalin injection). BPA-postnatal treatment induced a decrease in paw-jerk frequency in males and females during the second

part of the test (30-60 min after formalin injection). Plasma concentrations of corticosterone, estradiol and testosterone did not differ significantly between groups. These results indicate that exposure to BPA modified the activity of neural pathways and/or centers involved in nociception and pain in a sex-related and exposure-related manner.

Alonso-Magdalena, P., Morimoto1, S., Ripoll, C., Fuentes,E. and Nadal, A. (2005) The Estrogenic Effect of Bisphenol-A Disrupts the Pancreatic beta Cell Function *in vivo* and Induces Insulin Resistance. Environ Health Perspect. doi:10.1289/ehp.8451 (available at http://dx.doi.org/),. Online 20 September 2005.

The function of the pancreatic β-cell is the storage and release of insulin, the main hormone involved in blood glucose homeostasis. The results given in this work show that the widespread environmental contaminant, bisphenol-A (BPA) imitates 17β- estradiol (E2) effects *in vivo* on blood glucose homeostasis through genomic and nongenomic pathways. The exposure of adult mice to a unique low dose (10 µg/kg) of either E2 or BPA induces a rapid decrease in glycaemia that correlates with a rise of plasma insulin. Longer exposures to E2 and BPA induce an increase in the pancreatic β- cell insulin content in an estrogen receptor-dependent manner. This effect is visible after 2 days of treatment and starting at doses as low as 10 µg/kg/day. After four days of treatment with either E2 or BPA, these mice developed chronic hyperinsulinaemia and their glucose and insulin tolerance tests were altered. These experiments unveil the link between environmental estrogens and insulin resistance. Therefore, either abnormal levels of endogenous estrogens or environmental estrogen exposure enhance the risk of developing type 2 diabetes mellitus, hypertension and dyslipidaemia.

Arukwe, A., T. Celius, B. T. Walther and A. Goksoyr (2000). Effects of xenoestrogen treatment on *zona radiata* protein and vitellogenin expression in Atlantic salmon (*Salmo salar*). Aquatic Toxicology 49:159-170.

Zonagenesis (*zona radiata* protein synthesis) and vitellogenesis (yolk protein synthesis) are two reproductive responses that are integral aspects of fish oogenesis. This study examines the responses of eggshell zona radiata proteins (Zrp) and vitellogenin (Vtg) to five environmental pollutants; 4-nonylphenol (NP) and o,p'-DDT [both at 25 mg/kg], lindane (gamma-HCH) [10 mg/kg], a technical PCB mixture (Aroclor 1254; A1254) and bisphenol A (BPA) [both at 5 mg/kg] in juvenile Atlantic salmon (Salmo salar). Fish were given intraperitoneal injections of o,p'-DDT, gamma-HCH, A1254 or BPA; singly, in combination with NP, and as a cocktail of all five chemicals, and later compared to NP-treated and untreated fish. In a separate experiment, fish were exposed to BPA in a dose-response manner (1, 5, 25 and 125 mg/kg fish). Based on previous studies, blood and liver samples were collected 2 weeks after injection. Zrp and Vtg levels were analyzed in plasma using immunoblotting and enzyme-linked immunosorbent assay. Liver cytochrome P4501A was analyzed by 7-ethoxyresorufin O-deethylase (EROD) activity. NP caused pronounced elevations in plasma Zip and Vtg levels. In comparison, when NP was given in combination with gamma-HCH, Vtg levels was significantly reduced, compared to NP treatment alone. Using o,p'-DDT, A1254 and BPA, significant elevations of plasma Zrp and Vtg were seen when chemicals were given in combination with NP, but not when administered by themselves. An apparent dose-response induction of Vtg and Zrp levels were observed in BPA treated juvenile salmon. In immunoblots, one component of molecular weight approximating the Zrp-beta was detected when either o,p'-DDT, gamma-HCH, A1254 or BPA were given singly. A1254 significantly induced hepatic EROD activity when administered alone. However, when given as a mixture with all the other xenobiotics, reduction of EROD activity was observed. The data suggest a pattern of xenobiotics action which may complicate assessment of their reproductive effects. Zrp (the beta monomer) was more responsive to the xenoestrogens than Vtg, and provides a sensitive means of detecting exposure to environmental

estrogens.

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.

This study investigated whether neonatal exposure of male rats to estrogenic compounds altered pubertal spermatogenesis (days 18 and 25) and whether the changes observed resulted in long-term changes in testis size, mating, or fertility (days 90-100). Rats were treated neonatally with a range of doses (0.01-10 microg) of diethylstilbestrol (DES; administered on alternate days from days 2-12), a high dose of octylphenol (OP; 2 mg administered daily from days 2-12) or bisphenol A (Bis-A; 0.5 mg (37 mg/kg/day) administered daily from days 2-12), or vehicle, while maintained on a standard soy-containing diet. The effect on the same parameters of rearing control animals on a soy-free diet was also assessed as was the effect of administering such animals genistein (4 mg/kg/day daily from days 2-18). Testis weight, seminiferous tubule lumen formation, the germ cell apoptotic index (apoptotic/viable germ cell nuclear volume), and spermatocyte nuclear volume per unit Sertoli cell nuclear volume were used to characterize pubertal spermatogenesis. Compared with (soy-fed) controls, DES administration caused dose-dependent retardation of pubertal spermatogenesis on day 18, as evidenced by decreases in testis weight, lumen formation, and spermatocyte nuclear volume per unit Sertoli cell and elevation of the germ cell apoptotic index. However, the two lowest doses of DES (0.1 and 0.01 microg) significantly increased spermatocyte nuclear volume per unit Sertoli cell. Similarly, treatment with either OP or Bis-A significantly advanced this and some of the other aspects of pubertal spermatogenesis. Maintenance of control animals on a soy-free diet also significantly advanced lumen formation and spermatocyte nuclear volume per unit Sertoli cell compared with controls fed a soy-containing diet. Administration of genistein reversed the stimulatory effects of a soy-free diet and significantly retarded most measures of pubertal spermatogenesis. In general, plasma FSH levels in the treatment groups changed in parallel to the spermatogenic changes (reduced when pubertal spermatogenesis retarded, increased when pubertal spermatogenesis advanced). By day 25, although the changes in FSH levels largely persisted, all of the stimulatory effects on spermatogenesis seen on day 18 in the various treatment groups were no longer evident. In adulthood, testis weight was decreased dose dependently in rats treated neonatally with DES, but only the lowest dose group (0.01 microg) showed evidence of mating (3 of 6) and normal fertility (3 litters). Animals treated neonatally with OP or Bis-A had normal or increased (Bis-A) testis weights and exhibited reasonably normal mating/fertility. Animals fed a soy-free diet had significantly larger testes than controls fed a soy-containing diet, and this difference was confirmed in a much larger study of more than 24 litters, which also showed a significant decrease in plasma FSH levels and a significant increase in body weight in the males kept on a soy-free diet. Neonatal treatment with genistein did not alter adult testis weight, and although most males exhibited normal mating and fertility, a minority did not mate or were infertile. It is concluded that 1) neonatal exposure of rats to low levels of estrogens can advance the first wave of spermatogenesis at puberty, although it is unclear whether this is due to direct effects of the estrogen or to associated elevation of FSH levels; 2) the effect of high doses of OP and Bis-A on these processes is essentially benign; and 3) the presence or absence of soy or genistein in the diet has significant short-term (pubertal spermatogenesis) and long-term (body weight, testis size, FSH levels, and possibly mating) effects on males.

Berg, C., Halldin, K. and Brunstrom, B. (2001). Effects of bisphenol A and tetrabromobisphenol A on sex organ development in quail and chicken embryos. Environ. Toxicol. Chem. 20:2836-2840.

Bisphenol A was injected into quail and chicken embryos. At 200 µg/g egg bisphenol A resulted in malformation of the oviduct in female quail and the development of ovotestes in male chicken embryos. At doses of both 67 and 200 µg/g egg bisphenol A increased mortality in chickens but not quail, relative to controls. [The dose reaching the embryo in this study, and thus the dose per kg body weight of the embryo, is not known]

Biggers, W.J. and Laufer, H. (2004). Identification of juvenile hormone-active alkylphenols in the lobster Homarus americanus and in marine sediments. Biol Bull 206:13-24.

We have identified, by gas chromatography/mass spectrometry, four alkylphenols that are present in the hemolymph and tissues of the American lobster Homarus americanus and in marine sediments. These alkylphenols are used industrially in antioxidant formulations for plastic and rubber polymer manufacturing, and are similar in structure to a known endocrine disruptor, bisphenol A. The compound 2-t-butyl-4-(dimethylbenzyl)phenol was present at concentrations of 0.02 to 1.15 microg/ml in hemolymph and 8.95 to 21.58 microg/g in sediments. A second compound, 2,4-bis-(dimethylbenzyl)phenol, was present at concentrations between 0.07 and 19.78 microg/ml in hemolymph and 138.94 to 224.89 microg/g in sediment, while a third compound, 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol, was found at concentrations between 0.01 and 13.00 microg/ml in hemolymph, 2.55 and 6.11 microg/g in hepatopancreas, and 47.85 and 74.66 microg/g in sediment. A fourth compound, 2,4-bis-(dimethylbenzyl)-6-t-butylphenol, was found at concentrations of 0.20 to 70.71 microg/ml in hemolymph, 23.56 to 26.89 microg/g in hepatopancreas, and 90.68 to 125.58 microg/g in sediment. These compounds, along with bisphenol A, 4-dimethylbenzylphenol, and nonylphenol, display high juvenile hormone activity in bioassays. Alkylphenols at high concentrations are toxic to crustaceans and may contribute significantly to lobster mortality; at lower concentrations, they are likely to have endocrine-disrupting effects. Bisphenol A showed a very low EC50 of 0.05 µM (11.4 parts per billion) in a bioassay that detects an effect of juvenile hormone on larval metamorphosis, which is stimulated by juvenile hormone. This indicates that bisphenol A has very high juvenile hormone activity in lobsters.

Bindhumol, V., Chitra, K.C. and Mathur, P.P. (2003). Bisphenol A induces reactive oxygen species generation in the liver of male rats. Toxicol. 188:117-124.

Bisphenol A, an environmental contaminant, widely used as a monomer in polycarbonate plastics, has been shown to cause abnormalities in liver of rats and mice. The nature and mechanism of action of bisphenol A on liver is not clear. The aim of the present study was to investigate if bisphenol A induces oxidative stress in the liver of rats and if co-administration of vitamin C, an antioxidant, can prevent oxidative stress. Bisphenol A (0.2, 2.0 and 20 µg/kg body weight per day) and bisphenol A+vitamin C (0.2, 2.0, 20 µg+40 mg/kg body weight per day) was orally administered to rats for 30 days. After 24 h of the last treatment, rats were killed using overdose of anesthetic ether**. Body weights of the animals and the weights of liver showed no significant changes.** The activities of antioxidant enzymes, superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase were decreased by all doses of bisphenol A in mitochondrial and microsome-rich fractions of liver. The levels of hydrogen peroxide and lipid peroxidation increased in the treated rats when compared with the corresponding group of control animals. Activity of alanine transaminase, a marker enzyme of hepatic injury remained unchanged in the treated rats as compared with the corresponding control rats. Co-administration of bisphenol A and vitamin C showed no changes in the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase and in the levels of hydrogen peroxide and lipid peroxidation as compared with the

corresponding control groups. The results indicated that bisphenol A induces oxidative stress in the liver of rats by decreasing the antioxidant enzymes. Co-administration of vitamin C reversed the effects of bisphenol A-induced oxidative stress in the liver of rats.

Canesi, L., Betti, M., Lorusso, L.C., Ciacci, C. and Gallo, G. (2005). 'In vivo' effects of Bisphenol A in Mytilus hemocytes: modulation of kinase-mediated signalling pathways. Aquat. Toxicol. 71:73-84.

Endocrine disrupting chemicals (EDCs) include a variety of natural and synthetic estrogens, as well as estrogen-mimicking chemicals. We have previously shown that in the hemocytes of the mussel Mytilus galloprovincialis Lam. both natural and environmental estrogens in vitro can rapidly affect the phosphorylation state of components of tyrosine kinase-mediated cell signalling, in particular of mitogen activated protein kinases (MAPKs) and signal transducers and activators of transcription (STAT), that are involved in mediating the hemocyte immune response. These effects were consistent with the hypothesis that 'alternative' modes of estrogen action involving kinase-mediated pathways similar to those described in mammalian systems are also present in invertebrate cells. This possibility was investigated in vivo with Bisphenol A (BPA): mussels were injected with BPA and hemocytes sampled at 6, 12, and 24 h post-injection. The results show that BPA (25 nM or 5.7 ppb nominal concentration in the hemolymph) lead to a significant lysosomal membrane destabilisation at all times post-injection, indicating BPA-induced stress conditions in the hemocytes, whereas lower concentrations were ineffective. BPA induced significant changes in the phosphorylation state of MAPK and STAT members, as evaluated by SDS-PAGE and WB of hemocyte protein extracts with specific antibodies, although to a different degree at different exposure times. In particular, BPA induced a dramatic decrease in phosphorylation of the stress-activated p38 MAPK, whose activation is crucial in mediating the bactericidal activity. Moreover, BPA decreased the phosphorylation of a CREB-like transcription factor (cAMP-responsive element binding protein). The results demonstrate that BPA can affect kinase-mediated cell signalling in mussel hemocytes also in vivo, and suggest that EDCs may affect gene expression in mussel cells through modulation of the activity of transcription factors secondary to cytosolic kinase cascades. Overall, these data address the importance of investigating full range responses to EDCs in ecologically relevant marine invertebrate species.

Carr, R.L., Bertasi, F.R., Betancourt, A.M., Bowers, S.D., Gandy, B.S., Ryan, P.L., Willard, S.T. (2003). Effect of neonatal rat bisphenol A exposure on performance in the Morris water maze. J. Toxicol. Environ. Health A 66:2077-2088.

To investigate the effects of repeated postnatal exposure of male and female rats to BPA on spatial learning and memory were investigated using a Morris water maze. Breeders and offspring were maintained on a standard phytoestrogen-free diet. Oral administration of 72 µg/kg 17beta-estradiol (E-2), 100 µg/kg BPA (low BPA), 250 µg/kg BPA (high BPA), or the safflower oil vehicle was performed daily from postnatal d 1 (PND1) through PND14. There were no treatment-related effects on swimming ability or motivation (PND33) or on acquisition of maze solution (PND34-37). However, acquisition of maze performance was significantly better in control males than in control females. Treatment with E-2 and low BPA disrupted this normal gender-dependent pattern of acquisition, while treatment with high BPA did not. In a probe trial (PND40), females treated with high BPA spent significantly less time in the escape quadrant. These data indicate that E-2 and low dosages of BPA can alter the normal gender-dependent pattern of acquisition, while higher dosages of BPA alter the retention of spatial information without significantly affecting acquisition.

Chitra KC, Latchoumycandane C, Mathur PP. (2003). Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology. 185(1-2):119-127.

Bisphenol A was administered to adult male Wistar rats reared in Pondicherry, India for 45 days at doses of 0.2, 2 and 20 µg/kg/day. One day later organs were examined. Bisphenol A resulted in dose-related changes, with significant effects observed at all doses, including the lowest dose (0.2 µg/kg/day). The ventral prostate was significantly increased in size, while the testes and epididymis were significantly smaller relative to controls. Epididymal sperm count and motility were significantly reduced by bisphenol A, as were the activities of catalase, glutathione reductase, glutathione peroxicase and superoxide dismutase in epididymal sperm. In contrast, there was an increase in the levels of hydrogen peroxide and lipid peroxidation caused by bisphenol A. This is important since the formation of reactive oxygen species is related to infertility in men, and bisphenol A reduced by activity of enzymes that form part of the defense against formation of reactive oxygen species, while increasing the production of cytotoxic free radicals involved in oxidative stress. The finding of a decrease sperm in both testes and epididymis is consistent with Al-Hiyasat findings in adult mice, and is also consistent with the findings of Sakaue that bisphenol A decreases testicular sperm in adult rats. The finding that effects occur at 0.2 µg/kg/day is the current LOEL for bisphenol A in rats.

Colerangle, J.B. and Roy, D. Profound effects of the weak environmental estrogen-like chemical bisphenol A on the growth of the mammary gland of noble rats. (1997). J Steroid Biochem Molec 60:153-160

A 100 µg/kg/day dose of bisphenol A was administered to adult female Noble rats for 11 days via a minipump (tonic administration). Bisphenol A stimulated the mammary gland, with alterations in cell cycle markers. Markey showed that prenatal exposure to 25 and 250 ng/kg/day administered via minipump resulted in a marked hyperplasia in mammary ducts.

Dessi-Fulgheri, F., Porrini, S. and farabollini, F. (2002). Effects of perinatal exposure to bisphenol A on play behavior of female and male juvenile rats. Environ. Health Perspect. 110: (Suppl 3):403-407.

Bisphenol A was fed to pregnant and lactating Sprague-Dawley rats at doses of 40 and 400 µg/kg/day. Play behavior was examined in juvenile animals. Bisphenol A masculinized the play behavior of females in terms of their interaction with other females, and also altered the play behavior of males with females at both doses tested.

Duft M, Schulte-Oehlmann U, Weltje L, Tillmann M, Oehlmann J. (2003). Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum*. Aquatic Toxicology 64:437-449.

The effects of three suspected endocrine disrupting chemicals, the xeno-estrogens bisphenol A (BPA), 4-tertoctylphenol (OP) and 4-n-nonylphenol (NP), were investigated in a whole-sediment biotest with the freshwater mudsnail Potamopyrgus antipodarum (Gastropoda, Prosobranchia). Artificial sediments were spiked with five nominal concentrations (six for NP), ranging from 1-300 mug/kg dry weight (1-1000 pg/kg for NP). After 2, 4 and 8 weeks of exposure, the responses of the test species were analysed. P. antipodarum exhibited a distinct increase in the number of embryos sheltered in its brood pouch in a time- and concentration-dependent manner in comparison to the solvent control sediment for BPA and OP. The number of "new", still unshelled embryos turned out to be the most sensitive parameter. The lowest observed effect concentration (LOEC) was equivalent to the lowest administered concentration (1 mug/kg for each test compound) for most parameters after 8 weeks of exposure. The calculation of effect concentrations resulted in even lower values for BPA (unshelled embryos after 2 weeks: EC10 0.22 mug BPA/kg, EC50 24.5 mug BPA/kg; after 4 weeks: EC10 4 ng OP/kg, EC50 5.67 mug OP/kg) and OP (unshelled embryos after 4 weeks: EC10 4 ng OP/kg, EC50 0.07 mug OP/kg). For NP, there was no clear concentration-dependent response, and

therefore, no EC10 or EC50 could be estimated, but the data suggest an inverted u-shape type of curve. The LOEC in the experiments with NP was 10 pg/kg. Our results indicate that P. antipodarum is highly sensitive to the tested endocrine disruptors at environmentally relevant concentrations. Furthermore, the biotest with P. antipodarum is a useful tool for the identification of sediment-bound pollutants and for the assessment of sediment quality.

Evans, N. P., T. North, S. Dye and T. Sweeney (2004). Differential effects of the endocrine-disrupting compounds bisphenol-A and octylphenol on gonadotropin secretion, in prepubertal ewe lambs. Domest. Anim. Endocrinol. 26:61-73.

This study examined the effects of long-term exposure to the endocrine-disrupting compounds (EDCs) Bisphenol-A (BPA) and Octylphenol (OP) on gonadotrophin secretion in pre-pubertal female sheep. Four-week-old, female lambs were randomly allocated to four groups (n=6), and twice each week treated with i.m. injections of either corn oil (vehicle controls), diethylstilbestrol (DES; 0.175 mg/kg), BPA (3.5 mg/kg) or OP (3.5 mg/kg). After 5 weeks of treatment, animals were ovariectomized (ovx) and ovary weights recorded. Two weeks later, blood samples were collected from lambs every 15min for 6h, for LH pulse analysis. Animals were then euthanased and adrenal and kidney weight recorded. An age-related increase in tonic LH secretion was noted in Control, BPA- and OP-treated lambs, but was absent in DES-treated lambs. Following ovx, LH secretion increased in all except DES-treated lambs; FSH concentrations increased in all groups. BPA and DES significantly suppressed LH pulse frequency (C: 6.7+/-0.3pulses/6h, DES: 1.5+/-0.8pulses/6h, BPA: 2.3+/-0.8pulses/6h) and amplitude (C: 7.1+/-1.0ng/ml, DES: 1.9+/-0.6ng/ml, BPA: 1.6+/-0.4ng/ml). OP had no effect on LH secretion (Frequency: 5.8+/-0.5pulses/6h, amplitude: 8.0+/-2.0ng/ml). Ovary weight was similar among all groups. Results show that chronic in vivo exposure of prepubertal female lambs to BPA, at levels lower than those reported previously, can have significant effects on LH secretion that are comparable to those seen following exposure to a dose of DES 200-fold lower than bisphenol A. Exposure to an equal dose of the EDC OP, over the equivalent period of time was without effect on gonadotropin secretion in the prepubertal ewe lamb. These results indicate that exposure of prepubertal female lambs to the EDC BPA can induce significant effects on gonadotropin secretion. These results are consistent with findings in rats by Akingbemi et al. (2004) and Rubin et al. (2001).

Facciolo, R.M., Alo, F., Madeo, M. Canonaco, M. and Dessi-Fulgheri, F. (2002). Early cerebral activities of the environmental estrogen bisphenol A appear to act via the somatostatin receptor subtype sst_2. Environ. Health Perspect. 110 (Suppl 3): 397-402.

Sprague-Dawley rats were fed 40 or 400 µg/kg/day bisphenol A throughout pregnancy and lactating, and somatostatin receptor subtype sst_2 was examined in specific brain regions at different postnatal ages in offspring (only the high 400 µg/kg/day dose was examined for most endpoints). The high dose of bisphenol A resulted in changes in sst_2 binding in different regions of the brain of ligands known to exert analgesic action by acting as agonists for the GABA type A receptor. There was a shift caused by bisphenol A toward the high affinity form of the sst_2 receptor. Changes in neurotransmitters that regulate growth hormone as well as other aspects of neural function could impact many types of behavior as well as the functioing of the neuroendocrine system.

Farabollini, F., Porrini, S., and Dessi-Fulgheri, F. (1999). Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats. Pharmacol. Biochem. Behav. 64:687-694.

Effects were seen on male and female offspring at doses of 40 and 400 µg/kg/day adminisitered to pregnant and lactating Sprague-Dawley rats. Changes in behavior of both males and females in an elevated plus maze that is used to assess anxiety were noted at both doses.

Farabollini, F., Porrini, S., Setz, D.D., Bianchi, F. and Dessi-Fulgheri, F. (2002). Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. Environ. Health Perspect. 110 (Suppl 3): 409-414.

Sprague-Dawley rats were fed 40 µg/kg/day bisphenol A throughout pregnancy (prenatal exposure group) or throughout lactation (lactation exposed group), and male and female offspring were examined for effects on aggression and sexual behavior. Males exposed prenatally to bisphenol A showed an increase in defensive aggression, consistent with findings reported by Kawai et al. (2002) in mice. The length of time for the initiation of intromissions during sexual interactions with females was significantly increased in males exposed prenatally to bisphenol A, which is consistent with findings reported by Kubo et al. (2003). Interestingly, in females, fetal or lactational exposure to bisphenol A resulted in an enhancement of sexual behavior, recorded as an increase in the frequency of the receptive posture (lordosis) when paired with a male.

Fisher, J. S., K. J. Turner, D. Brown and R. M. Sharpe (1999). Effect of neonatal exposure to estrogenic compounds on development of the excurrent ducts of the rat testis through puberty to adulthood. Environ Health Perspect 107:397-405.

Neonatal exposure to diethylstilbestrol (DES) can alter the structure of the testicular excurrent ducts in rats. We characterized these changes according to dose and time posttreatment and established whether potent estrogens (ethinyl estradiol), environmental estrogens (genistein, octylphenol, bisphenol A, parabens), and tamoxifen induce such changes. Rats were administered these compounds neonatally and assessed at several time points during (day 10, or day 18 for some treatments) and after (days 18, 25, 35, and 75) the treatment period to detect any changes in testis weight, distension of the rete testis and efferent ducts, epithelial cell height in the efferent ducts, and immunoexpression of the water channel aquaporin-1 (AQP-1). Treatment with DES (10, 1, or 0.1 microg/injection; equivalent to 0.37, 0.037, or 0.0037 mg/kg/day, respectively) induced dose-dependent changes in testis weight and all parameters. These effects were most pronounced at days 18 and 25 and appeared to lessen with time, although some persisted into adulthood. Neonatal treatment with ethinyl estradiol (10 microg/injection; equivalent to 0.37 mg/kg/day) caused changes broadly similar to those induced by 10 mg DES. Administration of tamoxifen (2 mg/kg/day) caused changes at 18 days that were similar to those induced by 1 microg DES. Treatment with genistein (4 mg/kg/day), octylphenol (2 mg/injection; equivalent to 150 mg/kg/day), or bisphenol A (0.5 mg/injection; equivalent to 37 mg/kg/day) caused minor but significant ($p<0.05$) decreases in epithelial cell height of the efferent ducts at days 18 and/or 25. In animals that were followed through to 35 days and/or adulthood, these changes were no longer obvious; other parameters were either unaffected or were affected only marginally and transiently. Administration of parabens (2 mg/kg/day) had no detectable effect on any parameter at day 18. To establish whether these effects of estrogens were direct or indirect (i.e., resulting from reduced follicle-stimulating hormone/luteinizing hormone secretion), the above end points were assessed in animals in which gonadotropin secretion was suppressed neonatally by administration of a gonadotropin-releasing hormone antagonist. This treatment permanently reduced testis weight, but did not affect any of the other end points, apart from a minor transient reduction in efferent duct epithelial cell height at 18 days. This study suggests that structural and functional (expression of AQP-1) development of the excurrent ducts is susceptible to impairment by neonatal estrogen exposure, probably as a consequence of direct effects. The

magnitude and duration of adverse changes induced by treatment with a range of estrogenic compounds was broadly comparable to their estrogenic potencies reported from in vitro assays.

Fujimoto,T., Kubob, K. and Aou, S. (2006). Prenatal exposure to bisphenol A impairs sexual differentiation of exploratory behavior and increases depression-like behavior in rats. Brain Res. Online: November, 2005.

Perinatal exposure to bisphenol A (BPA, 0.1 and 1 ppm in drinking water applied to mother rats for 6 weeks) has been shown to impair the sexual differentiation in exploratory behavior, but the exact critical period of this disrupting effect is still unknown. In this study, we examined the effects of prenatal exposure to BPA (0.1 ppm in drinking water applied to dams during the final week of pregnant) on emotional and learning behaviors in addition to exploratory behavior. Estimated daily intake was 15 μg/kg/day, below the reference dose (RfD) in the United States and the daily tolerable intake (TDI) in Japan (50 μg/kg/day). The rats were successively tested in open-field test, elevated plus maze test, passive avoidance test and forced swimming test during development from 6 to 9 weeks of juvenile period. Prenatal exposure to BPA mainly affected male rats and abolished sex differences in rearing behavior in the open-field test and struggling behavior in the forced swimming test. BPA increased the immobility of male rats in the forced swimming test. The avoidance learning and behaviors in the elevated plus maze were not affected. The present study demonstrates that male rats at the final week of prenatal period are sensitive to BPA, which impairs sexual differentiation in rearing and struggling behavior and facilitate depression-like behavior.

Funabashi, T., M. Kawaguchi and F. Kimura (2001). The endocrine disrupters butyl benzyl phthalate and bisphenol A increase the expression of progesterone receptor messenger ribonucleic acid in the preoptic area of adult ovariectomized rats. Neuroendocrinol. **74**: 77-81.

A single 10 mg/kg/day dose of bisphenol A was injected sc into adult ovariectomized female Wistar rats, and preoptic area of the brain was examined for progesterone receptor mRNA. Other females were injected with 10 mg butyl benzyl phthalate or 10 μg estradiol. All 3 chemicals resulted in a similar significant increase in progesterone receptor mRNA levels in the preoptic area. Aloisi et al. 2001 showed that bisphenol A increased the number of estrogen receptor positive cells in the preoptic area in female rats.

Funabashi, T., A. Sano, D. Mitsushima and F. Kimura (2003). Bisphenol A increases progesterone receptor immunoreactivity in the hypothalamus in a dose-dependent manner and affects se J Neuroendocrinolxual behaviour in adult ovariectomized rats. J. Neuroendocrinol. **15**(2): 134-40.

The prior finding by this group was replicated in that they showed that a single 10 mg/kg/day dose of bisphenol A that was injected sc into adult ovariectomized female Wistar rats led to a significant increase in progesterone receptor mRNA levels in the preoptic area. They then showed in a separate experiment that there was a dose-related increase in progesterone receptor mRNA, with a significant increase being detected at a bisphenol A dose of 400 μg/kg/day.

Funabashi, T., T. J. Nakamura and F. Kimura (2004). p-Nonylphenol, 4-tert-octylphenol and bisphenol A increase the expression of progesterone receptor mRNA in the frontal cortex of adult ovariectomized rats. J. Neuroendocrinol. 16:99-104.

Alkylphenols [p-nonylphenol (NP) and 4-tert-octylphenol (OP)] and bisphenol A (BPA) were examine to determine whether they would affect progesterone receptor (PR) mRNA expression in the adult female Wistar rat neocortex. In one experiment, at 12.00 h, ovariectomized rats were given a subcutaneous injection of 10 mg of NP, 10 mg of OP or 10 mg of BPA, or sesame oil alone as control (all doses were ~40 mg/kg/day). Twenty-four hours after injection, the left side of the frontal cortex,

parietal cortex and temporal cortex was collected. In a second experiment to study the time-course of the effects of BPA on PR mRNA, the ovariectomized rats were given a subcutaneous injection of 10 mg of BPA and killed 0, 6, 12 and 24 h after injection. In addition to the frontal cortex and temporal cortex, the occipital cortex was also collected. Northern blotting revealed that, in the first experiment, injection of NP, OP or BPA significantly increased PR mRNA expression in the frontal cortex but not in the parietal cortex. In the temporal cortex, BPA significantly decreased PR mRNA, but NP and OP produced no significant changes. The second experiment revealed that, in the frontal cortex, BPA induced a significant increase in PR mRNA expression at 6 h after injection, which lasted until 24 h after injection. In the temporal cortex, PR mRNA expression was significantly decreased 6 h after injection of BPA and was still significantly low 24 h after injection. No significant change was observed in the occipital cortex. These results suggest that, even in adult rats, endocrine disrupters alter the neocortical function by affecting the PR system, although the physiological significance of PR in the affected area is unknown.

Funabashi, T., M. Kawaguchi, M. Furuta, A. Fukushima and F. Kimura (2004). Exposure to bisphenol A during gestation and lactation causes loss of sex difference in corticotropin-releasing hormone-immunoreactive neurons in the bed nucleus of the stria terminalis of rats. Psychoneuroendocrinol. 29:475-485.

It has been suspected that endocrine disrupters induce abnormal differentiation and development of reproductive organs. In the present study, we examined whether exposure to bisphenol A (BPA), a known endocrine disrupter, during gestation and lactation affects sex difference in the number of corticotropin-releasing hormone-immunoreactive neurons (CRH neurons) in the preoptic area (POA) and the bed nucleus of the stria terminalis (BST). For that purpose, pregnant female Wistar rats (n=8-11 per treatment group) were treated with either 0.1% ethanol (control group) or 10 mg/l BPA, equivalen to about 2.5 mg/kg/day (BPA group) dissolved in their drinking water until their offspring were weaned. In the control group, we confirmed a previous report that the POA of female rats contained significantly more CRH neurons than that of male rats ($p<0.05$). This significant sex difference was also evident in the BPA group, indicating that BPA exposure used in the present study had no effect on the sex difference in CRH neurons in the POA. We also found in the control group that the BST of female rats contained significantly more CRH neurons ($p<0.05$) than that of male rats. However, this significant sex difference was not observed in the BPA group ($p>0.05$), suggesting that BPA exposure affected the sex difference in CRH neurons in the BST. Since there was no statistically significant difference in the number of CRH neurons between the control and the BPA group, irrespective of the sex, the results suggested that a loss of sex difference in CRH neurons was due to both an increase in CRH neurons in male rats and a decrease in CRH neurons in female rats. The present study indicates that there is a significant sex difference in the number of CRH neurons in the BST as well as in the POA and that exposure to BPA during gestation and lactation causes a loss of this sex difference in the rat BST, but not in the POA. We suggest that CRH neurons in the BST are more susceptible to endocrine disrupters than those in the POA, irrespective of the sex.

Furuya M, Adachi K, Kuwahara S, Ogawa K, Tsukamoto Y. Inhibition of male chick phenotypes and spermatogenesis by Bisphenol-A. Life Sci. Online November 2005.

Bisphenol-A (BPA) has been reported to bind to the estrogen receptor (ER) and also to act as a xenoestrogen on the reproductive system of many species. In our previous study, a high dose of BPA disturbed the growth of the comb and testes of male chickens. In this study, the exposure of relatively low doses of BPA on the growth of the male chicken phenotypes was investigated. White Leghorn male chicks were orally administered various doses of BPA (2 µg to 200 mg/kg) from 2

weeks of age, and thereafter the comb, wattle and testes were examined at 5, 10, 15, 20 and 25 weeks of age. Although the body weight showed no significant difference among the birds of all ages, the growth of above organs was significantly affected in the chicks even with a minimal dose of 2-µg BPA. These inhibitory effects appeared in a dose-dependent manner. Histologically, the growth of the testes was negatively affected by exposure to over 20-µg/kg BPA: namely, the development of seminiferous tubuli and spermatogenesis were severely inhibited. The mRNA expressions of ER alpha and the aromatase gene (p450arom) increased in the testes in a dose-dependent manner after BPA administration. Accordingly, even low doses of BPA delayed the growth of the male chicken phenotype either by a direct effect or by an indirect response resulting in an increase in both of the endogenous estrogen levels and hyper-sensitivity to estrogen.

Goloubkova, T., M. F. Ribeiro, L. P. Rodrigues, A. L. Cecconello and P. M. Spritzer (2000). Effects of xenoestrogen bisphenol A on uterine and pituitary weight, serum prolactin levels and immunoreactive prolactin cells in ovariectomized Wistar rats. Archives of Toxicology 74:92-98.

Considerable attention has currently been focused on bisphenol A (BPA), an environmental endocrine disrupting chemical that has oestrogenic activity. In vitro and in vivo short-term assays have shown that BPA is weakly estrogenic. In addition, the issue of species- and strain-differences in susceptibility to BPA was raised. The treatment of ovariectomized (OVX) Wistar rats with BPA at doses of 11-250 mg/kg per day, s.c., for 7 days, resulted in significant dose-dependent regrowth of uterus in uterotrophic assay, with significant stimulation occurring at all doses. The Wistar rat thus appears to be more sensitive to the uterotrophic effect of BPA relative to Sprague-Dawley rats or CD-1 mice.

Additionally, the stimulation of anterior pituitary gland growth and induction of hyperprolactinaemia, as determined by wet organ weight and radioimmunoassay (RIA), respectively, were also dose-dependent (at 128 and 250 mg/kg per day, $P < 0.05$). Prolactin immunostaining of anterior pituitary glands revealed that BPA at a dose of 250 mg/kg per day increased the number of prolactin-immunopositive cells by 63% compared to OVX rats. These results demonstrate that the reproductive tract and neuroendocrine axis of Wistar rats are able to respond to BPA. Furthermore, the pituitary gland hypertrophy and hyperprolactinaemia can be mediated, at least partly, by increase in number of prolactin-immunoreactive cells. The long-term consequences of this proliferation are yet unknown but neoplasm formation is an obvious possibility.

Gould, J.C., Leonared, L.S., Maness, S.C., Wagner, B.L., Conner, K., Zacharewski, T., Safe, T., McDonnell, D.P., and Gaido, K.W. (1998). Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. Molecular and Cellular Endocrinology 142:203-214.

Bisphenol A at an oral dose of 5-150 mg/kg/day did not stimulate a uterine weight increase in prepubertal Sprague-Dawley rats, However, bisphenol A at all doses significantly increased uterine progesterone receptors, and at 100 and 150 mg/kg/day increased uterine peroxidase activity. Bisphenol A alone at a dose of 5 mg/kg/day inhibited peroxidase activity relative to negative controls. Co-administration of 5 mg/kg/day bisphenol A with estradiol (0.5 µg) resulted in an inhibition of peroxidase and progesterone receptor induction relative to estradiol alone, although not to negative control levels, while no effect on uterine weight was observed. This study shows the marked insensitivity to bisphenol A of the uterus using the standard uterotrophic assay in prepubertal Sprague-Dawley rats, while uterine peroxidase activity and progesterone receptors did show significant responses at the lowest bisphenol A dose tested.

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

Pregnant CD-1 mice were fed low and high doses of diethylstilbestrol, DES, (0.1 µg/kg/day and 200 µg/kg/day) and a low dose of bisphenol A (50 µg/kg/day). Fifteen male offspring per group were examined at 3, 21 and 60 days old. Bisphenol A and 0.1 µg/kg/day DES significantly increased prostate weight at all ages and also decreased epididymis weight (only examined at day 60). The 200 µg/kg/day dose of DES significantly decreased prostate weight at all ages. Bisphenol A and 0.1 µg/kg/day DES significantly increased prostate androgen receptors. When fetal prostate was placed in culture, a significant increase in prostate gland number and size and an increase in prostate androgen receptors occurred with a 50 pg/ml medium (50 ppt) dose of bisphenol A and a 0.5 ppt dose of DES (with a constant amount of testosterone). A 5 ppt dose of bisphenol A did not stimulate prostate growth. The *in vitro* LOAEL for stimulating growth of the fetal mouse prostate is 50 ppt. This demonstrates that effects of low-doses of bisphenol A and DES are on the prostate. There were opposite effects of low and high doses of DES on the prostate, replicating findings by vom Saal et al. 1997 and providing additional evidence for inverted-U dose-response curves for DES.

Hahn, T., Schenk, K. and Schultz, R. (2002) Environmental chemicals with known endocrine potential affect yolk protein content in the aquatic insect (Chironomus riparius). Environ. Pollut. 120: 525-528.

Vitellogenin and vitellin immunoreactivity was measured in freshly emerged midges. Although females responded only to the highest dose of BPA, males responded significantly at all doses tested (1, 100, and 3000 ug/L - ppb). The use of vitellogenesis as a marker for possible effects of endocrine disrupting agents on insects was tested in the aquatic midge Chironomus riparius. As test substances the synthetic ecdysoid tebufenozide, and the endocrine disruptors bisphenol a and 4-n-nonylphenol were applied in a semi-static manner. The yolk protein contents of freshly emerged (24 h) male and female midges were determined by an ELISA procedure. In males, where always low amounts of immunoreactivity were apparent, yolk concentrations were lowered by 10% after a 80 microg/l tebufenozide treatment, and by 20-25% after exposition to bisphenol a at concentrations of 1, 100, and 3,000 microg/l. 4-n-nonylphenol contamination caused an inverted dose-response curve. At low test concentrations (1.9-30 microg/l) reduced yolk immunoreactivity occurred, while at medium concentrations (120 and 500 microg/l) no significant effects were observable. In the most highly contaminated group (2,000 microg/l) yolk protein immunoreactivity was elevated to 107% of the control. Female yolk protein contents were affected only in the 3,000 microg bisphenol a/l contaminated group, where yolk immunoreactivity was reduced by ca. 10% compared to the control.

Haubruge, E., Petit, F. and Gage, M. J. G. (2000). Reduced sperm counts in guppies (Poecilia reticulata) following exposure to low levels of tributyltin and bisphenol A. Proc. Roy. Soc. Lond. 267:2333-2337.

Adult male guppies were exposed to bisphenol A at 2 concentrations: 274 ppb and 549 ppb. There was a doses related decrease in mature sperm stored in deferent testes canals prior to ejaculation. The 274 ppb dose reduced sperm by about 50%, and the 549 ppb dose reduced sperm by about 70%.

Honkanen JO, Holopainen IJ, Kukkonen JVK. 2004. Bisphenol A induces yolk-sac oedema and other adverse effects in landlocked salmon (Salmo salar m. sebago) yolk-sac fry. Chemosphere 55:187-196.

Accumulation and toxicity of waterborne bisphenol A were studied in landlocked salmon (Salmo salar m. sebago) yolk-sac fry. In a short-term (96 h) exposure to five bisphenol A concentrations yolk-sac fry had higher accumulation rates and bioconcentration factors (BCF96) than earlier studies have shown for salmon eggs. Furthermore, the conditional uptake rate constant tended

to decrease as exposure concentration increased. Fry were also exposed to bisphenol A for 42 days at three concentrations (10, 100 and 1000 µg/l - ppb), and changes in behaviour, morphology and histological structure were observed. After 6 days of exposure, the highest concentration (1000 mug/1) of bisphenol A caused fluid accumulation (oedema) in the yolk sac and haemorrhages in the front part of the yolk sac and in the head around the gill arches. Later on, the fry at 1000 mug/l showed phlegmatic behaviour and had darker skin coloration than the fry in the other treatments. At the two highest concentrations (100 and 1000 mug/1) histological changes were seen in liver cell nuclei, where strongly stained fragments were observed. In the control fry and the fry exposed to 10 mug/l the nucleolus was clearly visible and spherical in shape and no strongly stained fragments were present. This study shows that 100 and 1000 ppb concentrations of bisphenol A may have both morphological and histological effects on salmon yolk-sac fry.

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.

Bisphenol A was injected into pregnant ICR mice at doses of 2 and 20 µg/kg/day, and DES was injected at doses of 0.02, 0.2 and 2 µg/kg/day from gestation day 11-17. Age at vaginal opening and first vaginal estrus was advanced at all DES doses and at 20 µg/kg/day bisphenol A, and this also occurred at lower body weight relative to controls. These treatments did not alter the number of offspring produced when the treated females reached adulthood.

Hoshi, H., Y. Kamata and T. Uemura (2003). Effects of 17beta-estradiol, bisphenol A and tributyltin chloride on germ cells of Caenorhabditis elegans. J. Vet. Med. Sci. 65: 881-885.

Effects of a one-generation exposure to a natural estrogen, 17beta-estradiol (E2), and environmental pollutants such as bisphenol A (BPA) and tributyltin chloride (TBTCL) on the number of germ cells were investigated in the hermaphrodite Caenorhabditis elegans. The eggs of gravid adult worms isolated by alkaline hypochlorite treatment were seeded on a test chemical-containing NGM (nematode growth medium) agar plate without cholesterol. After incubation for 6 days at 16 degrees C, the germ cells of adult worms were stained with 4', 6-diamino-2-phenylindole dihydrochloride (DAPI). The staining procedure was completed within one hour and the stained germ cells were counted under a fluorescence microscope without dissection. The number of germ cells in the worms treated with E2 (10(-10)-10(-6) M) and BPA (10(-9)-10 (-5) M) was significantly increased. Maximal increases were observed at 10(-8) M E2 (156 +/- 15.3% of control) and 10(-5) M BPA (168 +/- 20.0 % of control). TBTCL (10(-9)-10(-6) M) significantly decreased the number of germ cells. The minimal decrease was observed at 10(-6) M TBTCL (30.2 +/- 3.51% of control). These results indicate that changes in the number of germ cells are a sensitive indicator of the effects of chemicals on the reproductive system. Since the method described in this paper is a novel, simple, time- and money-saving bioassay, C. elegans is an excellent model with which to determine the reproductive toxicity of chemicals including environmental pollutants.

Howdeshell, K.L., Hotchkiss, A.K., Thayer, K.A., Vandenbergh, J.G. and vom Saal, F.S. (1999). Exposure to bisphenol A advances puberty. Nature 401:763-764.

A 2.4 µg/kg/day dose of bisphenol A fed to pregnant CF-1 mice increased body weight of male and female offspring and resulted in early onset of puberty. The effect of bisphenol A was greatest in males and females with the highest background levels of estradiol during fetal life due to being positioned in utero between two female fetuses.

Hunt, P. A., K. E. Koehler, M. Susiarjo, C. A. Hodges, A. Hagan, R. C. Voigt, S. Thomas, B. F. Thomas and T. J. Hassold (2003). Bisphenol A causes meiotic aneuploidy in the female mouse. Current Biol. **13**:546-553.

An adverse effect of exposure to very low doses of bisphenol A is profound disruption of chromosomes during meiosis in oocytes in female mice exposed to bisphenol A that leached out of polycarbonate animal cages and water bottles after being washed in an alkaline detergent. Specifically, Hunt and colleagues report that after washing polycarbonate cages and water bottles in an alkaline detergent (which greatly accelerates the breakdown of the polycarbonate ester bonds), there was a dramatic increase in the incidence of abnormal alignment of chromosomes during the first meiotic division in oocytes. This results in aneuploidy, or abnormal numbers of chromosomes in oocytes, such as occurs in Down's syndrome; these authors thus refer to bisphenol A as a "potent meiotic aneugen". Aneuploidy is thought to be a major cause of embryonic mortality in humans; with the exception of chromosome 21 (Down's syndrome), abnormal numbers of chromosomes are typically lethal. Hunt reported that severe oocyte chromosome abnormalities increased in peripubertal female mice from a baseline frequency of 1.8% in control animals (not housed in damaged cages) to 20% due to housing females in damaged polycarbonate cages, 30% due to the use of damaged polycarbonate water bottles, and 41% due to combined use of both damaged cages and water bottles. In a subsequent experiment the researchers intentionally damaged polycarbonate water bottles by washing them different numbers of times in the detergent. The polycarbonate water bottles were found by gas chromatography-mass spectrometry (GC-MS) analysis to release between 100 (mild damage) and 260 µg/liter (severe damage) free bisphenol A into water placed into the bottles, resulting in daily exposure of the female mice ranging between 15-72 µg/kg/day. When peripubertal female mice housed in undamaged new cages were fed bisphenol A one time per day in oil at the very low doses of 20, 40 and 100 µg/kg/day to simulate exposure within the range released by the polycarbonate, there was a significant dose-related increase in the incidence of aneuploidy.

Imanishi S, Manabe N, Nishizawa H, Morita M, Sugimoto M, Iwahori M, Miyamoto H. 2003. Effects of oral exposure of bisphenol A on mRNA expression of nuclear receptors in murine placentae assessed by DNA microarray. Journal of Reproduction & Development 49:329-336.

Bisphenol A (BPA), a candidate endocrine disruptor (ED), is considered to bind to estrogen receptors and to regulate expressions of estrogen responsive genes. It has also shown evidence of affecting the reproductive, immunological and nervous systems of mammalian embryos. However, the effects of BPA on placentae, a central organ of feto-maternal interlocution, are still unclear. To reveal the mechanisms of BPA effects on placentae in mammals, we compared the mRNA expression of 20 nuclear receptors between placentae of vehicle controls and those of orally BPA exposed pregnant mice (2 µg/kg/day from GD 6-17) by a DNA microarray technique. In murine placentae, mRNAs of 11 nuclear receptors were not detected. However, greater than 1.5 fold changes in mRNA expression of nine nuclear receptors between vehicle control and BPA treated mice were noted. Moreover, remarkable changes in mRNA expression of six non-nuclear receptor proteins were induced by BPA exposure. There were various differences in the effects of BPA on the expression of these mRNAs between the placentae with male embryos and those with female embryos. Such embryo-sex dependent differences are interesting and important pointers to understanding of the endocrine disrupting effect of BPA. The present data indicate that BPA affects the expression of nuclear receptor mRNAs in placentae and may disrupt the physiological functions of placentae.

Ishibashi, H., Watanabe, N., Matsumura, N., Hirano, M., Nagao, Y, Shiratsuchi, H., Kohra, S., Yoshihara, S. and Arizono, K. (2005). Toxicity to early life stages and an estrogenic effect of a bisphenol A metabolite, 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene on the medaka (Oryzias latipes). Life Sci. ONLINE at sciencedirect.com.

In a recent study, it was reported that 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP), a metabolite of bisphenol A (BPA; 2,2-bis(4-hydroxyphenyl)propane), showed estrogenic activity in several in vitro assays, and the estrogenic activity of MBP was higher than that of BPA. In this study, we have investigated the early life stage toxicity and estrogenic effect of MBP on medaka (Oryzias latipes). The 96-h median lethal concentration value of MBP and BPA with 24-h-old larvae was estimated to be 1640 and 13,900 µg/l, respectively. The hatchability of fertilized eggs exposed to MBP and BPA over 14 days was significantly decreased at doses of 2500 µg/l and 12,500 µg/l, respectively. Moreover, to compare the potency of estrogenic activity in vivo, male medaka were exposed to various concentrations of MBP and BPA for 21 days. The lowest-observed-effect concentrations of MBP and BPA for hepatic vitellogenin induction in male medaka were estimated to be 4.1 and 1000 µg/l, respectively. These results suggest that MBP has high toxicity for early life stages of the medaka, and that the estrogenic activity of MBP was about 250-fold higher than that of BPA to male medaka.

Ishido, M., Y. Masuo, M. Kunimoto, S. Oka and M. Morita (2004). Bisphenol A causes hyperactivity in the rat concomitantly with impairment of tyrosine hydroxylase immunoreactivity. J. Neurosci. Res. 76:423-33.

A single intracisternal administration of bisphenol A (doses ranged from 0.02-20 µg/animal or about 3, 30, 300 and 3000 µg/kg)) into 5-day-old male Wistar rats caused significant hyperactivity at 4-5 weeks of age. Rats were about 1.6-fold more active in the nocturnal phase after administration of 30 – 3000 µg/kg of bisphenol A than were control rats, and the response was dose-dependent. There was no effect of bisphenol A on body weight. Based on DNA macroarray followed by RT-PCR analyses of the striatum and midbrain, bisphenol A decreased by more than two fold gene expression levels of the dopamine D4 receptor at 4 weeks of age and the dopamine transporter at 8 weeks of age. Bisphenol A inhibited central dopaminergic system activity, resulting in hyperactivity. A large reduction of tyrosine hydroxylase activity in the substantia nigra at the one bisphenol A dose tested (3000 µg/kg), indicating a marked reduction in the capacity to synthesize dopamine. This is important since the symptoms of ADHD are inattention, impulsiveness, and hyperactivity.

Jobling, S., D. Casey, T. Rodgers-Gray, J. Oehlmann, U. Schulte-Oehlmann, S. Pawlowski, T. Baunbeck, A. P. Turner and C. R. Tyler (2003). Comp0arative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat. Toxicol. **65**(2): 205-20.

This study concerned the relative sensitivities of different aquatic wildlife species to individual estrogenic chemicals [17alpha-ethinylestradiol (0.1 – 100 ppt), bisphenol-A (1-100 ppb), and 4-tert octylphenol (1-100 ppb)], which are within the range of doses present in the environment. Animals were also exposed to mixtures of these, and many other, chemicals present in treated sewage effluent, that was collected and administered (using serial dilution) in a continuous flow system for this study. Effects on embryo production in the prosobranch mollusc, Potamopyrgus antipodarum, were studied and compared with the effects on vitellogenin induction and egg production in various species of freshwater fish (fathead minnow; Pimaphales promelas, rainbow trout (Oncorhynchus mykiss); Cyprinus carpio, carp; Cyprinus carpio). The lab-based studies demonstrated that all of the tested chemicals (known to be estrogenic and to cause reproductive effects in fish) also affected embryo production in P. antipodarum. Exposure to ethinylestradiol induced similar reproductive responses in the snails as in the fathead minnow (Pimephales promelas), stimulating egg/embryo production at low doses (up to 1 ng/l (1 ppt) in the minnow and 25 ng/l in the snail) and causing inhibitory effects at higher doses. A similar pattern of embryo production occurred in P. antipodarum when it was exposed to a graded concentration of treated sewage effluent containing mixtures of estrogenic chemicals and hence, the total number of new embryos produced by the snails increased steadily over the 9 weeks exposure period in treated snails. In fact, for most endpoints in studies using the different

species exposed to the effluent mixture, the lowest dilution examined (12.5%) caused significant effects after one month of exposure. Plasma vitellogenin (an estrogen regulated liver protein) concentrations in two species of male fish (the rainbow trout and the carp) also increased over the same time period. These data indicate that both the nature of the response and the relative sensitivities to environmental estrogens in P. antipodarum and three different fish species fish are comparable. P. antipodarum is thus, potentially a sensitive test organism for assessing estrogenicity of chemicals with a relevance to their activity in vertebrates. These findings provide further evidence for inverted-U dose-response relationships for estrogenic chemicals, with low doses producing effects opposite to, and thus unpredicted by, high doses. For example, embryo production in snails was influenced by ethinylestradiol, which stimulated produccttion at 1 ppt but inhibited production at 100 ppt. At bisphenol A and octylphenol doses of 5 and 25 ppb, embryo production was stimulated, while a dose of 100 ppb inhibited embryo production. Similarly, at 5 and 25 ppb, bisphenol A stimulated growth in snails, but no effect was seen at higher doses. In fathead minnows, ethinylestradiol caused the same inverted-U effect on egg production, with significant stimulation at 0.1 ppt, maximum stimulation at 1 ppt, significant inhibition at 10 ppt, and complete inhibition at 100 ppt. The authors concluded that based on all data, that bisphenol A is approximately 100-fold less potent than ethinylestradiol, which is similar to the relationship seen in studies of these chemicals in rodents and in comparisons of bisphenol A and DES in rodents.

Kabuto, H., S. Hasuike, N. Minagawa and T. Shishibori (2003). Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. Environ. Res. **93**(1): 31-35.

Adult male ICR mice were injected ip for 5 days with 25 or 50 mg/kg/day bisphenol A and tissues were examined 6 hours after the last injection. Thiobarbituric acid-reaactive substance (TBARS) was measured as a peroxidation indicator. Also measured were the antioxidant (free radical scavenger), glutathione, as well as enzymes: superoxide dismutase (that catalyzes the dismutation of superoxide to hydrogen peroxide), and both glutathione peroxidase and catalyse (that convert hydrogen peroxide into water (hydrogen oxide). Oxidative stress reduces tissue levels of glutathione. Bisphenol A levels 6 hours after the last 50 mg/kg injection ranged from 0.19 (plasma) to 1.2 (fat) to 2 (kidney) ppb (ng/mg wet weight). There was a dose-related increase in liver superoxide dismutase activity, while liver catalyse decreased at 50 mg/kg/day bisphenol A, suggesting that bisphenol A produces an overproduction of hydrogen peroxide in the liver. The interesting aspect of this findings is that while bisphenol A, similar to other phenols, has antioxidant properties, it acts as a peroxidant via its other biological pathways. The 50 mg/kg/day dose of bisphenol A is the current LOAEL used by the EPA to calculate the reference dose of 50 µg/kg/day, which would not be affected by the results of this study. Significant effects were observed at the 25 µg/kg/day dose.

Kabuto, H., M. Amakawa and T. Shishibori (2004). Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. Life Sci. 74:2931-40.

Endogenous antioxidant capacity and oxidative damage in the brain, liver, kidney and testis in mice exposed to bisphenol A (BPA) was examined. Mice were exposed to BPA throughout embryonic/fetal life and during lactation by feeding their pregnant/lactating mothers BPA at 5 or 10 µg per milliliter of drinking water (approximately 2.5-5 µg/kg/day). At the age of four weeks, male mice were sacrificed. The 10 µg/ml dose of BPA resulted in bisphenol A at 10 –25 ng/g (ppb) wet weight in brain, kidney liver and testes. Oxidative stress due to accumulation of hydrogen peroxide. Superoxide and hydroxyl radicals results in tissue damage during aging. Thiobarbituric acid-reaactive substance (TBARS) was measured as a peroxidation indicator. Also measured were the antioxidant (free radical scavenger), glutathione, as well as enzymes: superoxide dismutase (that catalyzes the dismutation of superoxide to hydrogen peroxide), and

both glutathione peroxidase and catalyse (that convert hydrogen peroxide into water (hydrogen oxide). Oxidative stress reduces tissue levels of glutathione. Exposure to BPA increased the activity of catalase and glutathione peroxidase in the liver and kidney, respectively. It also increased thiobarbituric acid-reactive substances in the brain, kidney and testis, and decreased the wet weight of the brain, kidney and testis, while no effect on body weight was noted. These results suggest that exposure to BPA throughout embryonic/fetal life and during infancy induces tissue oxidative stress and peroxidation, ultimately leading to underdevelopment of the brain, kidney and testis.

Kashiwada S, Ishikawa H, Miyamoto N, Ohnishi Y, Magara Y. (2002). Fish test for endocrine-disruption and estimation of water quality of Japanese rivers. Water Research 36: 2161-2166.

The LC50 values (72 h) of 17beta-estradiol (E2), p-nonylphenol (NP) and bisphenol-A (BPA) to adult male and female medaka were 3.5 and 3.5, 0.85 and 0.87, and 6.8 and 8.3 mg L-1, respectively the LC50 values to embryos were 0.46, 0.13 and 5.1 mg L-1, respectively. The IC50 values for inhibition to egg hatching were 0.47, 0.85 and 9.0 mg L-1, respectively. These values were much higher than concentrations detected in river water in Japan and the chemicals were considered to have no lethal effect on the fish in an aquatic environment. Mature male medaka was continuously exposed to 0.005, 0.05 or 1.0 µg/L (ppb) of E2, or to 0.1, 10 or 100 µg/L (ppb) of NP or BPA. Female specific proteins (FSP) were induced in the blood of male medaka that were exposed for 5 weeks to E2 at doses above 0.005 ppb, to NP at doses above 0.1 ppb, or BPA at doses above 10 ppb. Based on these ESP inducible concentrations and reported concentrations of E2, NP and BPA in Japanese river water, some river water contaminated by E2 or NP could be estimated as the ESP inducible in male medaka.

Kawai, K., N. Takehiro, H. Nishikata, S. Aou, M. Takii and C. Kubo (2003). Aggressive behavior and serum testosterone concentration during the maturation process of male mice: The effects of fetal exposure to bisphenol A. Environ. Health Perspect. **111**:175-178.

Effects of bisphenol A on the behavior of offspring at very low doses (2 and 20 µg/kg/day) administered to pregnant female mice were examined. Specifically, the duration of time that male offspring spent interacting with other males in an aggressive manner was significantly increased by both doses of bisphenol A. Testis weight was decreased and serum T was lower in BPA treated males.

Khurana, S., S. Ranmal and N. Ben-Jonathan (2000). Exposure of newborn male and female rats to environmental estrogens: delayed and sustained hyperprolactinemia and alterations in estrogen receptor expression. Endocrinology 141:4512-4517.

The estrogenicity of two such compounds, bisphenol A (BPA) and octylphenol (OP), during development of the neuroendocrine system was investigated. The objective was to compare the effects of neonatal exposure to BPA, OP, and diethylstilbestrol (DES), a potent synthetic estrogen, on prepubertal serum PRL levels and estrogen receptor (ER) expression in the anterior pituitary and medial basal hypothalamus. Receptor expression in the uterus and prostate, two peripheral estrogen-responsive tissues, was also examined. Newborn male and female Fischer 344 rats were s.c. injected on days 1-5 after birth with corn oil (control), BPA and OP (100 or 500 microg/day; abour 15 and 75 mg/kg/day), or DES (5 microg/day; about 0.75 mg/kg/day). Rats were bled on days 15, 20, and 25 and on the day of death (day 30), and serum PRL was analyzed by RIA. Relative expressions of ERalpha and ERbeta were determined by RT-PCR. BPA and OP induced delayed, but progressive, increases in serum PRL levels, up to 3-fold above control levels, in both males and females. The low dose of either compound was equally or more effective as the high dose in eliciting and sustaining elevated serum PRL levels, namely hyperprolactinemia (NOTE: this is suggestive of an inverte-U dose response relationship). In contrast, the DES treatment resulted in a transient rise in serum PRL

levels. BPA, OP, and, to a lesser extent, DES increased the expression of both ERalpha and ERbeta in the anterior pituitary of males, but not females, whereas the hypothalamic ERs were less responsive to these compounds. DES treatment caused down-regulation of ERalpha expression in the uterus and up-regulation of ERbeta in the prostate, whereas BPA or OP was without effect. In conclusion, exposure of newborn rats of either sex to environmental estrogens results in delayed and sustained hyperprolactinemia and differential alterations in ER expression in the hypothalamus and pituitary. DES appears to target the lower reproductive tract more effectively than the neuroendocrine system.

Kloas, W. Lutz, I. And Einspanier, R. (1999). Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals in vitro and in vivo. The Science of the Total Environment 225:59-68.

In African frogs (Xenopus laevis), sex determination is influenced by estrogen, which can result in sex reversal (animals that would have become males instead become females). Exposure of Xenopus tadpoles for 12 weeks to 23 ppb bisphenol A significantly changed the sex ratio and increased the number of females, similar to a 2.8 ppb dose of estradiol.

Koehler, K. E., R. C. Voigt, S. Thomas, B. Lamb, C. Urban, T. J. Hassold and P. A. Hunt (2003). When disaster strikes: Rethinking caging materials. Lab Anim. 32:32-35.

These authors report that there was a significant increase in mortality in mice at the time the cages that caused aneuploidy in the Hunt et al. (2003) study were being used in their animal colony. They reported that some polycarbonate cage manufacturers are now only recommending use of the cages for 20 autoclave cycles.

Kubo, K., Arai, O., Ogata, R., Omura, M., Hori, T. and Aou, S. (2001). Exposure to Bisphenol A during the fetal and suckling periods disrupts sexual differentiation of the locus coeruleus and of behaviour in the rat. Neurosci. Lett. 304(1-2): 73-76.

Bisphenol A was administered in water at a dose of 1.5 mg/kg/day to pregnant and lactating Wistar rats. While there were sex differences in control males and females in activity, avoidance learning and the size of the locus coeruleus in the brain, bisphenol A exposure eliminated these sex differences.

Kubo, K., O. Arai, M. Omura, R. Watanabe, R. Ogata and S. Aou (2003). Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci. Res. **45**:345-356.

Bisphenol A was administered to pregnant and lactating Wistar rats in drinking water at doses of 30 and 300 µg/kg/day, and also administered DES as a positive control at 6.5 µg/kg/day. Open field behavior was sexually dimorphic in control animals, but sex differences were eliminated in animals exposed to bisphenol A or DES. While the lower dose of bisphenol A reduced the rate of intromission during coitus in males, other measures of male sexual behavior were not affected. DES reduced the size of the epididymis but no other inhibitory effects were noted on male reproductive organs; only the ventral prostate, not the dorsolateral, was weighed. There were no effects of bisphenol A on female sexual behaviors, and no significant effects on serum gonadotropins or gonadal steroids were found. In this study the reversal of the normal sex difference (F > M)) in the size of the locus coeruleus as a result of exposure to both the low and high dose of bisphenol A, as well as DES, was again found. This finding shows that bisphenol A can change brain structure at very low (30 µg/kg/day) doses and is similar to DES administered at a dose 5 – 50-fold lower than the bisphenol A dose. This confirms other findings that bisphenol A and DES show similar outcomes and that for some effets, the potency of DES relative to bisphenol A falls within 10-100 fold.

Kwak, H. I.; Bae, M. O.; Lee, M. H.; Lee, Y. S.; Lee, B. J.; Kang, K. S.; Chae, C. H.; Sung, H. J.; Shin, J. S.; Kim, J. H.; Mar, W. C.; Sheen, Y. Y., and Cho, M. H. (2001). Effects of nonylphenol, bisphenol A, and their mixture on the viviparous swordtail fish (*Xiphophorus helleri*). Environmental Toxicology & Chemistry. 20(4):787-795.

A number of fish species have been used for studies on endocrine disrupting chemicals (EDCs). However, despite the widespread use of oviparous fish, relatively little attention has been given to viviparous species. This study investigated the effects of EDCs in a viviparous fish and examined the possible usefulness of the fish as an alternative model for the studies on EDCs. Swordtails (Xiphophorus helleri) were exposed to nonylphenol (NP), bisphenol A (BPA), and their mixture. Both short-term (3-d) and relatively long-term (60-d) exposures were carried out using adult male and 30-d-old juvenile fish, respectively. The acute LC-50 for BPA was 18 ppm. Following short-term exposure, a mixture of 4 ppb NP and 400 ppb BPA caused vitellogenin mRNA expression. Flow cytometric analysis and terminal deoxynucleotidyl transferase assay on the testes of treated fish indicated reproductive damage at the lowest dose of BPA tested, which was 400 ppb. Histopathological analysis found degenerative and necrotic cells in seminiferous tubules following the exposure to 100 ppb NP but not BPA. The testes with lesions were also associated with highly suppressed spermatogenesis. Following the long-term (60 day) exposure, both NP and BPA exposures significantly affected the growth of swordtails; there was a dose-related inhibitory effect on sword growth between 0.2 and 20 ppb BPA). In all cases, the results showed that the mixture was always more potent than a single chemical and that swordtail fish can be a useful model for the study of endocrine disruptors.

Laviola, G., Gioiosa, L., Adriania, W. and Palanza, P. (2005) d-Amphetamine-related reinforcing effects are reduced in mice exposed prenatally to estrogenic endocrine disruptors. Brain Research Bulletin 65:235–240.

Estrogenic endocrine disruptors are hormonally active compounds that can bind to estradiol receptors. Central dopamine pathways have been reported to be affected by early developmental exposure to estrogenic endocrine disruptors. In the present study, pregnant female CD-1 mice were allowed to drink spontaneously either oil or environmentally relevant low doses of two estrogenic compounds, methoxychlor (20 µg/kg) or bisphenol-A (10 µg/kg) during gestation days 11–18. Their adult offspring were assessed for conditioned place preference produced by d-amphetamine (0, 1 or 2 mg/kg). Interestingly, prenatal treatment effects were sex-dependent and no changes in conditioned place preference emerged for the male offspring. Conversely, a clear-cut profile of d-amphetamine-induced conditioned place preference was only shown by oil-exposed females, whereas exposure to bisphenol-A or methoxychlor resulted in little or no place conditioning. Locomotor effects of acute d-amphetamine were not affected by prenatal exposure to bisphenol-A or methoxychlor. As a whole, prenatal exposure to estrogenic endocrine disruptors affected some steps in the organization of the brain dopaminergic systems in the female offspring, thus leading to long-term alterations in neurobehavioral function. These data confirm that exposure to weak environmental estrogens in the period of brain sexual differentiation can influence adult behavior.

Lee, M.H., Chung, S.W., Kang, B.Y., Park, J., Lee, C.H., Hwang, S.Y. and Kim, T.S. (2003). Enhanced interleukin-4 production in CD4+ T cells and elevated immunoglobulin E levels in antigen-primed mice by bisphenol A and nonylphenol, endocrine disruptors: involvement of nuclear factor-AT and Ca2+. Immunology 109:76-86.

Bisphenol A (BPA) and p-nonylphenol (NP) are representative endocrine disruptors (EDs) that may have adverse effects on human health. The influence of these compounds on allergic

immune responses remains unclear. In this study, we have examined the effects of BPA and NP on production of interleukin-4 (IL-4), a pro-inflammatory cytokine closely associated with allergic immune responses. Both BPA and NP significantly enhanced IL-4 production in keyhole limpet haemocyanin (KLH)-primed CD4+ T cells in a concentration-dependent manner. Treatment with BPA or NP in vivo resulted in significant increase of IL-4 production in CD4+ T cells and of antigen-specific immunoglobulin E (IgE) levels in the sera of KLH-primed mice. Furthermore, BPA and NP enhanced the activation of IL-4 gene promoter in EL4 T cells transiently transfected with IL-4 promoter/reporter constructs, and the enhancing effect mapped to a region in the IL-4 promoter containing binding sites for nuclear factor (NF)-AT. Activation of T lymphocytes by phorbol 12-myristate 13-acetate/ionomycin resulted in markedly enhanced binding activities to the NF-AT site, which significantly increased upon addition of BPA or NP, as demonstrated by the electrophoretic mobility shift assay, indicating that the transcription factor NF-AT was involved in the enhancing effect of BPA and NP on IL-4 production. The enhancement of IL-4 production by BPA or NP was significantly reduced by nitrendipine, a blocker of Ca2+ influx, and by FK506, a calcineurin inhibitor. FK506 inhibited the NF-AT-DNA binding activity and IL-4 gene promoter activity enhanced by BPA (10 or 50 μM) or NP (1 and 5 μM). These results represent the first report describing possible enhancement of allergic response by EDs through increasing IL-4 production in CD4+ T cells and antigen-specific IgE levels in the sera via the stimulation of Ca2+/calcineurin-dependent NF-AT activation.

Lemmen, J. G., R. J. Arends, P. T. van der Saag and B. van der Burg (2004). In vivo imaging of activated estrogen receptors in utero by estrogens and bisphenol A. Environ. Health Perspect. 112, 1544-1549.

Environmental estrogens are of particular concern when exposure occurs during embryonic development. Although there are good models to study estrogenic activity of chemicals in adult animals, developmental exposure is much more difficult to test. The weak estrogenic activity of the environmental estrogen bisphenol A (BPA) in embryos is controversial. We have recently generated transgenic mice that carry a reporter construct with estrogen-responsive elements coupled to luciferase. We show that, using this *in vivo* model in combination with the IVIS imaging system, activation of estrogen receptors (ERs) by maternally applied BPA and other estrogens can be detected in living embryos *in utero*. Eight hours after exposure to 1 mg/kg BPA, ER transactivation could be significantly induced in the embryos. This was more potent than would be estimated from *in vitro* assays, although its intrinsic activity is still lower than that of diethylstilbestrol and 17ß-estradiol dipropionate. On the basis of these results, we conclude that the estrogenic potency of BPA estimated using *in vitro* assays might underestimate its estrogenic potential in embryos. *Key words:* bisphenol A, estrogen receptor, *in utero*, *in vivo*, reporter mice.

Levy, G., I. Lutz, A. Kruger and W. Kloas (2004). Bisphenol A inducees feminization in Xenopus laevis tadpoles. Environ. Res. 94:102-111.

In this study Kloas and colleagues replicated and extended their initial findings in two separate experiments. Levy reported that, in addition to causing a change in gonadal development in *Xenopus* tadpoles that would have differentiated into males, bisphenol A and estradiol also significantly up-regulated estrogen receptor (ER) mRNA extracted from the whole organism. Bisphenol A significantly altered ER mRNA expression at a dose of 2.3 ppb. The authors previously showed that estrogen receptor protein is increased in tadpoles by estradiol. An increase in estrogen receptors induced by bisphenol A could serve to increase the responsiveness of tissues to any estrogenic chemical. The initial findings of significant effects of bisphenol A altering gonadal development were replicated by Levy (Levy, Lutz et al. 2003) at a dose of 23 ppb, while significant effects of estradiol were again observed at 2.8 ppb. The effect of bisphenol A and

estradiol on the gonads was complete, in that 99% of the gonads were histologically normal rather than having a mix of both ovarian and testicular tissue (intersex condition). It is interesting that the dose-response curve for bisphenol A was found to form an inverted U, with doses of 2.3 and 230 ppb not significantly altering sex ratio.

Lindholst, C., Pedersen, K. L., and Pedersen, S. N. (2000). Estrogenic response of bisphenol A in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology. 48:87-94.

Bisphenol A (BPA) previously shown to possess xenoestrogenic activities was administered to rainbow trout (Oncorhynchus mykiss) through a continuos flow system. The estrogenic response expressed as the induction of vitellogenin (VTG) synthesis was measured during 12 days of exposure, using a direct sandwich ELISA. Quantification of internal liver and muscle concentrations of non-metabolised BPA was performed by LC-MS at the end of the exposure period. A significant induction of the VTG synthesis was obtained at 500 microg BPA/l (500 ppb) exposure, although an increase in the ratio of responding animals was observed already between 40 and 70 microg BPA/l (40-70 ppb). An increase in VTG levels was observed for the 500 microg BPA/l group over the study period, whereas constant or decreasing levels could be detected in the low exposure groups between days 6 and 12. Average internal liver concentrations of BPA increased from 0.22 to 4.36 microg/g for the 10-500 microg BPA/l groups. However, BPA could not be detected in muscle tissue below an exposure level of 70 microg BPA/l. A dose response relationship was established between the internal liver concentrations of BPA and the corresponding VTG responses, with a $P<0.001$ and a correlation coefficient of 0.66.

Long, X., Steinmetz, R., Ben-Jonathan, N., Caperell-Grant, A., Young, P.C.M., Nephew, K.P., and Bigsby, R.M. (2000). Strain diffrences in vaginal responses to the xenoestrogen bisphenol A. Environ. Health Perspect. 108:243-247.

Adult female Fischer 344 rats are more sensitive to bisphenol A than are female Sprague-Dawley rats, but metabolic clearance rate, the binding of bisphenol A to ERα and induction of the c-fos gene in vaginal cells from both strains was identical. However, at a dose of 37 mg/kg/day vaginal epithelium DNA synthesis was increased in female F344 rats, while no response is S-D rats occurred at doses up to 150 mg/kg/day. The strain difference was not explained by metabolic clearance rate or affinity or number of estrogen reeeptors, and both strains showed a similar induction of the early response gene, c-fos. Thus, the absence of induction by bisphenol A of vaginal DNA synthesis in Sprague-Dawley rats represents a deficit in induction of the genetic cascade that occurs after binding of bisphenol A to the estrogen receptor and activation of immediate early response genes, such as c-fos.

The absence of response to a specific estrogen-receptor ligand in selected tissues is evidence that bisphenol A acts as a selective estrogen receptor modulator (SERM). This hypothesis is consistent with a rapidly growing literature. There is now considerable evidence that bisphenol A acts as a SERM, and relative to estradiol: 1) interacts differently within the ligand-binding domain of estrogen receptors (Gould, Leonard et al. 1998), 2) shows a different binding affinity for and regulation of ERα and ERß in target cells (Kuiper, Carlsson et al. 1997; Routhledge, White et al. 2000), and 3) interacts differently with transcriptional co-regulators (Routhledge, White et al. 2000). In addition, there is now evidence that similar to estradiol, bisphenol A can elicit rapid responses in cells through non-genomic signaling systems (Wade, Robinson et al. 2001; Sato, Matsuki et al. 2002).

MacLusky, N.J., Hajszan, T. and Leranth, C. (2005). The environmental estrogen bisphenol A inhibits estrogen-induced hippocampal synaptogenesis. Environ. Health Perspect. 113:675-679.

Because BPA leaches out of plastic food and drink containers, as well as the BPA-containing plastics used in dental prostheses and sealants, considerable potential exists for human exposure to this compound. Here we show that treatment of ovariectomized rats with BPA dose-dependently inhibits the estrogen-induced formation of dendritic spine synapses on pyramidal neurons in the CA1 area of the hippocampus. Significant inhibitory effects of BPA are observed at a dose of only 40 µg/kg, below the current EPA reference daily limit for human exposure, and the dose that reduced the estradiol effect by 50% (ED-50) was 117 µg/kg. Since synaptic remodeling has been postulated to contribute to the rapid effects of estrogen on hippocampus-dependent memory, these data suggest that environmental BPA exposure may interfere with the development and expression of normal sex differences in cognitive function, via inhibition of estrogen-dependent hippocampal synapse formation. It may also exacerbate the impairment of hippocampal function observed during normal aging, as endogenous estrogen production declines.

Markey, C. M., Luque, E. H., Munoz De Toro, M., Sonnenschein, C. and Soto, A. M. (2001). In utero exposure to bisphenol a alters the development and tissue organization of the mouse mammary gland. Biol Reprod **65**(4):1215-23.

Bisphenol A was administered to female CD-1 mice on gestation day 9 through parturition via Alzet osmotic pumps at doses of 25 and 250 ng/kg/day (the initial report stated that the dose was 25 and 250 µg/kg/day based on gestation day 9 body weight, and this was corrected in a subsequent erratum published in Biology of Reproduction 71(5): 1753, 2004). At 30 days old, the 25 ng/kg/day dose of bisphenol A resulted in a significant increase in mammary gland duct length, while the 250 ng/kg/day bisphenol A dose resulted in a reduction in length, relative to the 25 ng/kg dose. By 6 months old, females exposed to both doses of bisphenol A showed a significant increase in mammary gland ducts and alveolar structures relative to control females.

Markey, C. M., Michaelson, C. L., Veson, E. C., Sonnenschein, C. and Soto, A. M. (2001). The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A. Environ Health Perspect 109:55-60.

The prevalence of synthetic chemicals in our environment that are capable of mimicking the female hormone estrogen is a growing concern. One such chemical, bisphenol A (BPA), has been shown to leach from a variety of resin-based and plastic products, including dental sealants and food and beverage containers, in concentrations that are sufficient to induce cell proliferation in vitro. The response to BPA in vivo has been varied; thus the aims of this study were to investigate a) whether BPA has an estrogenic effect in CD-1 mice, a strain that is useful for developmental studies; and b) whether the uterotrophic assay is a valid means of determining the estrogenicity of BPA by comparing it with other end points measured in the uterus. Immature female CD-1 mice were exposed to BPA in concentrations ranging from 0.1, 0.5, 1, 5, 50, 75 and 100 mg/kg body weight for 3 days. Results showed that BPA induced a significant increase in the height of luminal epithelial cells within the uterus at concentrations of 5, 75, and 100 mg/kg and that BPA induced lactoferrin at concentrations of 75 and 100 mg/kg. A uterotrophic response (increase in uterine wet weight) was induced by 100 mg/kg BPA only. Further, the proportion of mice showing vaginal opening was greater after exposure to 0.1 and 100 mg/kg BPA, relative to the control animals and those receiving intermediate doses of BPA. The BPA dose of 0.5 mg/kg/day significantly increased body weight, which was slightly, but not statistically elevated at other low BPA doses up to 5 mg/kg/day. These results demonstrate that BPA induces changes in the mouse uterus that differ depending on the exposure dose and the end point measured, and reveal that certain tissue effects show a nonmonotonic relationship with dose. These data also demonstrate that BPA induces estrogenic changes in the uterus of the CD-1 mouse, and highlight the need to reevaluate the validity of the

mouse uterotrophic assay as an end point for determining the estrogenicity of suspected environmental estrogens.

Markey, C.M., Coombs M.A., Sonnenschein C., Soto, A.M. (2003). Mammalian development in a changing environment: exposure to endocrine disruptors reveals the developmental plasticity of steroid-hormone target organs. Evolut & Develop. 5 (1):67-75.

Impregnated CD-1 mice were implanted with Alzet osmotic pumps delivering 25 and 250 ng/kg/day BPA from GD 1 through 9 (as with the Markey et al., 1991 Biol. Reprod. Paper, the dose was initially incorrectly published as being µg/kg/day instead of ng/kg/day). At 9 months the body weight of their female pups was significantly higher ($p<0.0001$ based on age and $p<0.05$ based on treatment). There were no differences in age of VO compared with controls. Those with persistent cyclicity were increased significantly in both dose groups. At 6 months there was an increase in blood-filled ovarian bursae. Histologically there were increased alveolar buds and terminal end buds and lobuloalveioli in the mammary glands especially in the 25 ng BPA/kg/day group at 4 months.

Markey, C. M., P. R. Wadia, B. S. Rubin, C. Sonnenschein and A. M. Soto (2005). Long-Term Effects of Fetal Exposure to Low Doses of the Xenoestrogen Bisphenol-A in the Female Mouse Genital Tract. Biol Reprod. 72:1344-1351.

Developmental exposure to estrogenic chemicals induces morphological, functional and behavioral anomalies associated with reproduction. Humans are routinely exposed to bisphenol-A (BPA), an estrogenic compound that leaches from dental materials and plastic food and beverage containers. The aim of the present study was to determine the effects of in utero exposure to low, environmentally relevant doses of BPA on the development of female reproductive tissues in CD-1 mice. In previous publications, we have shown that this treatment alters the morphology of the mammary gland and affects estrous cyclicity. Here we report that in utero exposure to 25 and 250 ng BPA/kg body weight/day via osmotic pumps implanted into pregnant dams at gestational day 9 induces alterations in the genital tract of female offspring that are revealed during adulthood. They include: decreased wet weight of the vagina, decreased volume of the endometrial lamina propria, increased incorporation of bromodeoxyuridine into the DNA of endometrial gland epithelial cells, and increased expression of estrogen receptor-alpha (ER alpha) and progesterone receptor in the luminal epithelium of the endometrium and subepithelial stroma. Since ER alpha is known to be expressed in these estrogen-target organs at the time of BPA exposure, it is plausible that BPA may directly affect the expression of ER controlled genes involved in the morphogenesis of these organs. In addition, BPA-induced alterations that specifically affect hypothalamic-pituitary-gonadal axis function may further contribute to the anomalies observed at three months of age, long after the cessation of BPA exposure.

Masuo, Y., M. Ishido, M. Morita and S. Oka (2004). Effects of neonatal treatment with 6-hydroxydopamine and endocrine disruptors on motor activity and gene expression in rats. Neural Plast 11:59-76.

To investigate the mechanisms underlying motor hyperactivity, we performed intracisternal injection of 6-hydroxydopamine or endocrine disruptors in rats on postnatal day 5. 6-Hydroxydopamine (100 microg, 488 nmol) caused a significant increase in spontaneous motor activities at 4 weeks of age. Gene-expression profiling using a cDNA membrane array revealed alterations in several classes of gene at 8 weeks of age. In the midbrain, gene expression was enhanced in dopamine transporter 1; a platelet-derived growth factor receptor; dopamine receptor D4; galanin receptor 2; arginine vasopressin receptor 2; neuropeptide Y; tachykinin 2; and fibroblast growth factor 10. Expression was also enhanced in the glutamate/aspartate transporter gene in the

striatum. Rats received an endocrine disruptor (87 nmol), such as bisphenol A, nonylphenol, p-octylphenol, or diethylhexylphthalate, which also caused motor hyperactivity at 4 weeks. The effects of bisphenol A on motor activity were dose-dependent from 0.87 to 87 nmol per PND 5 male pup that weighed ~ 10 g). 8.7 mM, 8.7 micro mol / ml; 87 n mol / 10 microliter = 198 ng injected ~200ng / 10g animal; 20 ppb inj or 20 µM/kg. The phenols caused a deficit in dopamine neurons, similarly to the deficit caused by 6-hydroxydopamine. Gene-expression profiles after treatment with endocrine disruptors showed variation and differed from those of 6-hydroxydopamine. The results suggest that neonatal treatment with environmental chemicals can generate an animal model of attention-deficit hyperactivity disorder, in which clinical symptoms are pervasive.

Metcalfe, C. D., Metcalfe, T. L., Kiparissis, Y., Koenig, B. G., Khan, C., Hughes, R. J., Croley, T. R., March, R. E. and Potter, T. (2001). Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by *in vivo* assays with japanese medaka (*Oryzias latipes*). Environmental Toxicology & Chemistry **20**(2):297-308.

Effects of bisphenol A were examined in Japanese medaka at doses ranging from 10-200 ppb in water from 1-100 days after hatching. Effects observed at 10 ppb included the presence of ovo-testes, which was noted in a few males. At 50-200 ppb, testicular abnormalities were observed, including a decrease in the number of spermatozoa.

Mizuo, K., Narita, M., Miyagawa, K., Okuno, E. and Suzuki, T. (2004). Prenatal and neonatal exposure to bisphenol-A affects the morphine-induced rewarding effect and hyperlocomotion in mice. Neurosci Lett 356:95-98.

Bisphenol-A (BPA), one of the most common environmental endocrine disrupters, has been extensively evaluated for toxicity and carcinogenicity. However, little is still known about its action on the CNS. Here we found that prenatal and neonatal exposure to BPA (0.002 – 2 mg/g feed; ~ 3 – 300 mg/kg/day) resulted in the enhancement of the rewarding effect and hyperlocomotion induced by morphine in mice. Under these conditions, no change in the G-protein activation by morphine and mu-opioid receptor expression in the lower midbrain was observed by prenatal and neonatal exposure to BPA. These results suggest that chronic exposure to BPA produces the supersensitivity of the morphine-induced rewarding effect and hyperlocomotion without direct changes in mu-opioid receptor function in the lower midbrain. The present data provide further evidence that prenatal and neonatal exposure to BPA can directly influence the development of the central dopaminergic system.

Mizuo, K., Narita, M., Yoshida, T. and Suzuki, T. (2004). Functional changes in dopamine D3 receptors by prenatal and neonatal exposure to an endocrine disruptor bisphenol-A in mice. Addict Biol 9:19-25.

Bisphenol-A (BPA), one of the most common environmental endocrine disrupters, has been evaluated extensively for toxicity and carcinogenicity. However, little is still known about its action on the central nervous system (CNS). In the previous study, we found that prenatal and neonatal exposure to BPA markedly enhanced the rewarding effect induced by morphine. Here we found that prenatal and neonatal exposure to BPA (2 mg/g feed) resulted in the attenuation of dopamine D3 receptor-mediated G-protein activation by 7-OH-DPAT in the mouse limbic forebrain. This treatment also caused a significant decrease in the B(max) value of [(3)H]PD128907, a dopamine D3 receptor ligand, in this area. Under these conditions, no change in dopamine D3 receptor mRNA expression in the limbic forebrain and lower midbrain was observed by prenatal and neonatal exposure to BPA. The present data provide further evidence that prenatal and neonatal exposure to BPA leads to the

reduction of functional dopamine D3 receptors without affecting the new synthesis of dopamine D3 receptors in the mouse limbic forebrain.

Munoz-de-Toro, M., Markey, C., Wadia, P.R., Luque, E.H., Rubin, B.S., Sonnenschein C. and Soto, A.M. (2005). Perinatal exposure to bisphenol A alters peripubertal mammary gland development in mice. Endocrinol. Online: May 31.

Developmental exposure to estrogenic chemicals induces morphological, functional and behavioral anomalies associated with reproduction. Humans are exposed to bisphenol A (BPA), an estrogenic compound that leaches from dental materials and plastic food and beverage containers. The aim of the present study was to determine the effects of perinatal exposure to low, environmentally relevant doses of BPA (25 and 250 ng BPA/kg body weight (bw)/day) on the peripubertal development of the mammary gland. BPA exposure enhanced the mammary glands' sensitivity to estradiol in ovariectomized CD-1 mice. In their intact 30-day-old littermates, the area and numbers of terminal end buds relative to the gland ductal area increased while their apoptotic activity decreased. There was a positive correlation between ductal length and the age at first proestrus; the slope was steeper in the controls and reduced as the BPA dose increased, suggesting that BPA exposure slows down ductal invasion of the stroma. There was also a significant increase of progesterone receptor-positive ductal epithelial cells that were localized in clusters, suggesting future branching points. Indeed, lateral branching was significantly enhanced at 4 months of age in mice exposed to 25 ng BPA /kg bw/day. In conclusion, perinatal exposure to environmentally relevant BPA doses results in persistent alterations in mammary gland morphogenesis. Of special concern is the increased terminal end bud density at puberty as well as the increased number of terminal ends reported previously in adult animals, since these two structures are the sites where cancer arises in humans and rodents.

Nagel, S. C., vom Saal, F. S., Thayer, K. A., Dhar, M. G., Boechler, M. and Welshons, W. V. (1997). Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. Environmental Health Perspectives 105:70-6.

The lower binding of bisphenol A in plasma relative to estradiol predicted a greater potency of bisphenol A in vivo than predicted, particularly in fetuses when plasma binding restricts estrogen entry into tissues. As predicted, administration to pregnant CF-1 mice of low doses (2 and 20 µg/kg/day) of bisphenol A resulted in prostate enlargement in male offspring. The effect at 20 µg/kg/day was predicted by the finding of reduced binding of bisphenol A in human serum relative to estradiol in the RBA-SMA assay conducted with human breast cancer (MCF-7) cells. The effect of bisphenol A on the testes, daily sperm production, epididymides and seminal vesicles in these males was reported in: vom Saal, et al. 1998 (see below).

Nagel, S. C., J. L. Hagelbarger and D. P. McDonnell (2001). Development of an ER action indicator mouse for the study of estrogens, selective ER modulators (SERMs), and Xenobiotics. Endocrinology 142(11): 4721-4628.

A transgenic mouse that incorporated estrogen receptors linked to a reporter gene was used to assess the effects of estrogens in tissues that express estrogen receptors. This mouse provides a novel method of separating the binding capacity of chemical estrogens for the estrogen receptor from their ability to elicit responses in different tissues. Consistent with other findings (Long et al. 2000), bisphenol A bound to estrogen receptors in the uterus and stimulated transcriptional activity at doses of 25 and 800 µg/kg, yet there was no effect on uterine growth, demonstrating the unique inability for the female reproductive tract to respond to bisphenol A by showing the normal proliferative response.

This study revealed, however, that a transcriptional response could be detected in the uterus at the same low doses that initiate transcriptional activity as well as proliferative responses in other tissues. This provides additional evidence that bisphenol A acts as a SERM.

Negishi, T., K. Kawasaki, S. Suzaki, H. Maeda, Y. Ishii, S. Kyuwa, Y. Kuroda and Y. Yoshikawa (2004). Behavioral alterations in response to fear-provoking stimuli and tranylcypromine induced by perinatal exposure to bisphenol A and nonylphenol in male rats. Environ. Health Perspect. 112:1159-64.

The purpose of this study was to examine whether perinatal exposure to two major environmental endocrine-disrupting chemicals, bisphenol A (BPA; 0.1 mg/kg/day orally) and nonylphenol [NP; 0.1 mg/kg/day (low dose) and 10 mg/kg/day (high dose) orally] daily from gestational day 3 to postnatal day 20 (transplacental and lactational exposures) would lead to behavioral alterations in the male offspring of F344 rats. Neither BPA nor NP exposure affected behavioral characteristics in an open-field test (8 weeks of age), in a measurement of spontaneous motor activity (12 weeks of age), or in an elevated plus-maze test (14 weeks of age). A passive avoidance test (13 weeks of age) showed that both BPA- and NP-treated offspring tended to delay entry into a dark compartment. An active avoidance test at 15 weeks of age revealed that 0.1 mg/kg/day BPA-treated offspring showed significantly fewer avoidance responses and low-dose NP-treated offspring exhibited slightly fewer avoidance responses. Furthermore, low-dose BPA-treated offspring significantly increased the number of failures to avoid electrical unconditioned stimuli within 5-sec electrical shock presentation compared with the control offspring. Perinatal exposure to a low dose of BPA thus led to an impaired ability to learn that a stimulus (sound and light) would predict getting shocked. Rats exposed to BPA showed signs of fear (crouching in the corner of the shock box) without an attempt to escape, a finding that is consistent with a report by Aloisi et al. 2002 showing that BPA increased sensitivity to noxious stimuli in male rats. In a monoamine-disruption test using 5 mg/kg (intraperitoneal) tranylcypromine (Tcy), a monoamine oxidase inhibitor, both BPA-treated and low-dose NP-treated offspring at 22-24 weeks of age failed to show a significant increment in locomotion in response to Tcy, whereas control and high-dose NP-treated offspring significantly increased locomotion behavior after Tcy injection. The present results indicate that perinatal low-dose BPA or NP exposure irreversibly influenced the reception of fear-provoking stimuli (e.g., electrical shock), as well as monoaminergic neural pathways.

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.

The objective of this study was to examine the effects of maternal exposure to xenoestrogen, at levels comparable to or greater than human exposure, on development of the reproductive tract and mammary glands in female CD-1 mouse offspring. Effects of genistein (GEN), resveratrol (RES), zearalenone (ZEA), bisphenol A (BPA) and diethylstilbestrol (DES) were examined. Beginning on gestational day 15, pregnant CD-1 mice were administered four daily subcutaneous injections with 0.5 or 10 mg/kg/day of GEN, RES, ZEA or BPA, 0.5 or 10 microg/kg/day of DES dissolved in dimethylsulfoxide (DMSO), or DMSO vehicle (n = 6). Vaginal opening was monitored, 6 animals per group were autopsied at 4, 8, 12 and 16 weeks of age and estrous cyclicity was monitored from 9 to 11 weeks of age. Maternal exposure to xenoestrogen accelerated puberty onset (vaginal opening) and increased the length of the estrous cycle; mice treated with GEN, RES, BPA or DES spent more time in diestrus, and ZEA-treated mice spent more time in estrus. Lack of corpora lutea and vaginal cornification were observed at 4 weeks of age in the high-dose GEN (33%) and RES (17%) groups, and in the high- and low-dose BPA groups (33 and 50%, respectively) and DES groups (83 and

100%, respectively). Lack of corpora lutea and vaginal cornification was observed in the high-dose ZEA group at 4, 8, 12 and 16 weeks of age (83, 100, 83 and 33%, respectively). Mammary gland differentiation was accelerated in ZEA- and BPA-treated mice with corpora lutea at 4 weeks of age. ZEA-treated mice without corpora lutea showed mammary growth arrest at 8, 12 and 16 weeks of age; their mammary glands consisted only of a dilatated duct filled with secreted fluid. Mammary gland growth was similar with xenoestrogens other than ZEA or BPA to that of the controls at all time points. High-dose GEN and RES and high- and low-dose BPA and DES exerted transient effects on the reproductive tract and mammary glands, whereas ZEA exerted prolonged effects. All chemicals resulted in an increase in body weight by 4 months of age relative to negative controls. Similar effecs of DES at 0.5 µg/kg/day and BPA at 500 µg/kg/day were found.

Nishizawa H, Manabe N, Morita M, Sugimoto M, Imanishi S, Miyamoto H. 2003. Effects of in utero exposure to bisphenol A on expression of RARalpha and RXRalpha mRNAs in murine embryos. Journal of Reproduction & Development 49:539-545.

Retinoic acid receptor (RAR) alpha and retinoid X receptor (RXR) alpha are key factors in a nuclear receptor-dependent signal. To evaluate the effects of bisphenol A (BPA), a candidate endocrine disrupter (ED), on embryonic development, we examined the mRNA levels of RARalpha and RXRalpha in murine embryos, exposed in utero to BPA (2 µg/kg/day) at 6.5-17.5 days post-coitum (dpc), by the real-time reverse transcription-polymerase chain reaction (RT-PCR) method. Higher levels of RARalpha mRNA in cerebra of male and female embryos of control groups were detected at 14.5 dpc. In utero BPA reduced the RARalpha mRNA expression. Higher levels of RXRa mRNA in cerebra of male and female embryos were seen at 12.5 dpc. The exposure decreased RXRalpha mRNA expression in male but not female embryos. No remarkable change in the RARalpha mRNA expression level was noted in cerebella of male or female embryos of the control group during embryonic development. Exposure to BPA increased expression levels of RARalpha mRNA in cerebella of male and female embryos at 12.5 dpc. Higher levels of RXRalpha. mRNA in cerebella of male and female embryos were seen, but no remarkable changes were noted during embryonic development. BPA significantly decreased the expression levels of RXRalpha mRNA in cerebella of female embryos at 12.5,14.5 and 18.5 dpc. RARalpha and RXRalpha mRNAs were expressed in gonads (testes and ovaries) of murine embryos from 12.5 to 18.5 dpc. In utero exposure to BPA decreased levels of RARalpha mRNA in testes of 14.5- and 18.5-dpc-embryos, levels of RXRalpha mRNA in testes of 14.5-dpc-embryos, and levels of RXRalpha mRNA in ovaries of 14.5-dpc-embryos. The present findings indicate that RARalpha and RXRalpha play crucial roles in organogenesis, and the growth and development of murine embryos, and will contribute to the assessment of the toxic effects of BPA on retinoid signals in embryogenesis.

Nishizawa, H., M. Morita, M. Sugimoto, S. Imanishi and N. Manabe (2005). Effects of in utero exposure to bisphenol a on mRNA expression of arylhydrocarbon and retinoid receptors in murine embryos. J Reprod Dev 51(3):315-324.

To evaluate the effects of bisphenol A (BPA), a candidate endocrine disruptor (ED), on embryonic development, we examined the mRNA expression levels of the arylhydrocarbon receptor (AhR), which binds with many EDs and plays crucial roles in xenobiotic metabolism, and of the retinoic acid receptor (RAR) alpha and retinoid X receptor (RXR) alpha, key factors in nuclear receptor-dependent retinoid signal transduction, in murine embryos exposed in utero to BPA (0.02, 2, 200, and 20,000 µg/kg/day) at 6.5-13.5 or 6.5-17.5 days post coitum (dpc), using the real-time reverse transcription-polymerase chain reaction (RT-PCR) method. Extremely low-dose BPA (0.02 µg/kg/day; 1/100 the dose of environmental exposure) remarkably increased AhR mRNA expression in the cerebra, cerebella, and gonads (testes and ovaries) of male and female 14.5- and 18.5-dpc-

embryos. In utero exposure to BPA at 2, 200, and 20,000 µg/kg/day also increased levels of AhR mRNA. In gonads of 14.5-dpc-embryos, AhR mRNA levels were elevated and showed diphasic (U) dose-response curves following exposure to BPA, but inverted U dose-response curves were obtained for 18.5-dpc-embryos. Exposure to BPA increased expression levels of RARalpha and RXRalpha mRNAs in the cerebra, cerebella, and gonads of male and female 14.5- and 18.5-dpc-embryos. Extremely low-dose BPA (0.02 µg/kg/day) increased RARalpha mRNA expression in the cerebella of male and female 14.5- and 18.5-dpc-embryos and in the gonads of female 14.5-dpc-embryos, and significantly increased RXRalpha mRNA expression in the cerebra and cerebella of male and female 14.5-dpc-embryos. The present findings confirm that in utero exposure to an extremely low dose of BPA up-regulates the mRNA expression of AhR, RARalpha, and RXRalpha in murine embryos and disrupts the receptor-dependent signal transducing systems, and will contribute to the assessment of the toxic effects of BPA on xenobiotic metabolism and retinoid signals in embryogenesis.

Nunez, A.A., Kannan, K. Giesy, J.P., Fang, J. and Clemens, L.G. (2001). Effects of bisphenol A on energy balance and accumulation in brown adipose tissue in rats. Chemosphere 42:917-922.

Treatment of adult female Sprague Dawley rats (~220 g) with 1, 4 or 5 mg/day by Alza minipumps for 15 days. Food intake was not affected, but body weight gain was reduced by bisphenol A at 4 or 5 mg/day. The 5 mg/day dose of bisphenol A resulted in blood levels of bisphenol A ranging from 18 – 160 ng/ml plasma (measured by HPLC, fluorescence detection, limit 1 ng). Bisphenol A accumulated preferentially in brown adipose tissue.

Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M. and Markert, B. (2000). Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology 9:383-397.

At doses down to the lowest tested (1 ppb) bisphenol A caused abnormalities in the reproductive organs and abnormal oocyte production in freshwater and marine snails. Exposure throughout life of the freshwater snail Marisa cornuarietis resulted in development of additional female reproductive organs, which showed malformations, Bisphenol A resulted in a massive stimulation of oocyte production and spawning mass, associated with increased mortality of the females at the lowest dose tested (1 ppb). The marine snail Nucella lapillus was exposed in adulthood only, and males showed a decrease in penis and prostate gland length, as well as stored sperm, again at the lowest dose tested (1 ppb). They also saw inverted-U dose-responses between 1 – 100 ppb octylphenol.

Oehlmann, J., Schulte-Oehlmann, U., Bachmann, J., Oetken, M., Lutz, I., Kloas, W. and Ternes, T.A. (2005). Bisphenol A induces superfeminization in the Ramshorn snail Marisa cornuarietis (Gastropoda: Prosobranchia) at environmentally-relevant concentrations. Environ. Health Perspect. Online July 6, 2005.

Previous investigations have shown that bisphenol A (BPA) induces a superfeminization syndrome in the freshwater snail Marisa cornuarietis at concentrations as low as 1 µg/L. Superfemales are characterized by the formation of additional female organs, enlarged accessory sex glands, gross malformations of the pallial oviduct and a stimulation of egg and clutch production resulting in increased female mortality. However, these studies were challenged due to experimental incompletion. It was therefore the objective of the current approach to bridge several gaps in knowledge by additional experiments. In a first series the dependence of study results from the reproductive phase of snails was evaluated in the sub-µg/L range. Before and after the spawning season superfemale responses were observed (NOEC 7.9 ng/L, EC10 13.9 ng/L), which were absent during the spawning season. A further experiment investigated the temperature-dependence of BPA

responses by exposing snails at two temperatures in parallel. The adverse effect of BPA was at least partially masked at 27°C (EC10 998 ng/L) when compared with 20°C (EC10 14.8 ng/L). In M. cornuarietis, BPA acts as an estrogen receptor (ER) agonist, because effects were completely antagonized by a co-exposure to tamoxifen and faslodex®. Anti-androgenic effects of BPA, such as a significant decrease in penis length at 20°C, were also observed. Competitive receptor displacement experiments indicate the presence of androgen- and estrogen-specific binding sites. The affinity for BPA of the estrogen binding sites in M. cornuarietis is higher than that of the ER in aquatic vertebrates. The results underline that prosobranchs are affected by BPA at lower concentrations than other wildlife groups and highlight the importance of exposure conditions.

Palanza, P., Howdeshell, K.L., Parmigiani, S. and vom Saal, F.S. Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ. Health Perspect. 110:415-422, 2002.

Treatment of pregnant females with 10 µg/kg/day decreased their nursing behavior after delivery. In addition, treatment of pregnant female CD-1 mice with 10 µg/kg/day bisphenol A resulted in a decrease in nursing behavior in their female offspring when the offspring reached adulthood and produced offspring.

Papaconstantinou, A.D., Umbreit, Thomas H., Fisher, B. R., Goering, P.L., Lappas, N.T., and Brown, Ken M. (2000). Bisphenol A-induced Increase in Uterine Weight and Alterations in Uterine Morphology in Ovariectomized B6C3F1 Mice: Role of the Estrogen Receptor. Toxicological Sciences 56: 332-339.

Bisphenol A was administered to adult ovariectomized mice at doses of 1, 10, 50, 100 and 400 mg/kg/day. Uterine weight increase was observed at 40 mg/kg/day and showed a dose-related increase in uterine weight; bisphenol A was only a partial agonist, with maximum response approximately one-third that of estradiol at 1 µg/kg/day. Bisphenol A at a dose of 100 mg/kg/day increased luminal epithelial height, and these effects were blocked by 10 mg/kg/day ICI 182,780. Bisphenol A increased the thickness of the stromal and myometrial layers of the uterus, The effects of ICI on these parameters was unclear as ICI stimulated these responses at the dose used in the bisphenol A study, revealing an agonistic effect of this dose of ICI.

Papaconstantinou, A. D., B. R. Fisher, T. H. Umbreit, P. L. Goering, N. T. Lappas and K. M. Brown (2001). Effectss of ß-estradiol and bisphenol A on heat shock protein levels and localization in the mouse uterus are antagonized by the antiestrogen ICI 182,780. Tox, Sci. **63**: 173-180.

B6C3F1 hybrid mice were ovariectomized. A few weeks later the females were injected with estradiol (0.02-20 µg/kg/day)) or bisphenol A (1, 10, 40, 100, 400 mg/kg/day) for 4 days. The ICI dose was 0.1 and 1 mg/kg/day co-administered with 1 µg/kg/day estradiol, 100 mg/kg/day bisphenol A or alone. At all doses examined, bisphenol A stimulated a significant increase in uterine glucose regulated protein (GRP) 94. Heat shock protein (HSP) 90α was stimulated at 10 mg/kg/day and above with an efficacy similar to estradiol at a dose of 0.2 µg/kg/day and above. In contrast, HSP72 was stimulated in a dose—depeendent manner by estradiol at 0.02 µg/kg/day and above to a maximum of 6-fold, while bisphenol A LOEL for HSP72 was 40 mg/kg/day and at 400 mg/kg/day only increased HSP72 by 3 fold (efficacy was about 50% that of estradiol). These findings show that this hybrid mouse, along with the Fischer 344 rat, are more sensitive than the CD-1 mouse (Markey et al. 2001) or Sprague-Dawley rat (Steinmetz et al. 1998) to the uterine stimulating effects of bisphenol A. Co-administration of ICI reduced these responses, suggesting a role for estrogen receptors in these responses, which have been shown in other studies to be mediated by estrogen receptors (ERα).

Porrini, S., V. Belloni, D. D. Seta, F. Farabollini, G. Giannelli and F. Dessi-Fulgheri (2005). Early exposure to a low dose of bisphenol A affects socio-sexual behavior of juvenile female rats. Brain Res. Bull. 65(3):261-266.

Play behavior is affected by alteration of the hormonal environment during development. In fact, congenital adrenal hyperplasia or early administration of diethylstilbestrol are able to modify female play behavior in mammals. In this research, play behavior of female rats was used to explore the effects of perinatal exposure to low, environmentally relevant dose of bisphenol A (BPA), a xenoestrogen widely diffused in the environment. We used 18 females born to mothers exposed to 40 µg/kg/day BPA during pregnancy and lactation, and 18 control females. The subjects were observed in a heterosexual social situation from 35 to 55 days of age. Six main behaviors were identified by principal component analysis (PCA): exploration, defensive behavior to males, play behavior with males, play behavior with females, low-intensity mating behavior, social grooming. Early administration of BPA was responsible for a significant increase of exploration (including social investigation) ($p<0.001$), as well as a decrease of play with males ($p<0.02$) and social grooming ($p<0.01$) at 45 days of age, indicating a general decrease of playful interactions. In general our results suggest that BPA does not induce a clear masculinization of female behavior, but is able however to defeminize some aspects of female behavior. This result is compatible with the estrogenic properties of BPA, and suggests caution in the use of a chemical that, in the range of human exposure, is able to influence the development of the brain during a critical period, resulting in long-term effects on behavior.

Ramos, J. G., Varayoud, J., Sonnenschein, C., Soto, A. M., Munoz De Toro, M. and Luque, E. H. (2001). Prenatal exposure to low doses of bisphenol A alters the periductal stroma and glandular cell function in the rat ventral prostate. Biol. Reprod. 65:1271-1277.

Pregnant Wistar rats were implanted with Alza osmotic pumps on gestation day 8 that released bisphenol A at doses of 25 and 250 µg/kg/day. Prenatal exposure to both doses of bisphenol A increased the size of area occupied by fibroblasts but decreased the size of the area occupied by smooth muscle in the periductal stroma of the ventral prostate of males at 30-days old. These changes in the cytoarchitecture of the ventral prostate are associated with a decrease in the proportion of periductal stroma cells that were positive for androgen receptors in males exposed to both doses of bisphenol A.

Ramos, J. G., J. Varayoud, L. Kass, H. Rodriguez, L. Costabel, M. Munoz-De-Toro and E. H. Luque (2003). Bisphenol A induces both transient and permanent histofunctional alterations of the hypothalamic-pituitary-gonadal axis in prenatally exposed male rats. Endocrinol. 144(7): 3206-15.

Pregnant Wistar rats were administered bisphenol A via minipumps at doses of 25 and 250 µg/kg/day from gestation day 8 through parturition, when presumably the pump was removed, although this was not specifically stated; treated mothers nursed their pups. Male offspring were examined on postnatal day 15, 30 and 120. The ventral prostate and hypothalamus were examined by immunochemistry for proliferation and ERα and androgen receptor (AR) protein. ERα and ERß mRNA expression in tissues and plasma LH and prolactin were also examined. In the ventral prostate both doses of bisphenol A produced a transient increase in the fibroblast sheath around the prostatic ducts and a decrease in stromal AR only on PND 30, and a transient increase in stromal proliferation on PND 15. Both doses of bisphenol A induced a 4-fold increase in ERß (but not ERα) gene expression (mRNA levels) in the preoptic area of the hypothalamus on PND 30 and 120, showing that this effect was permanent. Plasma prolacttin was elevated by bisphenol A only on PND 30, while testosterone was increased only on PND 15. These findings show that some effects of prenatal

bisphenol A exposure are only observed during specific times in development, suggesting that the course of development is altered, which can then lead to other types of long-latency outcomes.

Razzoli, M., P. Valsecchi and P. Palanza (2005). Chronic exposure to low doses bisphenol A interferes with pair-bonding and exploration in female Mongolian gerbils. Brain Res Bull 65(3):249-254.

Estrogenic endocrine disruptors, synthetic or naturally occurring substances found in the environment, can interfere with the vertebrate endocrine system and, mimicking estrogens, interact with the neuroendocrine substrates of behavior. Since species vary in their sensitivity to steroids, it is of great interest to widen the range of species included in the researches on neurobehavioral effects of estrogenic endocrine disruptors. We examined socio-sexual and exploratory behavior of Mongolian gerbil females (Meriones unguiculatus), a monogamous rodent, in response to chronic exposure to the estrogenic endocrine disruptor bisphenol A. Paired females were daily administered with one of the following treatments: bisphenol A (2 or 20 µg/kg body weight/day); 17 alpha-ethynil estradiol (0.04 µg/kg body weight/day 17alphaE); oil (vehicle). Females were treated for 3 weeks after pairing. Starting on day of pairing, social interactions within pairs were daily recorded. Three weeks after pairing, females were individually tested in a free exploratory paradigm. Bisphenol A and 17 alphaE affected male-female social interactions by increasing social investigation. Bisphenol A reduced several exploratory parameters, indicating a decreased exploratory propensity of females. These results highlight the sensitivity of adult female gerbils to bisphenol A during the hormonally sensitive period of pair formation, also considering that the bisphenol A doses tested are well below the suggested human tolerable daily intake.

Rubin, B.S., Murray, M.K., Damassa, D.A., King, J.C., and Soto, A.M. (2001). Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environ. Health Perspect. 109:675-680.

Bisphenol A was fed to pregnant and lactating Sprague-Dawley rats in drinking water at a low dose of approximately 100 µg/kg/day and a high dose of 1.2 mg/kg/day. The low dose of bisphenol A increased body weight throughout postnatal life in offspring. Females exposed during development to the high dose had disrupted estrous cycles and lower LH levels than controls. Adult treatment of ovariectomized females with bisphenol A at a doses up to 17 mg/kg/day did not stimulate uterine growth.

Sakaue, M., Ohsako, S., Ishimura, R., Kurosawa, S., Kurohmaru, M., Hayashi, Y., Aoki, Y., Yonemoto, J. and Tohyama, C. (2001). Bisphenol A affects spermatogenesis in the adult rat even at low doses. Journal of Occupational Health **43**:185-190.

Bisphenol A was administered orally for 6 days to adult Sprague-Dawley (CLEA Japan) rats over a very wide 8-order-of-magnitude dose range (2 ng/kg/day to 200 mg/kg/day). At a dose of 20 µg/kg/day and above, daily sperm production was significantly reduced by about 25%, and changes in the pattern of testicular proteins occurred. This finding shows that short-term adult treatment with a 20 µg/kg/day dose of bisphenol A reduces daily sperm production by a similar percent of negative control values as was reported in male mice exposed during fetal life to a 20 µg/kg/day dose fed to the pregnant dam. Whether the effect in adults is permanent, as with fetal exposure, or only observed when animals are being treated with bisphenol A remains to be examined.

Sawai, C., K. Anderson and D. Walser-Kuntz (2003). Effect of bisphenol A on murine immune function: Modificattion of interferon-gamma, IgG2a, and disease symptoms in NZB x NZW F1 mice. Environ. Health Perspect. **111**(16): 1883-1887.

In the first experiment, splenic mononuclear cells from 6-week old control C57BL/6J mice were incubated with ConA (4 µg/ml) and increasing doses of bisphenol A (0.0005, 0.05 and 5 µM). Interferon (IFN) γ secretion was significantly inhibited at 0.05 and 5 µM bisphenol A. In the second experiment, a 2.5 µg/kg/day dose of bisphenol A was fed (in a cereal treat) to CF57BL/6J male and female mice as well as NZB x NZW F1 female mice for 7 days beginning at 5 weeks of age. This same experiment was done with splenic monocytes collected 2-4 days after the last treatment in male and female C57BL/6J control mice and mice treated with bisphenol A. ConA was again administered (4 µg/ml) to stimulate the splenocytes, and IFN-γ was found to be significantly inhibited in monocytes from bisphenol A treated males and females relative to untreated controls. The same inhibition due to prior bisphenol A exposure was observed when the splenocytes were stimulated with heat-killed, gram positive Staphlococcus epidermidis. While bisphenol A did not alter the distribution of splenic B and T-lymphocytes, the total number of splenic mononuclear cells was increased by about 25% in C57BL/6J mice exposed to bisphenol A. Studies have shown that IFN-γ contributes to the progression of lupus, and splenic monocyte IFN-γ as well as IL-10 were downregulated in NZB x NZW F1 female mice (examined when 10 weeks old) mice exposed to bisphenol A for 7 days when 5 weeks old. Whereas IFN-γ decreases overall as lupus progresses, IL-10 increases. Bisphenol A thus caused a greater decrease in IFN-γ and attenuated the increase in IL-10. The decrease in IFN-γ was associated with a reduction in complement-fixing antibody class IgG2a, which is associated with glomerulonephritis as lupus progresses. Bisphenol A resulted in a marked attenuation of IgG2a production by splenic cells without altering the proportion of B cells, although as above, overall number of mononuclear cells in the bisphenol A treated spleens was increased. Bisphenol A treatment resulted in an average of 7 week delay in the onset of proteinuria (indicative of lupus) relative to controls. These results show a long-term effect of exposure for only one week to bisphenol A at postnatal week 5 at the very low dose of 2.5 µg/kg/day, which is 20-fold lower than the acceptable daily intake dose of 50 µg/kg/day calculated from studies that used only very high doses.

Schönfelder, G., Flick, B., Mayr, L., Talsness, C., Paul, M. and Chahoud, I. (2002). In utero exposure to low doses of bisphenol A lead to long-term deleterious effects in the vagina. Neoplasia 4:98-102.

They examined the influence of BPA at low doses to address the questions of (a) whether *in utero* exposure affects the vagina of the offspring and (b) which mechanisms cause the toxic effects. Gravid Sprague-Dawley dams were administered either 0.1 (low dose) or 50 mg/kg per day BPA, the no observed effect level, or 0.2 mg/kg per day 17-ethinyl estradiol by gavage. Striking morphological changes were observed in the vagina of postpubertal offspring leading us to examine vaginal estrogen receptor (ER) expression because BPA binds to the ER, which is important for growth of the vaginal epithelium. They show that the full-length ER is not expressed during estrus in the vagina of female offspring exposed to either dose of BPA when compared to the control group, whereas ER expression does not differ from the control group during the diestrus stage. Both doses of bisphenol A thus altered vaginal morphology and expression of ERα during the estrous phase of the cycle in postpubertal female offspring. ER downregulation seems to be responsible for the observed altered vaginal morphology.

Schonfelder, G., Friedrich, K., Paul, M. and Chahoud, I. (2004). Developmental effects of prenatal exposure to bisphenol A on the uterus of rat offspring. Neoplasia 6(5):584-594.

They examined BPA to address the question of whether *in utero* exposure affects the uterus of the offspring and studied the expression and distribution of the estrogen receptors alpha (ERa) and beta (ERß), because estrogens influence the development, growth, and function of the uterus through

both receptors. Gravid Sprague-Dawley dams were administered by gavage either 0.1 or 50 mg/kg per day BPA or 0.2 mg/kg per day 17-ethinyl estradiol (EE2) as reference dose on gestation days 6 through 21. Female offspring were killed in estrus. Uterine morphologic changes as well as ERα and ERß distribution and expression were measured by immunohistochemistry and Western blot analysis. Striking morphologic changes were observed in the uterine epithelium of postpubertal offspring during estrus of the *in utero* BPA-treated animals (the thickness of the total epithelium was significantly reduced). ERα expression was increased in the 50-mg BPA and EE2-treated group. In contrast, we observed significantly decreased ERß expression in all BPA- and EE2-treated animals when compared with the control. In summary, these results clearly indicate that *in utero* exposure of rats to BPA promotes uterine disruption in offspring. We hypothesize that the uterine disruption could possibly be provoked by a dysregulation of ERα and ERß.

Schulte-Oehlmann, U., M. Tillmann, D. Casey, M. Duft, B. Markert and J. Oehlmann (2001). Xeon-estrogenic eeffects of bisphenol A in prosobranchs (Mollusca: Gaastropoda: prosbranchia). Z. Umweltchem. Okotox. 13: 319-333.

Effects at very low doses of bisphenol A in the low part per trillion (ppt) range were reported in this study of freshwater (*Marisa cornuarietis*) and marine (*Nucella lapillus*) snails There was a significant increase in the number of embryos produced in freshwater snails at bisphenol A doses of 5 and 25 ppb, but not at 100 ppb, and the dose-response curve formed an inverted-U. This finding demonstrates that bisphenol A, similar to other hormonally active chemicals (vom Saal, Timms et al. 1997), can produce inverted-U dose-response curves. Bisphenol A induced malformations in the female reproductive organs and an increase in mortality. Examination of doses between 1 ppt and 1 ppb revealed that the no-effect concentration in freshwater snails for these adverse effects was 7.9 parts per trillion (ppt) and the lowest effect concentration (LOEC) was 48 ppt. The marine snail was exposed in adulthood only, and males showed a decrease in penis and prostate gland length as well as stored sperm at the lowest dose tested (1 ppb), revealing that the no effect levels for these effects is in the part per trillion range. The authors concluded tahat their studies should be considered in conducting an ecological risk assessment on bisphenol A, since clear adverse effects occurred in the range that bisphenol A is found in aquatic environments.

Seta, D. D., I. Minder, F. Dessi-Fulgheri and F. Farabollini (2005). Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain Res Bull 65(3):255-260.

In mammals, endogenous estrogens are crucial for sexual differentiation during the perinatal period, and the modulation in adulthood of many neuroendocrine and behavioral functions involved in reproduction. In rats, the estrogenic environment during pregnancy and lactation affects directly maternal behavior. This experiment was aimed to test whether the exposure to the estrogenic compound bisphenol-A (BPA; 40 µg/kg/day, orally) of adult female rats, from mating to weaning of the pups, could alter maternal behavior. An appropriate methodology was applied to reveal differences in the behavior of dams directed to male and female pups, testing the dams on postnatal days 3-4 and 8-9. Results show different maternal behavioral patterns towards male and female pups of control mothers, with more ano-genital licking to males than to females. Exposure of mothers to BPA modified their behavior, reducing specific components of maternal behavior, both active and passive, irrespective of the sex of pups and the period of observation. This experiment shows that maternal behavior is affected by a prolonged exposure to a low dose of BPA during pregnancy and lactation, thus suggesting an effect on neural circuits in adulthood.

Shibata, N., J. Matsumoto, K. Nakada, A. Yuasa and H. Yokota (2002). Male-specific suppression of hepatic microsomal UDP-glucuronosyl transferase activities toward sex hormones in the adult male rat administered bisphenol A. 368:783-788.

This study reported that UDP-glucuronosyltransferase (UGT) activities towards bisphenol A, testosterone and oestradiol were significantly decreased in liver microsomes prepared from adult male Wistar rats fed the endocrine disruptor bisphenol A or DES (1 mg/2 days, 0.5 mg/day/300 g animal, 1.5 mg/kg/day; duration of treatment was 2 or 4 weeks). However, suppression of the transferase activities was not observed in female rats, even after bisphenol A treatment for 4 weeks. DES had the same effects, but p-cumylphenol had no effect on UGT activities towards sex hormones. Co-administration of an anti-oestrogen, tamoxifen (1 mg), inhibited the suppression of the transferase activities by bisphenol A. Western blotting analysis showed that the amount of UGT2B1, an isoform of UGT which glucuronidates bisphenol A, was decreased in the rat liver microsomes by the treatment. Northern blotting analysis also indicated that UGT2B1 mRNA in the liver was decreased by bisphenol A treatment. The suppression of UGT activities, UGT2B1 protein and UGT2B1 mRNA expression did not occur in female rats. The results indicate that bisphenol A treatment reduces the mRNA expression of UGT2B1 and other UGT isoforms that mediate the glucuronidation of sex hormones in adult male rats, and this suggests that the endocrine balance may be disrupted by suppression of glucuronidation.

Sohoni, P., Tyler, C. R., Hurd, K., Caunter, J., Hetheridge, M., Williams, T., Woods, C., Evans, M., Toy, R., Gargas, M. and Sumpter, J. P. (2001). Reproductive effects of long-term exposure to Bisphenol A in the fathead minnow (Pimephales promelas). Environ Sci Technol **35**(14):2917-25.

Bisphenol A concentrations of 1 - 1,280 ppb in water were tested. A reduction in number of live offspring produced by females occurred at 640 ppb. In adult males, a decrease in length and weight occurred at 640 ppb, and a significant (29%) decrease in spermatazoa occurred at 16 ppb after 164 days of exposure; significant effects on spermatogenesis were seen at 1 ppb bisphenol A in male offspring. This finding showed that there was a greater sensitivity to low doses of bisphenol A during development than in adulthood in terms of adverse effects on the testis.

Steinmetz, R., Brown, N.G., Allen, D.L., Bigsby, R.M. And Ben-Jonathan, N. (1997). The environmental estrogen bisphenol A stimulates prolactin release *in vitro* and *in vivo*. Endocrinol. 138:1780-1786.

Bisphenol A at a dose of about 250 µg/kg/day and estradiol at a dose 40-times lower (about 6 µg/kg/day) stimulated a 7-fold increase in pituitary prolactin secretion in Fischer 344 rats but not Sprague-Dawley rats.

Steinmetz, R., Mitchner, N. A., Grant, A., Allen, D. L., Bigsby, R. M. and Ben-Jonathan, N. (1998). The xenoestrogen bisphenol A induces growth, differentiation, and c-fos gene expression in the female reproductive tract. Endocrinol. 139:2741-2747.

A 37.5 mg/kg/day dose of bisphenol A injected ip increased uterine weight and vaginal keratinization in female Fischer 344 rats, while a 19 mg/kg/day dose did not lead to significant effects. c-fos gene was induced with a similar efficacy by 50 mg/kg/day bisphenol A and 10 µg/kg/day estradiol in the F344 uterus and vagina (no other doses were examined). Estradiol had similar effects in Sprague-Dawley rats, but at the 50 mg/kg/day dose, bisphenol A had no effect in Sprague-Dawley rats. A 300 µg/kg/day dose of bisphenol A (administered by a Silastic capsule) increased uterine luminal epithelial cell height in female Fischer 344 rats, similar to effects of a 6-µg/kg/day dose of estradiol. Estradiol also stimulated uterine growth but BPA did not in the F344. In F344 rats, bisphenol A was thus approximately 50-fold less potent than estradiol in this assays, and in terms of uterine cell height, showed an efficacy about two-thirds that of estradiol.

*Stoker, T. E., C. L. Robinette, B. H. Britt, S. C. Laws and R. L. Cooper (1999). Prepubertal exposure to compounds that increase prolactin secretion in the male rat: effects on the adult prostate. Biol. Reprod. 61:1636-1643.

To test the hypothesis that a transient increase in prolactin (PRL) secretion prior to puberty can result in an alteration of the adult prostate, male rats were exposed from postnatal Days (PND) 22 to 32 to compounds that increase PRL secretion. These compounds included pimozide at 20 mg/kg/day (a dopamine antagonist), estradiol-17beta, and bisphenol A, at 50 mg/kg/day. During dosing, pimozide (PIM), bisphenol A (BPA), and estradiol-17beta (E(2)) stimulated an increased secretion of PRL. At 120 days of age, the lateral prostate weight was increased in the PIM and BPA groups as compared to the vehicle-injected controls. Examination of the prostates revealed inflammation in the lateral lobes of all treated groups. Results of a myeloperoxidase assay, a quantitative assay to assess acute inflammation, indicated an increase in the percentage of males with neutrophil infiltrate in the lateral prostates of the PIM and E(2) treatment groups compared to their respective controls. The histological evaluations of these tissues confirmed an increase in luminal polymorphonuclear cells and interstitial mononuclear cells of the lateral prostates in all treatment groups. Administration of the dopamine agonist, bromocriptine, to the estradiol-implanted males from PND 22 to 32 reversed the induction of lateral prostate inflammation by estradiol, suggesting that PRL was necessary for the inflammatory effect. This study demonstrates that prepubertal exposures to compounds that increase PRL secretion, albeit through different mechanisms, can increase the incidence of lateral prostate inflammation in the adult. The 50 mg/kg/day dose of bisphenol A is the current LOAEL used by the EPA to calculate the reference dose of 50 µg/kg/day, which would not be affected by the results of this study.

Stoker, C., Rey, F., Rodriguez, H., Ramos, J.G., Sirosky, P., Larriera, A., Luque, E.H. and Munoz-de-Toro, M. (2003). Sex reversal effects on Caiman latirostris exposed to environmentally relevant doses of the xenoestrogen bisphenol A. Gen Comp Endocrinol 133:287-296.

Exposure to environmental contaminants known as endocrine disruptors (EDs) alters the development and function of reproductive organs in several species. Bisphenol A (BPA) is an estrogenic chemical that leaches from dental materials and plastic food and beverage containers. BPA has been found in sewage, surface and drinking water, and therefore poses a potentially significant risk for human and wildlife. Prenatal exposure of rodents to environmentally relevant doses of BPA alters the development of the reproductive organs of male and female offspring. Species with temperature dependent sex determination (TSD) could act as sentinels of ecosystem health by providing sensitive biomarkers of endocrine disruptor's effects. We selected Caiman latirostris as an animal model to study endocrine disruption caused by BPA. The aim of this study was to determine whether exposure in ovum to BPA could cause estrogen-like effects on the reproductive system of C. latirostris. Sex determination and gonadal histoarchitecture were the endpoints evaluated after in ovum exposure to different doses of BPA and 17beta-estradiol (E(2)). We confirmed that C. latirostris is a species with TSD and additionally demonstrated that BPA causes estrogen-like developmental effects by reversing gonadal sex and altering gonadal histoarchitecture. Differences in responses to BPA and E(2) in our in vivo system were on the order of 100-fold. In contrast published in vitro studies have reported differences on the order of 10,000x or more. These results support the utility of C. latirostris, a species in which sex determination is temperature dependent, as a tool in assessing estrogenic activity in vivo and as a sentinel to monitor EDs in aquatic environment.

Sugita-Konishi, Y., S. Shimura, T. Nishikawa, F. Sunaga, H. Naito and Y. Suzuki (2003). Effect of Bisphenol A on non-specific immunodefenses against non-pathogenic Escherichia coli. Toxicol. Lett. 136:217-27.

Bisphenol A was examined for effects on non-specific defense (neutrophils, natural killer cells tissue macophages involved in the early elimination of bacteria from infectious foci) in experiments with a non-pathogenic bacterium, Escherichia coli K-12. BALB/c mice were injected sc with BPA (5 mg/kg body weight) for 5 consecutive days, and 3 days after the last treatment, injected ip with E. coli K-12. BPA pretreatment caused a decrease of T and B cell populations in the spleen of treated mice. After the challenge with E. coli, the activity to eliminate bacteria from the peritoneal cavity in the early stage of infection (within 24 h) was diminished compared with non-treated mice; this one experiment involved multiple doses (0.005, 0.05, 0.5 and 5 mg/kg, and only the 5 mg/kg dose significantly delayed bacterial clearance). BPA induced the migration of excess neutrophils into the peritoneal cavity, but reduced their phagocytic activity against E. coli K-12. For macrophages and lymphocytes, BPA reduced the population in the spleen and the accumulation at infection foci. The production of MCP-1 was enhanced by BPA treatment, but that of IL-6 was suppressed after infection. These results suggest that adult exposure to BPA suppressed the non-specific host immune defense system.

Suzuki, A., A. Sugihara, K. Uchida, T. Sato, Y. Ohta, Y. Katsu, H. Watanabe and T. Iguchi (2002). Developmental effects of perinatal exposure to bisphenol A and diethylstilbestrol on reproductive organs in female mice. Reprod. Toxicol. **16**: 107-116.

ICR mice (CLEA, Japan) were injected sc with bisphenol A (10 and 100 mg/kg/day) or DES (0.0067 – 67 µg/kg/day) from gestation day 10-18, and fetuses were delivered by cesarean section and reared postnatally by foster mothers. At 30 days of age, the 10 mg/kg dose of bisphenol A reduced the number of females with corpora lutea relative to negative controls, similar to the effect of a 6.7 µg/kg/day dose of DES. No effect on fertility was noted, however.

Suzuki, T., K. Mizuo, H. Nakazawa, Y. Funae, S. Fushiki, S. Fukushima, T. Shirai and M. Narita (2003). Prenatal and neonatal exposure to bisphenol-A enhances the central dopamine D1 receptor-mediated action in mice: enhancement of the methamphetamine-induced abuse state. Neurosci. **117**(3): 639-44.

Prenatal and lactational exposure to BPA in ddy mice was via the maternal feed at 0.002, 0.5 and 2 mg/g food (the lowest dose migh be in the range of 300 µg/kg/day if ~5 g of food were eaten by a 35 g pregnant or lactating mouse). At all doses, bisphenol A resulted in the enhancement of the dopamine D1 receptor-dependent rewarding effect induced by methamphetamine, and also enhanced hyper-locomotion and its sensitization induced by methamphetamine (sc, 0.5 mg/kg). Bisphenol A produced an up-regulation of dopamine D1 receptor function to activate G-protein in the mouse limbic forebrain, which is thought to be a critical site for the expression of rewarding effects by amphetamine. Additionally, chronic BPA exposure produced a significant increase in levels of the dopamine D1 receptor mRNA in the whole brain. The present data provide the evidence that prenatal and neonatal exposure to BPA can potentiate brain dopamine D1 receptor-dependent neurotransmission, resulting in supersensitivity of methamphetamine-induced pharmacological actions. The authors conclude that bisphenol A can potentiate drug-seeking (dependency and addiction) behavior associated with the use of psychostimulants such as amphetamine. The authors report, but did not show data that these effects occurred at bisphenol A blood levels in the mice of 10 ng/ml, which is within the range of human blood levels of bisphenol A.

Tabata, A., Kashiwada, S., Ohnishi, Y., Ishikawa, H., Miyamoto, N., Itoh, M. and Magara, Y. (2001). Estrogenic influences of estradiol-17ß, o-nonylphenol, and bisphenol A on Japanese medaka (Oryzias latipes) at detected environmental concentrations. Water Science and Technology 43:109-116.

The effects of bisphenol A on Japanese medaka were studied. The LC50 for bisphenol A in embryos was 5.1 ppm and in adults was 7.5 ppm. Female specific proteins were induced in adults at 10 ppb, and gonadal abnormalities were seen at 100 ppb.

Tabata, A., Watanabe, N., Yamamoto, I., Ohnishi, Y., Itoh, M., Kamei, T., Magara, Y., Terao, Y. (2004). The effect of bisphenol A and chlorinated derivatives of bishenol A on the level of serum vitellogenin in Japanese medaka (Oryzias latipes). Water Science and Technology 50: 125-132.

Although BPA is easily chlorinated, very little is reported on the effect of chlorinated BPA to the aquatic organisms. In this study, the estrogenic activities of BPA (100, 200, 500 100 ppb) and its chlorinated derivatives were evaluated by the induction of vitellogenin (VTG) in the serum of mature male Japanese medaka. In addition, the effect of sodium hypochlorite on the decomposition of BPA was tested. The relative potencies of estrogenic activities of chlorinated BPA descended in the order 3,3'-diCIBPA>BPA> or =3-CIBPA>3,3',5-triCIBPA, and no estrogenic activity was observed in 3,3',5,5'-tetraCIBPA. Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) for both 3-CIBPA and 3,3'-diCIBPA were 500 microg/L and 200 microg/L, respectively. LOEC for 3,3',5-triCIBPA was >500 microg/L. When BPA was reacted with sodium hypochlorite (24 hours; residual chlorine at 1 ppm), however, complete decomposition of BPA and its chlorinated derivatives was observed. The decrease in BPA and its chlorinated derivatives paralleled the decrease in estrogenic potency evaluated by the induction of vitellogenin (VTG) in the serum of mature male Japanese medaka. Induction of VTG by BPA occurred at 200 ppb.

Takahashi, O. and S. Oishi (2003). Testicular toxicity of dietarily or parenterally administered bisphenol A in rats and mice. Food Chem Toxicol 41:1035-44.

Male Crj:Wistar rats, HsdHot:Holtzman SD rats, Crj:CD-1(ICR) mice and C57BL/6CrSlc mice were administered bisphenol A (BPA) in the diet at a level of 0 (control) and 0.25% for 8 weeks. Daily BPA intake was about 200 and 400 mg/kg for rats and mice, respectively. No conspicuous signs of general or reproductive toxicity were observed after administration in any strain of these animals. Serum testosterone concentrations were not decreased in BPA-fed rats and mice. Successive subcutaneous administration of BPA at a dose of 200 mg/kg/day for 4 weeks significantly decreased the testis, epididymis, prostate and seminal vesicle weights, and the testicular daily sperm production in Jcl:Wistar rats. Successive intraperitoneal administration of BPA at a dose of 20 mg/kg/day for 4 weeks decreased the prostate and seminal vesicle weights (but not the testis or epididymis weights) and also decreased serum testosterone and both liver and kidney weight. An intraperitoneal dose of 2 mg BPA/kg/day to Wistar rats did not cause any toxicity. These results indicate that dietarily administered BPA is less toxic to most strains of rats and mice, and the maximum non-toxic dose and/or minimum toxic dose may be about 200 mg/kg/day. Subcutaneous or intraperitoneal BPA is much more toxic on male reproductive and sex accessory organs than dietary.

Takai, Y., Tsutsumi, O., Ikezuki, Y., Hiroi, H., Osuga, Y., Momoeda, M., Yano, T. and Taketani, Y. (2000). Estrogen receptor-mediated effects of a xenoestrogen, bisphenol A, on preimplantation mouse embryos. Biochem Biophys Res Commun 270:918-921.

Bisphenol A at doses of 228 ppt and 684 ppt accelerated development of 2-cell mouse embryos to the 8-cell stage, while a 2.3 ppm dose of bisphenol A inhibited development. The stimulatory effects of low doses of bisphenol A were inhibited by the antiestrogen tamoxifen. These findings provide more evidence for inverted-U dose-response relationships for bisphenol A.

Takai, Y., Tsutsumi, O., Ikezuki, Y., Kamei, Y., Osuga, Y., Yano, T. and Taketan, Y. (2000). Preimplantation exposure to bisphenol A advances postnatal development. Reproductive Toxicology **15**:71-74.

At the 2 cell stage mouse embryos were cultured with a 230 ppt dose of bisphenol A, and rate of development to the blastocyst stage was accelerated. The control and bisphenol A-treated embryos were implanted into and then nursed by control females. At weaning, the animals treated with bisphenol A as embryos were 37% heavier than controls. Embryonic exposure to BPA thus increases postnatal body weight.

Takao, T., W. Nanamiya, I. Nagano, K. Asaba, K. Kawabata and K. Hashimoto (1999). Exposure with the environmental estrogen bisphenol A disrupts the male reproductive tract in young mice. Life Sci 65:2351-7.

Here we examine plasma hormone levels and histology in the testis of mice following either 4- or 8-week oral administration of bisphenol A. Bisphenol A was administered via drinking water at 0.5 and 50 µg//ml. Average water intake was about 6 ml per day and body weights were about 25 g. The daily doses were thus about 120 µg/kg/day and 12 mg/kg/day. Plasma free testosterone levels were dramatically decreased following 8 weeks of 12 mg/kg/day bisphenol A treatment compared with control group, and morphologically multinucleated giant cells having greater than three nuclei were found in seminiferous tubules in the testis following the 8-week bisphenol A treatment at both doses while no control showed this. No differences in plasma corticosterone and luteinizing hormone levels were seen between bisphenol A and control groups. Thus, exposure with bisphenol A around pubertal period may directly disrupt the male reproductive tract. These facts suggest that more detailed studies will warrant the assessment of the risk to the developing human testis from exposure to bisphenol A and other environmental estrogens in prepubertal and pubertal period.

Takao, T., W. Nanamiya, H. P. Nazarloo, R. Matsumoto, K. Asaba and K. Hashimoto (2003). Exposure to the environmental estrogen bisphenol A differentially modulated estrogen receptor-alpha and -beta immunoreactivity and mRNA in male mouse testis. Life Sci. **72**(10): 1159-69.

Bisphenol A at concentrations of 0.5 or 50 µg/ml in the drinking water was fed to young male mice (doses are likely in the range of 0.2 and 20 mg/kg/day). Effects on estrogen receptor (ER) alpha and beta proteins and mRNA in the testis following 8-weeks of oral administration of bisphenol A. ERß was localized in the nuclei of spermatogonia and/or spermatocytes, and the number of ERß containing cells (and mRNA) per testis were significantly decreased in the 50 microg/ml bisphenol A-treated group compared with controls. In contrast, ERα immunopositive cells (and mRNA) per testis were markedly increased in the 50 microg/ml bisphenol A-treated group compared with the controls. The existence of ER alpha and beta in the testis suggests that estrogens directly affect germ cells during testicular development and spermatogenesis, and differential modulation of ER alpha and beta in the testis could be involved in the effects of bisphenol A.

Talsness, C., O. Fialkowski, C. Gericke, H.-J. Merker and I. Chahoud (2000). The effects of low and high doses of bisphenol A on the reproductive system of female and male rat offspring. Congenital Anomalies **40**:S94-S107.

Bisphenol A was fed by gavage to Spragd-Dawley rats at doses of 0.1 or 50 mg/kg/day, and females were fed 0.2 mg/kg/day ethinylestradiol (a positive control) from gestation day 6-21. A wide range of outcomes on the reproductive organs and reproductive function in male and female offspring were examined. An interesting feature of the extensive findings reported is the occurrence of non-monotonic, inverted-U dose-response curves. For example, body weight of pups at weaning was depressed at the low, but not high dose of bisphenol A, and vaginal opening was delayed by the low

dose and accelerated by the high dose of bisphenol A. Disruption of the estrous cycle in female offspring occurred at the low but not high dose of bisphenol A. Anogenital distance at birth, a marker of prenatal masculinization, was significantly reduced in males at the low but not high dose of bisphenol A. On postnatal day 70, prostate weight was significantly increased and daily sperm production was decreased at the low dose but not by the high dose of bisphenol A. This latter finding replicates the findings in mice reported by Gupta (2000) and vom Saal et al. 1998).

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.

We examined the effects of maternal exposure to estrogens on platelet-derived growth factor (PDGF) receptor (PDGFR) expression in newborn rat testis. Pregnant rats were treated from gestation Day 14 to birth with corn oil containing diethylstilbestrol (0.01 – 2 µg/kg/day), bisphenol A (0.1 – 200 mg/kg/day), genistein, or coumestrol by gavage or subcutaneous injection. These treatments induced a dose-dependent increase in the expression of PDGFR alpha and beta mRNAs, determined by semiquantitative reverse transcription polymerase chain reaction, though diethylstilbestrol had a biphasic effect on both mRNAs. A significant effect of BPA occurred at doses of 1 – 200 mg/kg/day and for DES at doses of 0.01, 0.1 and 1 µg/kg/day, but ata DES dose 2 µg/kg/day, the response decreased back to control levels, forming an inverted-U dose-response curve. In situ hybridization analysis showed that PDGFRalpha mRNA increased mostly in the interstitium, while PDGFRbeta mRNA increased both in the interstitium and seminiferous cords. Immunohistochemical studies of PDGFRalpha and beta proteins revealed that both receptors were present in testis before and after birth and that they were upregulated upon treatment with estrogens in 3-day-old rats, with PDGFRbeta increasing dramatically in gonocytes. PDGFRalpha and beta mRNAs and proteins were also found in purified gonocytes. Our previous finding that PDGF and 17beta-estradiol induce gonocyte proliferation in vitro, together with the present finding that in vivo exposure to estrogens upregulates PDGF receptors in testis, suggest that PDGF pathway is a target of estrogens in testis. In addition, these data identify PDGFRbeta in gonocytes as a major target of gestational estrogen exposure, suggesting that estrogen may have a physiological interaction with PDGF during gonocyte development. These results, however, do not exclude the possibility that the effects of the compounds examined in this study might be due to estrogen receptor-independent action(s).

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.

Exposure of human fetuses to manmade estrogenic chemicals can occur through several sources. For example, fetal exposure to ethinylestradiol occurs because each year approximately 3% of women taking oral contraceptives become pregnant. Exposure to the estrogenic chemical bisphenol A occurs through food and beverages because of significant leaching from polycarbonate plastic products and the lining of cans. We fed pregnant CD-1 mice ethinylestradiol (0.1 µg/kg/day) and bisphenol A (10 µg/kg/day), which are doses below the range of exposure by pregnant women. In male mouse fetuses both ethinylestradiol and bisphenol A produced an increase in the number and size of dorsolateral prostate ducts and an overall increase in prostate duct volume. Histochemical staining of sections with proliferating cell nuclear antigen and mouse keratin 5 antibodies indicated that this was due to a marked increase in proliferation of basal epithelial cells located in the primary ducts. The urethra was malformed in the colliculus region and significantly constricted where it enters the bladder, which could contribute to urine flow disorders. These effects were identical to those caused by a similar 0.1 µg/kg/day dose of the estrogenic drug, diethylstilbestrol (DES), a

known human developmental teratogen and carcinogen. In contrast, a 2000-fold higher DES dose completely inhibited dorsolateral prostate duct formation, revealing opposite effects of high and low doses of estrogen. Acceleration in the rate of proliferation of prostate epithelium during fetal life by small amounts of estrogenic chemicals could permanently disrupt cellular control systems and predispose the prostate to disease in adulthood.

Tohei, A., S. Suda, K. Taya, T. Hashimoto and H. Kogo (2001). Bisphenol A inhibits testicular functions and increases luteinizing hormone secretion in adult male rats. Exp. Biol. Med. **226**:216-221.

Adult male Wistar rats were given subcutaneous injections of bisphenol A for 2 weeks at a dose of about 3 mg/kg/day. Bisphenol A treatment resulted in significant decrease in plasma testosterone, which was associated with an increase in plasma LH. Testicular content, but not plasma levels, of inhibin were also decreased. When challenged with an iv injection of 10 IU human chorionic gonadotropin (hCG), bisphenol A exposed males showed significantly depressed levels of plasma progesterone and testosterone, demonstrating a direct inhibitory effect of bisphenol A on the testicular response to gonadotropin stimulation.

Toyama, Y. and S. Yuasa (2004). Effects of neonatal administration of 17beta-estradiol, beta-estradiol 3-benzoate, or bisphenol A on mouse and rat spermatogenesis. Reprod Toxicol 19:181-8.

Bisphenol A (BPA) is a global environmental contaminant that has been implicated as a potential endocrine disruptor. In the present study, newborn rats and mice were injected subcutaneously with BPA (doses approximately 30, 300, 1500 and 3000 µg/kg/day) to determine the potential developmental effects on the testis. Testes were examined by light and electron microscopy at 15 weeks of age. Other groups of newborn mice and rats were injected with 17beta-estradiol (E(2)) or beta-estradiol 3-benzoate (E(2)B) in a similar manner. BPA, E(2), and E(2)B had similar effects on testes. When treated animals reached puberty and spermiogenesis began, the first sign of the effects was detected in the steps 2-3 spermatids: the acrosomal granule and nucleus were deformed. Henceforth, abnormalities in the acrosome and nucleus were observed in older spermatids and spermatozoa. Ectoplasmic specialization between the Sertoli cell and spermatids was also affected: some specializations were partially or totally deleted. These abnormalities occurred beginning at 300 µg/kg/day bisphenol A and 1 µg/kg/day E2 and E2B. When animals fully matured, the effects of the agents were not found in the testes, and the animals were found to be fertile. The results of the present study show that BPA acts as an estrogen with approximately 300-fold lower potency that estradiol.

Trudeau, V.L., Turque, N., Le Mevel, S., Alliot, C., Gallant, N., Coen, L., Pakdel, F. and Demeneix, B. (2005). Assessment of estrogenic endocrine-disrupting chemical actions in the brain using in vivo somatic gene transfer. Environ Health Perspect 113:329-334.

Estrogenic endocrine-disrupting chemicals abnormally stimulate vitellogenin gene expression and production in the liver of many male aquatic vertebrates. However, very few studies demonstrate the effects of estrogenic pollutants on brain function. We have used polyethylenimine-mediated in vivo somatic gene transfer to introduce an estrogen response element-thymidine kinase-luciferase (ERE-TK-LUC) construct into the brain. To determine if waterborne estrogenic chemicals modulate gene transcription in the brain, we injected the estrogen-sensitive construct into the brains of Nieuwkoop-Faber stage 54 Xenopus laevis tadpoles. Both 0.5 nM ethinylestradiol (EE2; $p < 0.002$) and 50 nM (11.4 ppb) bisphenol A (BPA; $p < 0.03$) increased luciferase activity by 1.9- and 1.5-fold, respectively. In contrast, low physiologic levels of 17ss-estradiol had no effect ($p > 0.05$). The mixed antagonist/agonist tamoxifen was estrogenic in vivo and increased ($p < 0.003$) luciferase activity in

the tadpole brain by 2.3-fold. There have been no previous reports of somatic gene transfer to the fish brain; therefore, it was necessary to optimize injection and transfection conditions for the adult goldfish (Carassius auratus). Following third brain ventricle injection of cytomegalovirus (CMV)-green fluorescent protein or CMV-LUC gene constructs, we established that cells in the telencephalon and optic tectum are transfected. Optimal transfections were achieved with 1 microg DNA complexed with 18 nmol 22 kDa polyethylenimine 4 days after brain injections. Exposure to EE2 increased brain luciferase activity by 2-fold in males ($p < 0.05$) but not in females. Activation of an ERE-dependent luciferase reporter gene in both tadpole and fish indicates that waterborne estrogens can directly modulate transcription of estrogen-responsive genes in the brain. We provide a method adaptable to aquatic organisms to study the direct regulation of estrogen-responsive genes in vivo.

vom Saal, F. S., Cooke, P. S., Buchanan, D. L., Palanza, P., Thayer, K. A., Nagel, S. C., Parmigiani, S. and Welshons, W. V. (1998). A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Ind Health **14**(1-2):239-60.

Bisphenol A was administered orally to pregnant CF-1 mice. A 2 µg/kg/day dose resulted in enlarged prostate and preputial glands, but smaller seminal vesicles and epididymides, while a 20 µg/kg/day dose resulted in a decrease in daily sperm production per g testis in male offspring. The increase in prostate weight and decrease in epididymis weight was replicated by Gupta (2000) using a 50 µg/kg/day dose of bisphenol A. The effect of bisphenol A on the prostate in these male mice was reported in Nagel et al. 1997 (see above.

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.

We previously reported that gonocytes from 3-day-old rat testes proliferate in response to estradiol. In the present study, we found that purified gonocytes contained the mRNAs of estrogen receptor beta (ERbeta) and the chaperones Hsp90, p23, and Cyp40, but no inducible Hsp70. Immunoblot analysis showed high levels of ERbeta, Hsp90, p23, Cyp40, and the constitutive Hsc70 in gonocytes. Prenatal (maternal) exposure of Sprague-Dawley rats from GD 14-parturition to the estrogenic compounds diethylstilbestrol (DES) (0.01-1 µg/kg/day by injection), and by gavage bisphenol A (0.1-200 mg/kg/day), genistein (0.1-10 mg/kg/day), and coumestro (1-100 mg/kg/day), led to significantly increased Hsp90 mRNA levels in testis (BPA significant at 10 mg/kda/day), but not p23 and Cyp40 (significant inhibition by BPA at 1 mg/kg/day). Effects were seen in response to BPA at doses of 1 –200 mg/kg/day and to DES at doses of 0.01 –1 µg/kg/day. In situ hybridization analysis indicated that Hsp90 mRNA was prominent in gonocytes, where it was increased following phytoestrogen exposure, whereas bisphenol A induced a more generalized increase throughout the testis. Immunoblot analysis of testicular extracts demonstrated that Hsp90 protein levels were significantly increased following estrogen exposure, and immunohistochemical analysis indicated that this increase occurred predominantly in gonocytes. By contrast, no change was observed in the expression of Cyp40, p23, and ERbeta, whereas Hsc70 was increased by bisphenol A only. Using an antibody and reverse transcriptase-polymerase chain reaction probes specific for Hsp90alpha, we subsequently confirmed that Hsp90alpha was primarily expressed in gonocytes, and that it was increased following estrogen exposure. Hsp90 immunolocalization in fetal and prepubertal testes showed an increased expression in fetal gonocytes upon estrogen exposure, but no difference in the subsets of Hsp90-positive germ cells in prepubertal testes. These results demonstrate that prenatal estrogen exposure specifically affects Hsp90 expression in gonocytes. Considering the interaction of

Hsp90 with several signaling molecules, changes in its expression levels may lead to subsequent changes in gonocyte development.

Watanabe, M., Mitani, N., Ishii, N. and Miki, K. (2005). A mutation in a cuticle collagen causes hypersensitivity to the endocrine disrupting chemical, bisphenol A, in Caenorhabditis elegans. Mutat Res 570:71-80.

A novel mutant gene, bis-1 (bisphenol A sensitive) has been isolated in the nematode, Caenorhabditis elegans, that affects the response to endocrine disrupting chemicals (EDC). The bis-1(nx3) allele is hypersensitive to bisphenol A (BPA), is allelic to a collagen gene (col-121), and is expressed in hypodermal cells. Among the collagen mutants so far studied, bis-1(nx3), dpy-2(e8), dpy-7(e88) and dpy-10(e128) showed BPA sensitivity. The isolated mutant may work as a useful tool for the assay of EDC toxicity since the physiological effect of the collagen mutation (glycine substitution) indicates an increased sensitivity to BPA.

Watts, M. M., Pascoe, D. and Carroll, K. (2001). Chronic exposure to 17alpha-ethinylestradiol and bisphenol A - Effects on development and reproduction in the freshwater invertebrate Chironomus riparius (Diptera: Chironomidae). Aquat. Toxicol. 55:113-124.

The freshwater invertebrate (the nonbiting midge) Chironomus riparius was examined. At doses ranging from 78 ng/L to 750 µg/L (78 ppt to 750 ppb) the emergence of male and female adults in the second generation of exposed animals was significantly delayed.

Watts, M. M., D. Pascoe and K. Carroll (2003). Exposure to 17 alpha-ethinylestradiol and bisphenol A--effects on larval moulting and mouthpart structure of Chironomus riparius. **54**(2): 207-15.

The effects of the endocrine-disrupting chemicals 17alpha-ethinylestradiol and bisphenol A at doses ranging from 10 ng/L-1mg/L (10 ppt – 1 ppm) on the development of the aquatic life-cycle stages (eggs to pupa) of Chironomus riparius were investigated. In addition, three mouthpart structures (mentum, mandibles, and pecten epipharyngis) present on the head capsules of fourth-instar larvae were examined for structural deformities, which were observed at very lowest exposure concentration of 10 ng/L (10 ppt); the incidence of deformities was greater in the chironomids exposed to EE than BPA. Effects were mainly associated with the mentum, with statistically significant differences in median deformity score (Kruskal-Wallis $P<0.001$) recorded for both chemicals. At similar effect concentrations, an increased percentage of exposed animals had deformities of the pecten epipharyngis; however, little evidence of deformity was noted for the mandibles. At high concentrations, where moulting and wet weight were affected, no incidence of mouthpart deformity was noted (see Fig 8). Thus, as with other findings, effects at low doses were not predicted by effects at much higher doses due to inverted-U dose-response functions.

Williams, K., C. McKinnell, P. T. Saunders, M. Walker, J. S. Fisher, K. Turner, N. Atanassova and R. M. Sharpe (2001). Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man. Human Reproduction Update 7:236-247.

The effects on reproductive tract development in male rats, of neonatal exposure to potent (reference) oestrogens, diethylstilboestrol (DES) and ethinyl oestradiol (EE), with those of two environmental oestrogens, octylphenol and bisphenol A (at a dose of 37 µg/kg/day) were systematically compared when injected in rats on postnatal days 2-12. Other treatments, such as administration of a gonadotrophin-releasing hormone antagonist (GnRHa) or the anti-oestrogen tamoxifen or the anti-androgen flutamide, were used to aid interpretation of the pathways involved. All treatments were administered in the neonatal period before onset of puberty. The cellular sites of

expression of androgen receptors (AR) and of oestrogen receptor-alpha (ERalpha) and ERbeta were also established throughout development of the reproductive system. The main findings were as follows: (i) all cell types that express AR also express one or both ERs at all stages of development; (ii) Sertoli cell expression of ERbeta occurs considerably earlier in development than does expression of AR; (iii) most germ cells, including fetal gonocytes, express ERbeta but not AR; (iv) treatment with high, but not low, doses of potent oestrogens such as DES and EE, induces widespread structural and cellular abnormalities of the testis and reproductive tract before puberty; (v) the latter changes are associated with loss of immunoexpression of AR in all affected tissues and a reduction in Leydig cell volume per testis; (vi) none of the effects in (iv) and (v) can be duplicated by treating with high-dose octylphenol or bisphenol A; (vi) none of the reproductive tract changes in (iv) and (v) can be induced by simply suppressing androgen production (GnRHa treatment) or action (flutamide treatment); and (vii) the adverse changes induced by high-dose DES (iv and v) can be largely prevented by co-administration of testosterone. Thus, it is suggested that many of the adverse changes to the testis and reproductive tract induced by exposure to oestrogens result from a combination of high oestrogen and low androgen action. High oestrogen action or low androgen action on their own are unable to induce the same changes.

Williams, K., Fisher, J.S., Turner, K.J., McKinnell, C., Saunders, P.T. and Sharpe, R.M. (2001). Relationship between expression of sex steroid receptors and structure of the seminal vesicles after neonatal treatment of rats with potent or weak estrogens. Environ Health Perspect 109:1227-1235.

In this study we evaluated the effect of manipulating the estrogen and androgen environment of the neonatal male rat on subsequent immunoexpression of sex steroid receptors in the seminal vesicles (SVs) at age 18 days. The aim was to establish to what extent such changes were associated with and predictive of changes in SV structure/composition. Treatments were either diethylstilbestrol (DES; 10, 1, or 0.1 microg/injection), ethinyl estradiol (EE; 10 microg/injection), tamoxifen (2 mg/kg/day), flutamide (50 mg/kg), a gonadotropin-releasing hormone antagonist (GnRHa; 10 mg/kg), genistein (4 mg/kg/day), octylphenol (2 mg/injection), or bisphenol A (0.5 mg/injection). Compared with controls, treatment with DES (10 microg) induced loss of epithelial and stromal androgen receptor (AR) immunoexpression coincident with induction of stromal progesterone receptor (PR) immunoexpression and upregulation of stromal immunoexpression of estrogen receptor-alpha (ERalpha). These changes were associated with gross distortion (increase) of the normal stromal:epithelial tissue proportions in the SVs. DES (1 microg) and EE induced similar but less pronounced changes, and DES (0.1 microg) had no noticeable effect. Tamoxifen and flutamide induced PR and slightly upregulated ERalpha immunoexpression but had only a minor or no effect on AR expression and the stromal:epithelial ratio, though flutamide retarded normal development of the SVs. The latter was also evident in GnRHa-treated males, but otherwise this treatment had no effect on AR and PR immunoexpression. None of the foregoing treatments had any detectable effect on the immunoexpression of ERss in stromal or epithelial cells. The major treatment-induced changes in immunoexpression of AR, PR, and ERalpha and lack of change in ERss were confirmed by Western blots of SV protein extracts. None of the three weak (environmental) estrogens tested caused any detectable change in sex steroid receptor immunoexpression or SV tissue composition. We conclude that treatment-induced loss of AR is a prerequisite for altered stromal:epithelial proportions in the SVs and that such loss is always associated with induction of PR and upregulation of ERalpha; the latter two changes are insufficient on their own to bring about such a change. Nevertheless, induction of PR expression was always associated with altered SV development and is a potentially useful marker because it is not normally expressed in male reproductive tissues.

Wistuba, J., M. H. Brinkworth, S. Schlatt, I. Chahoud and E. Nieschlag (2003). Intrauterine bisphenol A exposure leads to stimulatory effects on Sertoli cell number in rats. Environ. Res. **91**(2): 95-103.

Bisphenol A was fed by gavage to Spragd-Dawley rats at doses of 0.1 or 50 mg/kg/day, and females were fed 0.02 mg/kg/day ethinylestradiol (a positive control) from gestation day 6-21. Testicular histology was examined in male offspring at 9-12 month of age. Spermatogenesis was qualitatively normal in all groups. Both doses of bisphenol A increased Sertoli cell number per organ, but not when expressed as per gram testis, since there was also a significant increase in testicular weight in males exposed to bisphenol A at both doses. Ethinylestradiol did not affect cell number per organ, but did affect numbers on a per gram testis, due to a lowered testis weight. Intrauterine exposure to a low dose of bisphenol A thus resulted in an increase by about 10-15% in Sertoli cell number per testis.

Yamaguchi, A., H. Ishibashi, S. Kohra, K. Arizono and N. Tominaga (2005). Short-term effects of endocrine-disrupting chemicals on the expression of estrogen-responsive genes in male medaka (Oryzias latipes). Aquat Toxicol 72(3):239-49.

To evaluate the estrogenic activities of selected estrogenic compounds such as estradiol-17beta (E2), nonylphenol (NP), 4-(1-adamantyl)phenol (AdP), bisphenol A (BPA), BPA metabolite 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP) and 4,4'-dihydroxy-alpha-methylstilbene (DHMS) in the shortest possible time, we investigated the expression of estrogen-responsive genes such as vitellogenin I, vitellogenin II and alpha-type estrogen receptor genes in the liver of male medaka (Oryzias latipes) using reverse transcription-polymerase chain reaction (RT-PCR) techniques. These estrogen-responsive genes responded rapidly to selected estrogenic compounds after 8 h exposure, and the expression of hepatic vitellogenin II and estrogen receptor alpha mRNA was found to be more responsive than that of vitellogenin I mRNA. As a result, the relative estrogenic potencies of tested chemicals descended in the order of E2 (100)>MBP (0.38)>AdP (0.25)>DHMS (0.05)>NP (0.02)>BPA (0.001). Moreover, this preliminary study indicates that AdP and DHMS should be considered as candidate estrogenic compounds with the potential to induce hepatic estrogen-responsive genes in male medaka. In previous studies, the estrogenic activity of either bisphenol A or bisphenol B was increased by several folds after incubation with the S9 fraction from rat liver. This active metabolite was confirmed to be MBP by various instrumental analyses, and they suggested that the estrogenic activity of MBP was much more potent than that of the parent BPA in several in vitro bioassays. DHMS is one of the major metabolic intermediates of the gram-negative aerobic bacterium strain MV1, which is capable of growth on BPA as the sole source of carbon. The results of the present study suggest that vitellogenin I, vitellogenin II and estrogen receptor alpha gene expression patterns alter in male medaka treated with selected estrogenic compounds, and that these genes may be useful molecular biomarkers for screening estrogenic endocrine-disrupting chemicals in the shortest possible time.

Yoshino, S., K. Yamaki, R. Yanagisawa, H. Takano, H. Hayashi and Y. Mori (2003). Effects of bisphenol A on antigen-specific antibody production, proliferative responses of lymphoid cells, and TH1 and TH2 immune responses in mice. Br J Pharmacol 138:1271-6.

This study investigated the effect of bisphenol A (BPA), which binds estrogen receptors, on immune responses including production of antigen-specific antibodies, proliferative responses of lymphoid cells, and Th1 and Th2 responses. For this investigation, mice were p.o. given varying doses including 3, 30, 300, and 3000 µg/kg of BPA immediately after immunization with hen egg lysozyme (HEL) (day 0) and then daily by day 20. On day 21, anti-HEL IgG antibodies in sera and proliferative responses of spleen cells to the antigen were measured. Anti-HEL IgG2a antibodies and IFN-gamma secreted from splenic lymphocytes were also measured as indicators of Th1 immune

responses, while anti-HEL IgG1 antibodies and IL-4, as those of Th2 responses. The results showed that treatment with 3000 µg/kg of BPA was followed by a significant increase in anti-HEL IgG as well as the antigen-specific cell proliferation. Anti-HEL IgG2a production and IFN-gamma secretion were significantly enhanced in mice treated with 300 and 30 µg/kg of BPA, respectively, while anti-HEL IgG1 production and IL-4 secretion were augmented in animals given 3000 and 300 µg/kg of the chemical, respectively. Augmentation of these immune responses was also observed in mice exposed to 0.3-30 µg/kg of estradiol, although Th1 responses appeared to be more sensitive to the sex hormone than Th2 responses. These results suggest that BPA may play a role in augmenting immune responses, especially Th1 responses.

Yoshino, S., K. Yamaki, X. Li, T. Sai, R. Yanagisawa, H. Takano, S. Taneda, H. Hayashi and Y. Mori (2004). Prenatal exposure to bisphenol A up-regulates immune responses, including T helper 1 and T helper 2 responses, in mice. Immunology 112:489-95.

The effect of prenatal exposure to bisphenol A (BPA) via feeding pregnant females 3, 30, 300 or 3000 µg/kg/day doses from gestation day 0 – 17 on the immune system in mice was investigated. On postnatal day 77, their male offspring were immunized with hen egg lysozyme (HEL). Three weeks later, anti-HEL immunoglobulin G (IgG) in sera, and proliferative responses of spleen cells to the antigen, were measured. Anti-HEL IgG2a and interferon-gamma (IFN-gamma), secreted from splenic lymphocytes, were measured as indicators of T helper 1 (Th1) immune responses, while anti-HEL IgG1 and interleukin-4 (IL-4) were measured as indicators of Th2 responses. The results showed that fetal exposure to BPA was followed by significant dose-related increases in anti-HEL IgG (significant for 300 and 3000 µg/kg/day) as well as antigen-specific cell proliferation (significant at 3000 µg/kg/day). Both Th1 responses (including anti-HEL IgG2a, significant at 30 µg/kg/day and above) and IFN-gamma production, significant at 300 µg/kg/day and above) and Th2 responses (including anti-HEL IgG1, significant at 3000 µg/kg/day, and IL-4, significant at 300 µg/kg/day, production) were augmented by prenatal exposure to BPA, although the augmentation of Th1 responses appeared to be greater than that of Th2 responses. Two-colour flow cytometric analysis showed that mice exposed prenatally to BPA had 29% and 100% more splenic CD3(+) CD4(+) and CD3(+) CD8(+) cells, respectively, than control animals. Similar results were obtained from females whose mothers had consumed BPA during pregnancy. These results suggest that prenatal exposure to BPA may result in the up-regulation of immune responses, especially Th1 responses, in adulthood.

Yurino, H., Ishikawa, S., Sato, T., Akadegawa, K., Ito, T., Ueha, S., Inadera, H. and Matsushima, K. (2004). Endocrine disruptors (environmental estrogens) enhance autoantibody production by B1 cells. Toxicol Sci 81:139-147.

Accumulating data suggest that endocrine disruptors affect not only the reproductive system, but also the immune system. We demonstrate here that endocrine disruptors including diethylstilbestrol (DES) and bisphenol-A (BPA) enhance autoantibody production by B1 cells both in vitro and in vivo. BWF1 mice, a murine model for systemic lupus erythematosus (SLE), implanted with Silastic tubes containing DES after orchidectomy developed murine lupus characterized by immunoglobulin G (IgG) anti-DNA antibody production and IgG deposition in the glomeruli in the kidney as well as those implanted with 17beta-estradiol (E2). Plaque-forming cells (PFC) producing autoantibodies specific for bromelain-treated red blood cells were significantly increased in mice implanted with DES and BPA. Blood levels of BPA were measured and at 2 - 3 months after implanting the Sialstic capsules were 25-30 ng/ml. IgM antibody production by B1 cells in vitro was also enhanced in the presence of endocrine disruptors including DES and BPA. Estrogen receptor (ER) expression was upregulated in B1 cells in aged BWF1 mice that developed lupus nephritis.

These results suggest that endocrine disruptors are involved in autoantibody production by B1 cells and may be an etiologic factor in the development of autoimmune diseases.

Zoeller, R. T., R. Bansal and C. Parris (2005). Bisphenol-A, an Environmental Contaminant that Acts as a Thyroid Hormone Receptor Antagonist In Vitro, Increases Serum Thyroxine and Alters RC3/Neurogranin Expression in the Developing Rat Brain. Endocrinol. 146:607-612.

Considering the importance of thyroid hormone (TH) in brain development, it is of potential concern that a wide variety of environmental chemicals can interfere with thyroid function or, perhaps of greater concern, with TH action at its receptor (TR). Recently, bisphenol-A (BPA, 4,4' isopropylidenediphenol) was reported to bind to the rat TR and act as an antagonist in vitro. BPA is a high production volume chemical, with over 800 million kg of BPA produced annually in the United States alone. It is detectable in serum of pregnant women and in cord serum taken at birth, is 5-fold higher in amniotic fluid at 15-18 weeks gestation compared with maternal serum, and was found in concentrations of up to 100 ng/g in placenta. Thus, the human population is widely exposed to BPA and it appears to accumulate in the fetus. Dietary exposure to each dose (1, 10 and 50 mg/kg/day) of BPA of Sprague Dawley rats from gestation day 6 of pregnancy throughout lactation caused a significant increase in serum total T4 in pups on postnatal day 15, but serum TSH was not different from controls. The expression of the TH-responsive gene RC3/Neurogranin, measured by in situ hybridization, was significantly up-regulated by all BPA doses in the dentate gyrus. These findings suggest that BPA acts as a TH antagonist on the beta TR, which mediates the negative feedback effect of TH on the pituitary gland, but that BPA is less effective at antagonizing TH on the alpha TR, leaving TRalpha-mediated events to respond to elevated T4.

Zsarnovsky, A., Lee, H.H., Wang, H-S. and Belcher, S.M. (2005). Ontogeny of rapid estrogen-mediated ERK1/2 signaling in the rat cerebellar cortex in vivo: potent non-genomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. Endocrinol. Online August 25, 2005; doi:10.1210/en.2005-0565.

In addition to regulating estrogen receptor-dependent gene expression, 17β-estradiol (E2) can directly influence intracellular signaling. In primary cultured cerebellar neurons, E2 was previously shown to regulate growth and oncotic cell death via rapid stimulation of extracellular regulated kinase 1 and 2 (ERK1/2) signaling. Here we show that ERK1/2- signaling in the cerebellum of neonatal and mature rats was rapidly responsive to E2 and during development to the environmental estrogen bisphenol A (BPA). In vivo dose response analysis for each estrogenic compound was performed by brief (6 min) intracerebellar injection, followed by rapid fixation and phosphorylation-state specific immunohistochemistry to quantitatively characterize changes in activated-ERK1/2 (pERK) immunopositive cell numbers. Beginning on postnatal day 8 (P8), E2 significantly influenced the number of pERK-positive cells in a cell-specific manner that was dependent on concentration and age, but not sex. In cerebellar granule cells on P10, E2 or BPA increased pERK-positive cell numbers at low-doses (10^{-12}-10^{-10}M) and at higher (10^{-7}-10^{-6}M) concentrations. Intermediate concentrations of either estrogenic compound did not modify basal ERK-signaling. Rapid E2-induced increases in pERKimmunoreactivity were specific to the ERK1/2 pathway as demonstrated by co-injection of the MEK1/2 inhibitor U0126. Co-administration of BPA (10^{-12} to 10^{-10}M) with 10^{-10}M E2 dose-dependently inhibited rapid E2-induced ERK1/2 activation in developing cerebellar neurons. The ability of BPA to act as a highly potent E2-mimetic and to also disrupt the rapid actions of E2 at very low concentrations during cerebellar development highlights the potential low-dose impact of xenoestrogens on the developing brain.

VIII. 41 FINDINGS OF SIGNIFICANT EFFECTS FROM THE ABOVE LIST USING DOSES AT AND BELOW THE REFERENCE DOSE OF 50 µg/kg/day

Adriani, W., Della Seta, D., Dessi-Fulgheri, D., Farabollini, F., Laviola, G. (2003). Altered profiles of spontaneous novelty seeking, impulsive behavior, and response to D-amphetamine in rats perinatally exposed to bisphenol A. Environ. Health Perspect. 111:395-401.
40 µg/kg/day

Akingbemi, B.T., Soitas, C.M., Koulova, A.I., Kleinfelter, G.R. nd Hardy, M.P. (2004). Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig Cells. Eodocrinol. 145: 592-603.
2.4 µg/kg/day

Al-Hiyasat, A. S., H. Darmani and A. M. Elbetieha (2002). Effects of bisphenol A on adult male mouse fertility. Eur. J. Oral Sci. **110**:163-167.
5, 25 and 100 µg/kg/day

Al-Hiyasat AS, Darmani H, Elbetieha AM. 2004. Leached components from dental composites and their effects on fertility of female mice. Eur J Oral Sci 112:267-272.
5, 25 and 100 µg/kg/day

Aloisi AM, Della Seta D, Rendo C, Ceccarelli I, Scaramuzzino A, Farabollini F. (2002). Exposure to the estrogenic pollutant bisphenol A affects pain behavior induced by subcutaneous formalin injection in male and female rats. Brain Res 937:1-7.
40 µg/kg/day

Alonso-Magdalena, P., Morimoto1, S., Ripoll, C., Fuentes,E. and Nadal, A. (2005) The Estrogenic Effect of Bisphenol-A Disrupts the Pancreatic β- Cell Function *in vivo* and Induces Insulin Resistance. Environ Health Perspect. doi:10.1289/ehp.8451 (available at http://dx.doi.org/),. Online 20 September 2005.
10 µg/kg

Bindhumol, V., Chitra, K.C. and Mathur, P.P. (2003). Bisphenol A induces reactive oxygen species generation in the liver of male rats. Toxicol. 188:117-124.
0.2, 2.0 and 20 µg/kg/day

Chitra KC, Latchoumycandane C, Mathur PP. (2003). Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology. 185(1-2):119-127.
0.2, 2 and 20 µg/kg/day

Dessi-Fulgheri, F., Porrini, S. and farabollini, F. (2002). Effects of perinatal exposure to bisphenol A on play behavior of female and male juvenile rats. Environ. Health Perspect. 110: (Suppl 3):403-407.
40 and 400 µg/kg/day

Farabollini, F., Porrini, S., and Dessi-Fulgheri, F. (1999). Perinatal exposure to the estrogenic pollutant bisphenol A affects behavior in male and female rats. Pharmacol. Biochem. Behav. 64:687-694.

40 and 400 µg/kg/day

Farabollini, F., Porrini, S., Setz, D.D., Bianchi, F. and Dessi-Fulgheri, F. (2002). Effects of perinatal exposure to bisphenol A on sociosexual behavior of female and male rats. Environ. Health Perspect. 110 (Suppl 3): 409-414.
40 µg/kg/day

Fujimoto,T., Kubob, K. and Aou, S. (2006). Prenatal exposure to bisphenol A impairs sexual differentiation of exploratory behavior and increases depression-like behavior in rats. Brain Res. Online: November, 2005.
Bisphenol A = 15 µg/kg/day

Gupta, C. (2000). Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. Proc. Soc. Exp. Biol. Med. 224:61-68.
bisphenol A = 50 µg/kg/day
DES = 0.1 µg/kg/day and 200 µg/kg/day

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.
Bisphenol A = 2 and 20 µg/kg/day
DES = 0.02, 0.2 and 2 µg/kg/day

Howdeshell, K.L., Hotchkiss, A.K., Thayer, K.A., Vandenbergh, J.G. and vom Saal, F.S. (1999). Exposure to bisphenol A advances puberty. Nature 401:763-764.
2.4 µg/kg/day

Hunt, P. A., K. E. Koehler, M. Susiarjo, C. A. Hodges, A. Hagan, R. C. Voigt, S. Thomas, B. F. Thomas and T. J. Hassold (2003). Bisphenol A causes meiotic aneuploidy in the female mouse. Current Biol. **13**:546-553.
Leaching from polycarbonate bottles = 15-72 µg/kg/day.
Oral dose = 20, 40 and 100 µg/kg/day

Imanishi S, Manabe N, Nishizawa H, Morita M, Sugimoto M, Iwahori M, Miyamoto H. 2003. Effects of oral exposure of bisphenol A on mRNA expression of nuclear receptors in murine placentae assessed by DNA microarray. Journal of Reproduction & Development 49:329-336.
2 µg/kg/day

Ishido, M., Y. Masuo, M. Kunimoto, S. Oka and M. Morita (2004). Bisphenol A causes hyperactivity in the rat concomitantly with impairment of tyrosine hydroxylase immunoreactivity. J. Neurosci. Res. 76:423-33.
about 3, 30, 300 and 3000 µg/kg

Kabuto, H., M. Amakawa and T. Shishibori (2004). Exposure to bisphenol A during embryonic/fetal life and infancy increases oxidative injury and causes underdevelopment of the brain and testis in mice. Life Sci. 74:2931-40.
5 or 10 µg per milliliter of drinking water (approximately 2.5-5 µg/kg/day).

Kawai, K., N. Takehiro, H. Nishikata, S. Aou, M. Takii and C. Kubo (2003). Aggressive behavior and serum testosterone concentration during the maturation process of male mice: The effects of fetal exposure to bisphenol A. Environ. Health Perspect. **111**:175-178.
2 and 20 µg/kg/day

Kubo, K., O. Arai, M. Omura, R. Watanabe, R. Ogata and S. Aou (2003). Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci. Res. **45**:345-356.
Bisphenol A = 30 and 300 µg/kg/day
DES = 6.5 µg/kg/day

Laviola, G., Gioiosa, L., Adriania, W. and Palanza, P. (2005.) d-Amphetamine-related reinforcing effects are reduced in mice exposed prenatally to estrogenic endocrine disruptors. Brain Research Bulletin 65:235–240.
10 µg/kg/day

MacLusky, N.J., Hajszan, T. and Leranth, C. (2005). The environmental estrogen bisphenol A inhibits estrogen-induced hippocampal synaptogenesis. Environ. Health Perspect. 113:675-679.
Bisphenol A = 40 µg/kg/day.

Markey, C. M., Luque, E. H., Munoz De Toro, M., Sonnenschein, C. and Soto, A. M. (2001). In utero exposure to bisphenol a alters the development and tissue organization of the mouse mammary gland. Biol Reprod **65**(4):1215-23.
25 and 250 ng/kg/day administered by Alzet pump
(initially incorrectly reported as µg/kg/day and subsequently corrected to ng/kg/day)

Markey, C.M., Coombs M.A., Sonnenschein C., Soto, A.M. (2003). Mammalian development in a changing environment: exposure to endocrine disruptors reveals the developmental plasticity of steroid-hormone target organs. Evolut & Develop. 5 (1):67-75.
25 and 250 ng/kg/day by Alzet pump
(initially incorrectly reported as µg/kg/day and subsequently corrected to ng/kg/day)

Markey, C. M., P. R. Wadia, B. S. Rubin, C. Sonnenschein and A. M. Soto (2005). Long-Term Effects of Fetal Exposure to Low Doses of the Xenoestrogen Bisphenol-A in the Female Mouse Genital Tract. Biol Reprod. Online Feb 2.
25 and 250 ng BPA/kg body weight/day via osmotic pumps implanted into pregnant dams

Masuo, Y., M. Ishido, M. Morita and S. Oka (2004). Effects of neonatal treatment with 6-hydroxydopamine and endocrine disruptors on motor activity and gene expression in rats. Neural Plast 11:59-76.
0.87 to 87 nmol per PND 5 male pup that weighed ~ 10 g). 8.7 mM, 8.7 micro mol / ml; 87 n mol / 10 microliter = 198 ng injected ~200ng / 10g; animal 20 ppb inj or 20 µM/kg (4.5 µg/kg).

Monica Munoz-de-Toro, M., Markey, C., Wadia, P.R., Luque, E.H., Rubin, B.S., Sonnenschein C. and Soto, A.M. (2005). Perinatal exposure to bisphenol A alters peripubertal mammary gland development in mice. Endocrinol. Online: May 31.
25 and 250 ng BPA/kg body weight/day via osmotic pumps implanted into pregnant dams

Nagel, S. C., vom Saal, F. S., Thayer, K. A., Dhar, M. G., Boechler, M. and Welshons, W. V. (1997). Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. Environmental Health Perspectives 105:70-6.
2 and 20 µg/kg/day

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25 and 800 µg/kg

Nishizawa H, Manabe N, Morita M, Sugimoto M, Imanishi S, Miyamoto H. 2003. Effects of in utero exposure to bisphenol A on expression of RARalpha and RXRalpha mRNAs in murine embryos. Journal of Reproduction & Development 49:539-545.
2 µg/kg/day

Nishizawa, H., M. Morita, M. Sugimoto, S. Imanishi and N. Manabe (2005). Effects of In Utero Exposure to Bisphenol A on mRNA Expression of Arylhydrocarbon and Retinoid Receptors in Murine Embryos. J Reprod Dev. 51:315-324.
0.02, 2, 200, 20,000 µg/kg/day

Palanza, P., Howdeshell, K.L., Parmigiani, S. and vom Saal, F.S. (2002). Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ. Health Perspect. 110:415-422.
10 µg/kg/day

Porrini, S., V. Belloni, D. D. Seta, F. Farabollini, G. Giannelli and F. Dessi-Fulgheri (2005). Early exposure to a low dose of bisphenol A affects socio-sexual behavior of juvenile female rats. Brain Res. Bull. 65(3):261-266.
40 µg/kg/day

Ramos, J. G., Varayoud, J., Sonnenschein, C., Soto, A. M., Munoz De Toro, M. and Luque, E. H. (2001). Prenatal exposure to low doses of bisphenol A alters the periductal stroma and glandular cell function in the rat ventral prostate. Biol. Reprod. 65:1271-1277.
25 and 250 µg/kg/day

Ramos, J. G., J. Varayoud, L. Kass, H. Rodriguez, L. Costabel, M. Munoz-De-Toro and E. H. Luque (2003). Bisphenol A induces both transient and permanent histofunctional alterations of the hypothalamic-pituitary-gonadal axis in prenatally exposed male rats. Endocrinol. **144**(7): 3206-15.
25 and 250 µg/kg/day

Razzoli, M., P. Valsecchi and P. Palanza (2005). Chronic exposure to low doses bisphenol A interferes with pair-bonding and exploration in female Mongolian gerbils. Brain Res Bull 65(3):249-254.
2 and 20 µg/kg/day

Sakaue, M., Ohsako, S., Ishimura, R., Kurosawa, S., Kurohmaru, M., Hayashi, Y., Aoki, Y., Yonemoto, J. and Tohyama, C. (2001). Bisphenol A affects spermatogenesis in the adult rat even at low doses. Journal of Occupational Health **43**:185-190.
dose range (2 ng/kg/day to 200 mg/kg/day, significant effect at 20 µg/kg/day and above

Sawai, C., K. Anderson and D. Walser-Kuntz (2003). Effect of bisphenol A on murine immune function: Modificattion of interferon-gamma, IgG2a, and disease symptoms in NZB x NZW F1 mice. Environ. Health Perspect. **111**(16): 1883-1887.
2.5 µg/kg/day

Seta, D. D., I. Minder, F. Dessi-Fulgheri and F. Farabollini (2005). Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain Res Bull 65(3):255-260.
40 µg/kg/day, orally to adult female rats

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.
10 µg/kg/day orally to pregnant CD-1 mice

Yoshino, S., K. Yamaki, R. Yanagisawa, H. Takano, H. Hayashi and Y. Mori (2003). Effects of bisphenol A on antigen-specific antibody production, proliferative responses of lymphoid cells, and TH1 and TH2 immune responses in mice. Br J Pharmacol 138:1271-6.
3, 30, 300, and 3000 µg/kg; effect at 30 µg/kg/day

Yoshino, S., K. Yamaki, X. Li, T. Sai, R. Yanagisawa, H. Takano, S. Taneda, H. Hayashi and Y. Mori (2004). Prenatal exposure to bisphenol A up-regulates immune responses, including T helper 1 and T helper 2 responses, in mice. Immunology 112:489-95.
3, 30, 300, and 3000 µg/kg; effect at 30 µg/kg/day

Yurino, H., Ishikawa, S., Sato, T., Akadegawa, K., Ito, T., Ueha, S., Inadera, H. and Matsushima, K. (2004). Endocrine disruptors (environmental estrogens) enhance autoantibody production by B1 cells. Toxicol Sci 81:139-147.
Blood levels of BPA were measured and at 2 - 3 months after implanting the Sialstic capsules were 25-30 ng/ml.

Zsarnovsky, A., Lee, H.H., Wang, H-S. and Belcher, S.M. (2005). Ontogeny of rapid estrogen-mediated ERK1/2 signaling in the rat cerebellar cortex in vivo: potent non-genomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. Endocrinol. Online August 25, 2005; doi:10.1210/en.2005-0565.
0.23 ppt intracerebellar injection

IX. INVERTED-U DOSE-RESPONSE CURVES REPORTED FOR BISPHENOL A

(Different effects found at low doses and at higher doses)

Akingbemi, B.T., Soitas, C.M., Koulova, A.I., Kleinfelter, G.R. nd Hardy, M.P. (2004). Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig Cells. Eodocrinol. 145: 592-603.

Carr RL, Bertasi FR, Betancourt AM, Bowers SD, Gandy BS, Ryan PL, Willard ST. (2003). Effect of neonatal rat bisphenol A exposure on performance in the Morris water maze. Journal of Toxicology & Environmental Health A 66:2077-2088.

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

Hahn, T., Schenk, K. and Schultz, R. (2002) Environmental chemicals with known endocrine potential affect yolk protein content in the aquatic insect (Chironomus riparius). Environ. Pollut. 120: 525-528.

Jobling, S., D. Casey, T. Rodgers-Gray, J. Oehlmann, U. Schulte-Oehlmann, S. Pawlowski, T. Baunbeck, A. P. Turner and C. R. Tyler (2003). Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. **65(**2): 205-20.

Levy, G., I. :Lutz, L. A. and W. Kloas (2003). Bisphenol A inducees feminization in Xenopus laevis tadpoles. Environ. Res.: online, in press.

Markey, C. M., Michaelson, C. L., Veson, E. C., Sonnenschein, C. and Soto, A. M. (2001). The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A. Environ Health Perspect 109:55-60.

Nishizawa, H., M. Morita, M. Sugimoto, S. Imanishi and N. Manabe (2005). Effects of In Utero Exposure to Bisphenol A on mRNA Expression of Arylhydrocarbon and Retinoid Receptors in Murine Embryos. J Reprod Dev. 51:315-324.

Oehlmann, J., Schulte-Oehlmann, U., Bachmann, J., Oetken, M., Lutz, I., Kloas, W. and Ternes, T.A. (2005). Bisphenol A induces superfeminization in the Ramshorn snail Marisa cornuarietis (Gastropoda: Prosobranchia) at environmentally-relevant concentrations. Environ. Health Perspect. Online July 6, 2005.

Schulte-Oehlmann, U., M. Tillmann, D. Casey, M. Duft, B. Markert and J. Oehlmann (2001). Xeon-estrogenic eeffects of bisphenol A in prosobranchs (Mollusca: Gaastropoda: prosbranchia). Z. Umweltchem. Okotox. **13**: 319-333.

Song, K.-H., K. Lee and H.-S. Choi (2002). Endocrine disrupter bisphenol A induces orphan nuclear receptor Nur77 gene expression and steroidogenesis in mouse testicular Leydig cells. Endocrinol. 143:2208-2215.

Takai, Y., Tsutsumi, O., Ikezuki, Y., Hiroi, H., Osuga, Y., Momoeda, M., Yano, T. and Taketani, Y. (2000). Estrogen receptor-mediated effects of a xenoestrogen, bisphenol A, on preimplantation mouse embryos. Biochem Biophys Res Commun 270:918-921.

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Watts, M. M., D. Pascoe and K. Carroll (2003). Exposure to 17 alpha-ethinylestradiol and bisphenol A--effects on larval moulting and mouthpart structure of Chironomus riparius. **54**(2): 207-15.

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Zsarnovsky, A., Lee, H.H., Wang, H-S. and Belcher, S.M. (2005). Ontogeny of rapid estrogen-mediated ERK1/2 signaling in the rat cerebellar cortex in vivo: potent non-genomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. Endocrinol. Online August 25, 2005; doi:10.1210/en.2005-0565.

Related Inverted-U and Mechanisms Findings Not Directly Concerning Bisphenol A

Bulayeva, N.N., Gametchu, B. and Watson, C.S. (2004). Quantitative measurement of estrogen-induced ERK 1 and 2 activation via multiple membrane-initiated signaling pathways. Steroids 69:181-192.

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

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Oehlmann, J., Schulte-Oehlmann, U., Tillmann, M. and Markert, B. (2000). Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. Ecotoxicology **9**(6):383-397.

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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.

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.

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X. DES SHOWS SAME *IN VIVO* EFFECTS AS BISPHENOL A
(BPA typically within 100-1000-fold of the potency of DES based on *in vivo* studies)
(NOTE: There is a very large literature based on in vitro studies showing that BPA and DES have the same effects, and for some responses, BPA and DES have the same potency)

Evans, N. P., T. North, S. Dye and T. Sweeney (2004). Differential effects of the endocrine-disrupting compounds bisphenol-A and octylphenol on gonadotropin secretion, in prepubertal ewe lambs. Domest. Anim. Endocrinol. 26:61-73.

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.

Kubo, K., O. Arai, M. Omura, R. Watanabe, R. Ogata and S. Aou (2003). Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci. Res. **45**:345-356.

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.

Suzuki, A., A. Sugihara, K. Uchida, T. Sato, Y. Ohta, Y. Katsu, H. Watanabe and T. Iguchi (2002). "Developmental effects of perinatal exposure to bisphenol A and diethylstilbestrol on reproductive organs in female mice." Reprod. Toxicol. **16**: 107-116.

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.

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.

XI. INSENSITIVITY OF THE UTEROTROPHIC ASSAY TO LOW DOSES OF BISPHENOL A WHEN USING SOME STRAINS OF RATS AND MICE

Ashby, J. and Odum, J. Gene Expression Changes in the Immature Rat Uterus: Effects of Uterotrophic and Sub-Uterotrophic Doses of Bisphenol A. Toxicol Sci. 2004 Oct 29. Advance Access, 9-29-04.

Gould et al. (1998) have reported that administration of 5-150mg/kg/day BPA to immature rats leads to increases in uterine peroxidase activity and progesterone receptor (PR) protein levels in the absence of an uterotrophic response. These observations are of interest given current concerns regarding the adequacy of the uterotrophic assay to act as a sentinel for the estrogenic activity of chemicals in vivo. Therefore, the uterotrophic activity of BPA to the immature rat has been re-evaluated over the dose range 2 micro g/kg-800mg/kg/day. Expression levels of three estrogen responsive uterine genes were determined using real-time RT-PCR - namely, complement component 3, lipocalin 2 and PR. 18S rRNA and RNA polymerase II large subunit acted as control genes. Observations of gene expression were made 4h and 72h after the first of three daily oral administrations of BPA. Increases in gene expression were observed over the uterotrophic dose range (approximately 200-800mg/kg BPA). Over the dose range 2 micro g/kg-20mg/kg BPA there was no uterotrophic response and no increase in gene expression. We conclude that BPA does not produce reproducible changes in gene expression in the uterus of immature rats at dose levels that are not also uterotrophic. Therefore, in the present study, the no effect level for uterotrophic activity for BPA coincided with the no transcriptional effect level for uterine genes.
FUNDED BY THE CHEMICAL INDUSTRY

Diel, P., S. Schmidt, G. Vollmer, P. Janning, A. Upmeier, H. Michna, H. M. Bolt and G. H. Degen (2004). Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. Arch. Toxicol. 78:183-93.

The rat uterotrophic assay is a widely used screening test for the detection of estrogenic, endocrine-disrupting chemicals. Although much attention has been paid to identifying protocol variables and reproducibility between laboratories the question whether toxicodynamic and toxicokinetic variations of different strains may affect their sensitivity to estrogenic stimuli has been rarely addressed. We have compared the estrogenic activity of the environmental chemicals genistein (GEN), bisphenol A (BPA) and p- tert-octylphenol (OCT) in DA/Han (DA), Sprague-Dawley (SD) and Wistar (WIS) rats after repeated oral application. Rats were ovariectomized and 4 days later were treated by gavage for 3 days with different doses of these weakly estrogenic compounds and the potent reference estrogen ethinylestradiol (EE) at 100 µg/kg/day. Then uterine wet weight, thickness of the uterine epithelium, uterine gene expression of clusterin (CLU), and thickness of the vaginal epithelium were examined as parameters for estrogenic potency of the test compounds in the three strains of rats. The uterotrophic response to treatment with BPA (5, 50, 200 mg/kg/day), OCT (5, 50, 200 mg/kg/day) and GEN (25, 50, 100 mg/kg/day) was similar in the three strains, and allowed us to rank them as GEN being more potent than OCT, and BPA being the weakest estrogen. This was

confirmed by analysis of other biological endpoints, despite some differences in the magnitude of their response among strains and to distinct compounds. For instance, the uterus wet weight response to EE treatment indicated lower sensitivity of SD rats than that of DA and WIS rats, but this was not observed for responses of the uterine or vaginal epithelium. Moreover, blood concentrations were assessed at the time of killing and related to biological responses: plasma levels of total and unconjugated BPA and GEN depended upon the dose administered and varied to some extent within treatment groups and among the three rat strains. However, there was no good correlation in the three strains between individual compound concentrations analysed 24 h after the last dose and the uterotrophic wet weights. Summarising our results, we conclude that the sensitivity of various biological endpoints can differ slightly between strains of rats. On the other hand, our data demonstrate that the choice of the rat strain does not lead to pronounced differences in the evaluation of estrogenic activities of chemicals, especially when different biological endpoints are included in the analysis.

These findings are consistent with those of Ferguson et al. (Neurotoxicol. Teratol. 25:491, 2003) that a sub-line of CD Sprague-Dawley rats purchased in the 1970s from Charles River and then maintained in a closed colony by the FDA are not sensitive to the positive control estrogen, ethinylestradiol (pregnant and lactating mothers and offspring show very few significant effects) relative to DA/Han rats. DA/HAN rats show profound physiological and behavioral disruption due to exposure just during mid pregnancy (when these strains are compared at about 15 µg/kg/day ethinylestradiol). The ethinylestradiol dose of 15 µg/kg/day is 30-fold higher than the typical dose of ethinylestradiol in oral contraceptives, which is about 0.5 µg/kg/day. The dose of EE used in the Diel study above is 200-fold higher than the dose of EE in oral contraceptives.

Gould, J.C., Leonared, L.S., Maness, S.C., Wagner, B.L., Conner, K., Zacharewski, T., Safe, T., McDonnell, D.P., and Gaido, K.W. (1998). Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. Molecular and Cellular Endocrinology 142:203-214.

Bisphenol A at an oral dose of 5-150 mg/kg/day did not stimulate a uterine weight increase in prepubertal Sprague-Dawley rats, However, bisphenol A at all doses significantly increased uterine progesterone receptors, and at 100 and 150 mg/kg/day increased uterine peroxidase activity. Coadministration of bisphenol A with estradiol (0.5 µg), and relative to estradiol alone, bisphenol A resulted in an inhibition of peroxidase and progesterone receptor induction, although not to negative control levels, while no effect on uterine weight was observed. This study shows the marked insensitivity to bisphenol A of the uterus using the standard uterotrophic assay in prepubertal Sprague-Dawley rats.

Laws, S.C., Carey, S.A., Ferrell, J.M., Bodman, G.J., and Cooper, R.L. (2000). Estrogenic Activity of Octylphenol, Nonylphenol, Bisphenol A and Methoxychlor in Rats. Tox. Sci. 54:154-167.

A dose of 200 mg/kg/day was required to stimulate uterine weight increase in prepubertal Long Evans rats, while a significant effect was not observed at 100 mg/kg/day. A 100 mg/kg/day dose (the only dose tested) to intact adult females lengthened the estrous cycle.

Markey, C. M., Michaelson, C. L., Veson, E. C., Sonnenschein, C. and Soto, A. M. (2001). The mouse uterotrophic assay: a reevaluation of its validity in assessing the estrogenicity of bisphenol A. Environ Health Perspect 109:55-60.

The uterus in prepubertal CD-1 mice requires 100 mg/kg/day to show an increase in size. When compared to the effects of bisphenol A on mammary glands, these findings reveal that the mammary glands are 4000-times more sensitive to bisphenol A than is the uterus in CD-1 mice, confirming a similar difference in sensitivity of these tissues to DES reported in prior studies.

Mehmood, Z., A. G. Smith, M. J. Tucker, F. Chuzel and N. G. Carmichael (2000). The development of methods for assessing the in vivo oestrogen-like effects of xenobiotics in CD-1 mice. Food Chem Toxicol 38:493-501.

The increasing awareness and concern about the potential health risks posed to the ecosystem and to man by endocrine disrupting chemicals with oestrogen-like activity in the environment has focused attention on the need for developing sensitive and specific methods for identifying these xenobiotics and to evaluate their degrees of toxic effects. We have conducted dose response studies in immature (21 days old) CD-1 female mice treated with four compounds, diethylstilboestrol (DES) (0.1 microg to 25 mg/kg body weight), alpha-zearalanol (0.5 mg to 25 mg/kg body weight), methoxychlor (0.5 mg to 500 mg/kg body weight) and bisphenol A (10 microg to 100 mg/kg body weight) administered subcutaneously daily for 3 days, and measured a number of uterine markers in treated and control (vehicle treated) mice. These were, in addition to the commonly measured changes in relative uterus weight and histopathological examination of uterine tissue, three other markers indicative of uterotrophic effects, namely, uterine luminal epithelium BrdU labelling index over the last 24 hr, peroxidase activity and lactoferrin expression. All of these markers showed clear dose-related increases in DES- and methoxychlor-treated animals. In the case of alpha-zearalanol treatment, relative uterine weight, peroxidase activity and lactoferrin expression showed dose-related increases at all the doses investigated. BrdU incorporation (an index of cell proliferation) also progressively increased at dose levels ranging from 0.1 mg to 5.0 mg/kg body weight, but apparently decreased at 25 mg/kg body weight. In contrast to these findings, bisphenol-A treatment showed no consistent changes in any of the four markers at the dose levels investigated. Additionally, studies were also conducted on a number of chemicals in CD-1 mice at one dose level. The chemicals investigated were: bisphenol A (1 g/kg body weight/day), naringenin (1 g/kg body weight/day) o,p'-DDT (500 mg/kg body weight/day), genistein (1 g/kg/day), coumestrol (0.5 mg/kg/day) and chlordecone (20 mg/kg/day) administered subcutaneously daily for 3 days. There was some variability in response of the markers perhaps indicating that the chemicals did not all act in the same way. The findings of our exploratory in vivo studies in CD-1 mice suggest that the measurement of a range of uterine markers, in addition to organ weight and histopathology, would provide useful information on the potential oestrogenicity of chemicals.
NOTE: While the magnitude of the increase in uterine weight (expressed as a percent of body weight) was about 4 fold for DES amd methoxychlor, it was only about 20% relative to controls for bisphenol A between 0.01 and 1 mg/kg/day, after which the response dropped to baseline at 10 and 100 mg/kg/day, forming an inverted-U dose-response curve. If the authors did not conduct a statistical analysis appropriate for these non-monotonic data, the possibility that the results are statistically significant could be missed. Neverrtheless, the magnitude of the uterine response to bisphenol A is a small fraction of the response observed for other chemicals.

Tinwell, H., R. Joiner, I. Pate, A. Soames, J. Foster and J. Ashby (2000). Uterotrophic activity of bisphenol A in the immature mouse. Regul Toxicol Pharmacol 32:118-26.

Bisphenol A (BPA) has been evaluated in eight independent immature mouse uterotrophic assays using the subcutaneous route of administration, and in a single study employing oral gavage. The dose range covered was from 0.02 microg to 300 mg/kg BPA and some experiments were supplemented by assessments of uterine hypertrophy and hyperplasia. Pooling of the test data indicates no uterotrophic activity for the chemical. However, in a subset of the subcutaneous injection studies, where control uterine weights were relatively low, significant, but weak, uterotrophic activity was observed over a range of dose levels, but in the complete absence of a dose relationship. In the oral gavage study, no increases in uterine weight were seen, but there were increases in uterine

labeling with bromodeoxyuridine at 200-300 mg/kg BPA. The present study illustrated that when a large number of observations are made, a certain level of chance observations may be made, and that surrogates for an increase in uterine weight do not necessarily enhance assay sensitivity, albeit such data may complement uterine weight data. The data indicate that reducing control uterine weights may enhance assay sensitivity, but that animal body weight is an imperfect indicator of control uterine weight. The data also show that it is possible for individual investigators to be unable to confirm their own observations. It is concluded that BPA may be weakly uterotrophic to the mouse under specific conditions of test, and in the complete absence of a dose-response relationship to this activity. However, overall, we have failed to define BPA as reproducibly active in the immature mouse uterotrophic assay, and in that sense, our data are broadly consistent with those reported earlier by Coldham et al. (Environ. Health Perspect. 105, 734-742, 1997) in 1997 using a similar assay.

While there are positive effects reported in this study, the conclusion is that bisphenol A is essentially without effect, which is the consistent message of all studies funded by the chemical industry.
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Taken together, the above studies show that relative to the B6C3F1 hybrid mouse (Papaconstantinou et al. 2000, 2001) or F344 rat (Steinmetz et al., 1998), the CD-1 mouse and Sprague-Dawley rat show a markedly lower sensitivity to the uterotrophic action of bisphenol A, revealing marked strain differences in uterine sensitivity to this chemical.

XII. CHEMICAL INDUSTRY FUNDED STUDY OF BISPHENOL A WITH POSITIVE RESULTS (DETERMINED BY A NTP REVIEW PANEL) BUT REPORTED TO THE US-EPA AND THE PUBLIC AS NEGATIVE RESULTS

(For the critique of this article see NTP, 2001, p. A88-A91)
NTP, National Toxicology Program (2001). Endocrine Disruptors Low Dose Peer Review, Raleigh, NC,
http://ntp-server.niehs.nih.gov/htdocs/liason/LowDoseWebPage.html

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.
FUNDED BY THE CHEMICAL INDUSTRY

The NIH Low Dose peer review panel stated that the reasons for the conclusion drawn by the authors that there was no effect of bisphenol A on the prostate were “misleading”, "flawed" and "illogical" (NTP, 2001, p. A-90).
The conclusion by the NTP panel was that with regard to prostate weight in response to doses bisphenol A administered in the drinking water of pregnant and lactating rats:“The 0.05 mg/l [10 µg/kg/day] ($p<0.01$), the 5 mg/l [1 mg/kg/day] ($p<0.0001$) and the 50 mg/l [10 mg/kg/day] ($p<0.02$) [doses] were significantly increased relative to control.” p. A-86.

Letter from the American Chemistry Council to the US-EPA on the NTP-Low Dose Peer Review Final Report
(Appendix C of NTP report on Low Dose Effects, Public Comments, p. C-89)

"A very large study conducted by Welsch (Elswick and Welsch [and Janszen], 2000) using multiple pups per litter also found no BPA effects on prostate weight at 0.005, 0.05, 0.5 or 5 mg/L drinking water (0.001 to 10 mg/kg/day)."

In an official letter to the US-EPA, the American Chemistry Council (ACC) ignored the above statement by the NIH peer review that significant effects were found in the study.

XIII. CHEMICAL INDUSTRY FUNDED STUDIES INCORRECTLY DESCRIBED AS REPLICATING PROCEDURES BY VOM SAAL (DIFFERENT FEED WAS USED) THAT REPORTED NEGATTIVE RESULTS FOR BOTH BISPHENOL A AND THE POSITIVE CONTROL CHEMICAL, DES

Ashby, J. Tinwell, H. and 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.

Prenatal exposure to bisphenol A significantly increased body weight of offspring when they reached adulthood. In this study the positive control chemical, DES, at a dose of 0.2 µg/kg/day did not differ from the negative controls. The authors did not accurately identify that DES was included as the positive control. Failure to find a positive effect of the positive control estrogenic drug group relative to the negative control group would normally be considered to indicate that the experimental system being used was not able to detect an effect of any estrogen, regardless of potency. Also, all animals in this study were obese relative to CF-1 mice maintained on Purina 5008 pregnancy and Purina 5001 maintenance diets. Bisphenol A significantly increased body weight in offspring relative to negative controls.
FUNDED BY THE CHEMICAL INDUSTRY

Cagen SZ, Waechter, JM, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE and Harris LR. (1999) Normal reproductive organ development in CF-1 mice following prenatal exposure to Bisphenol A. Tox. Sci. 11:15-29.

In this study the positive control chemical, DES, at a dose of 0.2 µg/kg/day did not differ from the negative controls. Similar to the Ashby study above, these authors did not accurately identify that DES was included as the positive control. Failure to find a positive effect of the positive control estrogenic drug group relative to the negative control group would normally be considered to indicate that the experimental system being used was not able to detect an effect of any estrogen, regardless of potency. Importantly, the CF-1 mice in this study were fed Purina 5002 animal feed, whereas the CF-1 mice in the vom Saal laboratory were fed Purina 5008 pregnancy/lactation and Purina 5001 maintenance diets. Purina 5002 feed has highly variable levels of estrogens that can mask the effects of very potent estrogenic drugs, such as DES, based on a study by J. Thigpen at NIH. Since Cagen did not find any effect of DES, the findings of Thigpen et al. (2003) lead to the prediction that the 5002 feed masked the effect of both DES and bisphenol A. Studies that include a positive control group that shows no difference from the negative control group are properly determined to have failed, and such studies should be repeated. When positive effects of positive control chemicals cannot be shown, negative findings for test chemicals such as bisphenol A are meaningless.
FUNDED BY THE CHEMICAL INDUSTRY
REFERENCED ARTICLE: Thigpen, J., Haseman, J., Sandres, H., Grant, M. and Forsythe, D. (2003). Dietaray phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. Comparative Medicine 53(6): 477-485.

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

Elswick et al. 2000
Ema et al. 2001
Kato et al. 2005
Kobayashi et al. 2002
Kwon et al. 2000
Masutomi et al.. 2004
Nagao et al. 1999
Takagi et al. 2004
Tyl et al. 2002
Yamasaki et al. 2002

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.

Elswick reported that there were no significant effects of bisphenol A in their paper. However, the NIH Low Dose peer review panel stated that with regard to prostate weight in response to doses bisphenol A administered in the drinking water of pregnant and lactating rats:"The 0.05 mg/l [10 µg/kg/day] ($p<0.01$), the 5 mg/l [1 mg/kg/day] ($p<0.0001$) and the 50 mg/l [10 mg/kg/day] ($p<0.02$) [doses] were significantly increased relative to control." This is discussed in more detail below.

FUNDED BY THE CHEMICAL INDUSTRY

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.

This study was conducted to determine the low-dose effects of bisphenol A (BPA) in a rat two-generation reproduction study. Groups of 25 male and 25 female Crj: CD (SD) IGS rats were given BPA at 0.2, 2, 20, or 200 microg/kg/day by gastric intubation throughout the study beginning at the onset of a 10- and 2-week premating period, in F0 males and females, respectively, and continuing through the mating, gestation, and lactation periods, for two generations. There were adult (F0, F1, F2) and postnatal day (PND) 22 (F1, F2) necropsies: the oldest F2 males and females being killed at postnatal weeks 7 and 14, respectively. No compound-related clinical signs or effects on body weight or food consumption were observed in any generation. There were no compound-related changes in surface righting reflex, negative geotaxis reflex, mid-air righting reflex, pinna detachment, incisor eruption, eye opening, testes descent, preputial separation, or vaginal opening in F1 and F2 generations, or behavior in the open field or water filled multiple T-maze in the F1 generation. No test compound-related changes in estrous cyclicity, copulation index, fertility index, number of implantations, gestation length, litter size, pup weight, pup sex ratio, pup viability, or other functional reproductive measures were noted in any generation. A few significant changes in the anogenital distance (AGD) per cube root of body weight ratio were found at 0.2 and 20 microg/kg in F1 males, at 2, 20, and 200 microg/kg in F1 females, and at 20 and 200 microg/kg in F2 females. However, the changes in the AGD were consistently small (within 5% of control values), and no continuous changes in the AGD or AGD/cube root of body weight ratio were detected. There were no compound-related changes in epididymal sperm counts or motility in F0 and F1 males. No compound-related necropsy findings or effects on organ weight including the reproductive organs were found in any generation. Histopathologic examinations revealed no evidence of compound-

related changes in any organs including the reproductive organs of both sexes. The data indicate that oral doses of BPA of between 0.2 and 200 microg/kg over 2 generations did not cause significant compound-related changes in reproductive or developmental parameters in rats.

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.

Bisphenol A (BPA) was evaluated at concentrations of 0, 0.015, 0.3, 4.5, 75, 750, and 7500 ppm (approximately 0.001, 0.02, 0.3, 5, 50, and 500 mg/kg/day of BPA) administered in the diet ad libitum to 30 CD((R)) Sprague-Dawley rats/sex/dose for 3 offspring generations, 1 litter/generation, through F3 adults. Adult systemic toxicity at 750 and 7500 ppm in all generations included: reduced body weights and body weight gains, reduced absolute and increased relative weanling and adult organ weights (liver, kidneys, adrenals, spleen, pituitary, and brain), and female slight/mild renal and hepatic pathology at 7500 ppm. Reproductive organ histopathology and function were unaffected. Ovarian weights as well as total pups and live pups/litter on postnatal day (PND) 0 were decreased at 7500 ppm, which exceeded the adult maximum tolerated dose (MTD). Mating, fertility, gestational indices; ovarian primordial follicle counts; estrous cyclicity; precoital interval; gestational length; offspring sex ratios; postnatal survival; nipple/areolae retention in preweanling males; epididymal sperm number, motility, morphology; daily sperm production (DSP), and efficiency of DSP were all unaffected. At 7500 ppm, vaginal patency (VP) and preputial separation (PPS) were delayed in F1, F2, and F3 offspring, associated with reduced body weights. Anogenital distance (AGD) on PND 0 was unaffected for F2 and F3 males and F3 females (F2 female AGD was increased at some doses, not at 7500 ppm, and was considered not biologically or toxicologically relevant). Adult systemic no observed adverse effect level (NOAEL) = 75 ppm (5 mg/kg/day); reproductive and postnatal NOAELs = 750 ppm (50 mg/kg/day). There were no treatment-related effects in the low-dose region (0.001-5 mg/kg/day) on any parameters and no evidence of nonmonotonic dose-response curves across generations for either sex. BPA should not be considered a selective reproductive toxicant, based on the results of this study.

NOTE: This study also used a type of animal feed (Purina 5002) that contains contaminants that can mask the effects of DES.

FUNDED BY THE CHEMICAL INDUSTRY

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.

This study reports that Charles River Sprague-Dawley derived (CD-SD) strain of rat requires a very high 200 µg/kg/day dose of ethinyl estradiol (the potent estrogenic drug used in birth control pills at a dose of 0.5 µg/kg/day) in order to show a response. This rat is thus extremely insensitive to estrogen relative to women.

Review of the Ema, Tyl and Yamasaki Studies

The CD-SD rat strain was selected over a 40-year period by Charles River for very large litters and large body size, and are no longer similar in phenotype to the Sprague-Dawley rats that were purchased by Charles River in 1950. The male CF-1 mouse used in the vom Saal laboratory studies responds to ethinyl estradiol with significant changes in sperm production and prostate size at a dose of 0.002 µg/kg/day (Thayer, et al. Human Reproduction 16:988, 2001) and is thus 100,000-times more sensitive to ethinyl estradiol than is the CD Sprague Dawley rat used in the studies listed

above by: 1. Ema, 2. Tyl, and 3. Yamasaki. Yamasaki also reported that a 600 mg/kg/day dose of bisphenol A was required to see an effect. This dose is approximately 100,000-times higher than the bisphenol A doses used in the studies conducted in the vom Saal laboratory. The insensitivity of the CD Sprague Dawley rat to bisphenol A was predicted by its insensitivity to the positive control drug ethinylestradiol. Only by including in the experiment a positive control estrogenic chemical, such as DES or ethinyl estradiol, can the reason for the failure too find low dose effects of bisphenol A be determined. Since Tyl used this insensitive rat as well as a type of feed (Purina 5002) that Thigpen et al. (2003) reported can mask the effects of estrogenic chemicals, the Tyl study cannot be interpreted as showing that bisphenol is "safe" based on purely negative findings. A positive control (DES or ethinylestradiol) to establish the sensitivity of the animals used in the experiments conducted by Ema and Tyl was not included, but because Yamasaki did include ethinylestradiol as a positive control, the insensitivity of the CD Sprague Dawley rat to any estrogenic chemical, not just bisphenol A, was revealed.

Other Studies Showing No Effect of Low-High Doses of Bisphenol A and Other Estrogenic Chemicals in CD-SD Rats

Kato, H., Furuhashi T., Tanaka, M., Katsu, Y., Watanabe, H., Ohta, Y., Iguchi, T. (2005). Effects of bisphenol A given neonatally on reproductive functions of male rats. Reprod. Toxicol. Nov. 32, 2005 ONLINE. PMID: 16311018.

Male Sprague-Dawley rats (Crj:CD (IGS) were treated neonatally with bisphenol A (BPA) to evaluate effects on reproductive parameters. Animals were given BPA subcutaneously in corn oil to dosages of 0.002-97mg/kg body weight, or 0.9mg/kg 17beta-estradiol (E2) once a day from postnatal day (PND) 0 to PND 9. Preputial separation, copulatory rate, fertility rate, sperm analysis, serum testosterone levels, and gene expression in the testis were assessed. Males in the E2 group showed a decrease in testis weight and alterations of estrogen-mediated gene expression in the testis on PND 10, and by PND 150 incomplete preputial separation, decreases in the copulatory rate, testicular and accessory organ weights and number of sperm. In contrast, males in all BPA groups showed normal reproductive parameters. These results indicate that in male rats, BPA given during the neonatal period neither affected reproductive function nor evoked estrogen-mediated gene responses in the testis.

This finding is consistent with other findings that the CD-SD (Crj:CD (IGS) strain of rat is insensitive to low doses of estrogen. The positive ccontrol 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.

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.

There were no significant changes in body weight, liver weight, kidneys weight, testes weight, AGD, the ratio of AGD to BW, or the ratio of AGD to the cube root of BW in BPA exposed CD-SD rat pups compared to the vehicle-exposed control. This suggests that prenatal and postnatal exposure (indirect exposure) to BPA (4-40 mg/kg/day, GD 6-PND 20) does not affect on somatic growth or AGD of F1 generation of male and female rats.

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

Bisphenol A (BPA) is used on a large scale in the manufacture of polycarbonate plastics. BPA has been shown to bind weakly to both estrogen receptor (ER)alpha and ERbeta, and to transactivate reporter genes in vitro. The purpose of the present study was to determine whether exposure of rats to BPA during pre- and postnatal development affects estrogen-mediated end points related to pubertal development and reproductive functions. BPA was administered to pregnant Crl:CD BR Sprague-Dawley (CD-SD) rats by gavage at 0, 3.2, 32, or 320 mg/kg/day from gestation day (GD) 11 through postnatal day (PND) 20. Diethylstilbestrol (DES) at 15 microg/kg/day was used as a reference chemical with known estrogenic effects. Female pubertal development was not affected by indirect BPA exposure of the offspring at any of the dose levels. Treatment with this chemical also did not produce detectable effects on the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA), estrous cyclicity, sexual behavior, or male reproductive organ weights of F(1) offspring. However, DES at 15 microg/kg/day increased the volume of the SDN-POA of female offspring and affected their normal estrous cyclicity following puberty. In this study, pre- and postnatal exposure of rats to BPA at 3.2, 32, or 320 mg/kg/day from GD 11 through PND 20 did not have any apparent adverse effects on female rat pubertal development and reproductive functions.
FUNDED BY THE CHEMICAL INDUSTRY

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.

Masutomi administered estrogenic chemicals to pregnant and lactating CD-Sprague-Dawley (Japan) rats. Bisphenol A was mixed with the feed at concentrations of 60, 600 and 3000 ppm and was provided to maternal CD Sprague-Dawley [Charles River Kanagawa, Japan, Crj: CD(SD)IGS] rats from gestational day (GD) 15 to postnatal day (PND) 10 (gestation doses = 5, 50 and 230, lactation doses = 8, 80 and 380 mg/kg/day). Ethinylestradiol (EE) at 0.5 ppm was used as an estrogenic reference drug (gestation dose 27 and lactation dose 63 µg/kg/day). Among the chemicals MXC alone showed typical estrogenic effects only at the maternally toxic 1200 ppm. The present study was performed to examine the sensitivity of immunohistochemical analysis of pituitary cells of offspring similarly exposed to each chemical for detection of endocrine-disrupting effects. For this purpose, ratios of pituitary cells expressing luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin (PRL), were measured at 3 and 11 weeks of age. Ethinylestradiol (EE) at 0.5 ppm was used as a reference chemical. At week 3, decrease in the relative proportions of LH, FSH, and PRL cells in males and LH cells in females was evident with MXC at 1200 ppm. At week 11, increase was found for PRL cells from 240 ppm MXC, and FSH cells at 1200 ppm in females. On the other hand, EE increased the PRL cell percentage in females at week 3 but no effects were apparent at week 11. The other chemicals were without influence at either time point. The lack of sensitivity of the CD Sprague-Dawley rat to all of these estrogens is evident. The only significant effect at the lowest doses of BPA (all other doses were in the high-dose range) was s decrease in body weight for males.

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 (this is a very high dose) or 2 mg/kg estradiol benzoate from postnatal Day 1 to 5 was examined after puberty as well as histolopathogic changes in reproductive organs. All male and

female rats treated postnatally with estradiol benzoate showed poor reproductive capability, including adverse effects on masculine sexual behavior, and marked histopathologic alterations of the reproductive organs. In addition, estradiol benzoate markedly reduced the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in males. On the other hand, all male and female rats treated postnatally with bisphenol A showed normal reproductive function and no histopathologic abnormalities of reproductive organs. Bisphenol A did not affect the volume of the SDN-POA. These results indicated that neonatal exposure to estradiol benzoate affects reproductive function in male and female rats, and treatment with bisphenol A at a fairly high dose was ineffective if given postnatally to male and female rats.

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.

Bisphenol A and nonylphenol were mixed with diet at concentrations of 60, 600 and 3000 ppm and was provided to maternal (n = 5-6 mothers/group) CD Sprague-Dawley [Charles River Kanagawa, Japan, Crj: CD(SD)IGS] rats from gestational day (GD) 15 to postnatal day (PND) 10 (gestation doses = 5, 50 and 230, lactation doses = 8, 80 and 380 mg/kg/day). Ethinylestradiol (EE) at 0.5 ppm was used as an estrogenic reference drug (gestation dose 27 and lactation dose 63 µg/kg/day). During pregnancy and lactation, including the exposure period, a soy-free rodent diet was provided to eliminate possible modification of the study results by plant-derived phytoestrogens. The animals were housed in polycarbonate cages. Effects on endocrine/reproductive systems were evaluated by examining the anogenital distance, organ weights before puberty, onset of puberty, estrous cyclicity, and organ weights and histopathology of adult endocrine organs (at 11 weeks of age), as well as the volume of the sexually dimorphic nucleus of preoptic area. Both NP and BPA, at high doses, caused decreases in maternal body weights and retardation of offspring growth (reduced body weight), but neither affected any of the endocrine/reproductive endpoints of offspring, whereas EE at about 50 µg/kg/day induced irreversible changes in estrous cyclicity and histopathology of ovaries and uterus of adult females. The results indicated that maternal dietary exposure to NP or BPA at concentrations up to 3000 ppm (230-380 mg/kg/day) from GD 15 through PND 10 do not exert any apparent adverse effects on the endocrine/reproductive systems of offspring.

XV. FINDINGS OF NO SIGNIFICANT EFFECTS AT DOSES OF BISPHENOL A BELOW 50 MG/KG/DAY OR AT LOW DOSES IN AQUATIC ANIMALS

Ashby, J. Tinwell, H. and 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.

Prenatal exposure to bisphenol A significantly increased body weight of offspring when they reached adulthood. In this study the positive control chemical, DES, at a dose of 0.2 µg/kg/day did not differ from the negative controls. The authors did not accurately identify that DES was included as the positive control. Failure to find a positive effect of the positive control estrogenic drug group relative to the negative control group would normally be considered to indicate that the experimental system being used was not able to detect an effect of any estrogen, regardless of potency. Also, all animals in this study were obese relative to CF-1 mice maintained on Purina 5008 pregnancy and Purina 5001 maintenance diets. Bisphenol A significantly increased body weight in offspring relative to negative controls.

FUNDED BY THE CHEMICAL INDUSTRY

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 74:129-138.

M. Sakaue et al. (2001,J. Occup. Health vol. 43, pp. 185-190) have described how oral exposure of sexually mature male rats to bisphenol A (BPA) between postnatal days (PND) 91-97 led to a reduction in daily sperm production (DSP) 5 weeks later (18 weeks of age). Activity was observed over the dose range 20 microgram/kg-200 mg/kg BPA, with an absence of activity over the dose range 2 ng/kg-2 microgram/kg BPA. There was no evidence of a dose response relationship over the active dose range (five orders of magnitude range). The observation of endocrine disruption (ED) effects for BPA at such low doses, and in sexually mature animals, was unexpected. It was therefore decided to mount an independent repeat of their study. A total of four independent studies were conducted according to the protocol used by Sakaue et al. Doses of 20 microgram/kg, 2 mg/kg, or 200 mg/kg BPA were administered to adult Sprague-Dawley (SD) rats over PND 91-97, and the studies were terminated when the rats reached the age of 18 weeks. Three different rodent diets were employed (RM3, Purina 5002, and CE2), the last of which had been used by Sakaue et al. BPA failed to give any evidence of ED activities, including the changes in DSP reported by Sakaue et al. 2001.

During the course of these studies, the test protocol was adapted to coincide more precisely with that used by Sakaue et al.; this included restricting the number of animals per cage, removing bedding from the cages, and changing to the use of glass water bottles in the cages. The only thing of interest to emerge from our studies was the observation of a significant difference in DSP between the control groups of our first and second study. As the change in diet from RM3 to Purina 5002 was the major difference between those two studies, we conducted a repeat of the second study, but we were unable to confirm the differences seen between the first and second study. The probability that those differences arose either by chance, or as the result of intrinsic study-to-study variability, was strengthened by the absence of significant differences in the sperm parameters in a final (fifth) study where the sperm parameters for control animals maintained on the three different diets were compared under the conditions of the main experiments. No explanation for our failure to replicate the effects reported by Sakaue et al. is evident. A review of DSP values reported in the recent literature is provided and discussed, and it is concluded that use of the term DSP/g testis rather than DSP/testis could increase the sensitivity of DSP assessments.

This study was funded by the chemical industry. The data for the control animals in this study are at the level of the animals exposed to bisphenol A in the Sakaue study that this study was supposed to replicate. This is exactly what happened when the Ashby et al (1999) study was compared with the Nagel et al (1997) study, where the data for the control animals in the Ashby study were at the level of the bisphenol A treated animals in the Nagel study. These comparisons suggest that the animals in the Ashby lab were already maximally estrogenized by some other source. The inclusion of a positive control in the experiment is required to determine whether the experiment is able to detect a response to any estrogenic chemical. The Ashby et al (1999) study included a positive control (DES) that showed no effect, but the authors ignored this failure and still concluded that the experiment was valid, which violates traditional standards of experimental design and analysis.
FUNDED BY THE CHEMICAL INDUSTRY

Ashby, J. and Odum, J. Gene Expression Changes in the Immature Rat Uterus: Effects of Uterotrophic and Sub-Uterotrophic Doses of Bisphenol A. Toxicol Sci. 2004 Oct 29. Advance Access, 9-29-04.

Gould et al. (1998) have reported that administration of 5-150mg/kg/day BPA to immature rats leads to increases in uterine peroxidase activity and progesterone receptor (PR) protein levels in

the absence of an uterotrophic response. These observations are of interest given current concerns regarding the adequacy of the uterotrophic assay to act as a sentinel for the estrogenic activity of chemicals in vivo. Therefore, the uterotrophic activity of BPA to the immature rat has been re-evaluated over the dose range 2 micro g/kg-800mg/kg/day. Expression levels of three estrogen responsive uterine genes were determined using real-time RT-PCR - namely, complement component 3, lipocalin 2 and PR. 18S rRNA and RNA polymerase II large subunit acted as control genes. Observations of gene expression were made 4h and 72h after the first of three daily oral administrations of BPA. Increases in gene expression were observed over the uterotrophic dose range (approximately 200-800mg/kg BPA). Over the dose range 2 micro g/kg-20mg/kg BPA there was no uterotrophic response and no increase in gene expression. We conclude that BPA does not produce reproducible changes in gene expression in the uterus of immature rats at dose levels that are not also uterotrophic. Therefore, in the present study, the no effect level for uterotrophic activity for BPA coincided with the no transcriptional effect level for uterine genes.
FUNDED BY THE CHEMICAL INDUSTRY

Cagen SZ, Waechter, JM, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE and Harris LR. (1999) Normal reproductive organ development in CF-1 mice following prenatal exposure to Bisphenol A. Tox. Sci. 11:15-29.

In this study the positive control chemical, DES, at a dose of 0.2 µg/kg/day did not differ from the negative controls. Similar to the Ashby study above, these authors did not accurately identify that DES was included as the positive control. Failure to find a positive effect of the positive control estrogenic drug group relative to the negative control group would normally be considered to indicate that the experimental system being used was not able to detect an effect of any estrogen, regardless of potency. Importantly, the CF-1 mice in this study were fed Purina 5002 animal feed, whereas the CF-1 mice in the vom Saal laboratory were fed Purina 5008 pregnancy/lactation and Purina 5001 maintenance diets. Purina 5002 feed has highly variable levels of estrogens that can mask the effects of very potent estrogenic drugs, such as DES, based on a study by J. Thigpen at NIH. Since Cagen did not find any effect of DES, the findings of Thigpen et al. (2003) lead to the prediction that the 5002 feed masked the effect of both DES and bisphenol A. Studies that include a positive control group that shows no difference from the negative control group are properly determined to have failed, and such studies should be repeated. When positive effects of positive control chemicals cannot be shown, negative findings for test chemicals such as bisphenol A are meaningless.
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REFERENCED ARTICLE: Thigpen, J., Haseman, J., Sandres, H., Grant, M. and Forsythe, D. (2003). Dietaray phytoestrogens accelerate the time of vaginal opening in immature CD-1 mice. Comparative Medicine 53(6): 477-485.

Cagen S.Z, Waechter J.M, Dimond S.S, Breslin W.J, Butala J.H, Jekat F.W, Joiner R.L, Shiotsuka R.N, Veenstra G.E and Harris L.R (1999). Normal reproductive organ development in Wistar rats exposed to bisphenol A in the drinking water. Reg. Tox. Pharmacol. 30, 130-139.

Bisphenol A (BPA) is a chemical used primarily as a monomer in the manufacture of numerous chemical products, such as epoxy resins and polycarbonate. The objective of this study was to evaluate potential effects of BPA on sexual development of male rats and was designed to clarify low-dose observations reported as preliminary results by Sharpe et al. (1996). The protocol for the present study followed the same treatment schedule as reported by Sharpe et al. (1995, 1996), but included more treatment groups, a greater number of animals per group, and a more comprehensive number of reproductive endpoints. Groups of 28 female Han-Wistar albino rats were exposed to drinking water that contained 0, 0.01, 0.1, 1.0, or 10 ppm BPA or 0.1 ppm diethylstilbestrol (DES), 7

days per week, for a total of 10 weeks. Treatment of the females began at 10 weeks of age and continued throughout a 2-week premating period, 2 weeks of mating (to untreated males), 21-22 days of gestation, and 22 days of lactation. Offspring weanling males were given untreated drinking water and maintained until 90 days of age when evaluations were made of various reproductive organs. Consistent with Sharpe et al. (1996) the female offspring were not evaluated. No treatment-related effects on growth or reproductive endpoints were observed in adult females exposed to any concentration of BPA. Similarly, no treatment-related effects were observed on the growth, survival, or reproductive parameters (including testes, prostate and preputial gland weights, sperm count, daily sperm production, or testes histopathology) of male offspring from dams exposed to BPA during gestation and lactation. DES administered in the drinking water at 0. 1 ppm resulted in decreased body weight, body weight change, and food consumption in adult females. In addition, an increase in the duration of gestation and a decrease in the number of pups delivered and number of live pups were also observed in animals exposed to DES. In conclusion, these results do not confirm the previous findings of Sharpe et al. (1996) and show that low doses of BPA had no effects on male sexual development in the rat. Copyright
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Diel, P., S. Schmidt, G. Vollmer, P. Janning, A. Upmeier, H. Michna, H. M. Bolt and G. H. Degen (2004). Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. Arch. Toxicol. 78:183-93.

The rat uterotrophic assay is a widely used screening test for the detection of estrogenic, endocrine-disrupting chemicals. Although much attention has been paid to identifying protocol variables and reproducibility between laboratories the question whether toxicodynamic and toxicokinetic variations of different strains may affect their sensitivity to estrogenic stimuli has been rarely addressed. We have compared the estrogenic activity of the environmental chemicals genistein (GEN), bisphenol A (BPA) and p- tert-octylphenol (OCT) in DA/Han (DA), Sprague-Dawley (SD) and Wistar (WIS) rats after repeated oral application. Rats were ovariectomized and 4 days later were treated by gavage for 3 days with different doses of these weakly estrogenic compounds and the potent reference estrogen ethinylestradiol (EE) at 100 µg/kg/day. Then uterine wet weight, thickness of the uterine epithelium, uterine gene expression of clusterin (CLU), and thickness of the vaginal epithelium were examined as parameters for estrogenic potency of the test compounds in the three strains of rats. The uterotrophic response to treatment with BPA (5, 50, 200 mg/kg/day), OCT (5, 50, 200 mg/kg/day) and GEN (25, 50, 100 mg/kg/day) was similar in the three strains, and allowed us to rank them as GEN being more potent than OCT, and BPA being the weakest estrogen. This was confirmed by analysis of other biological endpoints, despite some differences in the magnitude of their response among strains and to distinct compounds. For instance, the uterus wet weight response to EE treatment indicated lower sensitivity of SD rats than that of DA and WIS rats, but this was not observed for responses of the uterine or vaginal epithelium. Moreover, blood concentrations were assessed at the time of killing and related to biological responses: plasma levels of total and unconjugated BPA and GEN depended upon the dose administered and varied to some extent within treatment groups and among the three rat strains. However, there was no good correlation in the three strains between individual compound concentrations analysed 24 h after the last dose and the uterotrophic wet weights. Summarising our results, we conclude that the sensitivity of various biological endpoints can differ slightly between strains of rats. On the other hand, our data demonstrate that the choice of the rat strain does not lead to pronounced differences in the evaluation of estrogenic activities of chemicals, especially when different biological endpoints are included in the analysis.

These findings are consistent with those of Ferguson et al. (Neurotoxicol. Teratol. 25:491, 2003) that a sub-line of CD Sprague-Dawley (CD-SD) rats purchased in the 1970s from Charles River and then maintained in a closed colony by the FDA are not sensitive to the positive control estrogen, ethinylestradiol (pregnant and lactating mothers and offspring show very few significant effects) relative to DA/Han rats. DA/HAN rats show profound physiological and behavioral disruption due to exposure just during mid pregnancy (when these strains are compared at about 15 µg/kg/day ethinylestradiol). The ethinylestradiol dose of 15 µg/kg/day is 30-fold higher than the typical dose of ethinylestradiol in oral contraceptives, which is about 0.5 µg/kg/day. The dose of EE used in the Diel study above is 200-fold higher than the dose of EE in oral contraceptives.
JOINTLY FUNDED BY THE CHEMICAL INDUSTRY AND GERMAN EPA

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.

Elswick reported that there were no significant effects of bisphenol A in their paper. However, the NIH Low Dose peer review panel stated that with regard to prostate weight in response to doses bisphenol A administered in the drinking water of pregnant and lactating rats:"The 0.05 mg/l [10 µg/kg/day] ($p<0.01$), the 5 mg/l [1 mg/kg/day] ($p<0.0001$) and the 50 mg/l [10 mg/kg/day] ($p<0.02$) [doses] were significantly increased relative to control." This is discussed in more detail below.
FUNDED BY THE CHEMICAL INDUSTRY

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.

This study was conducted to determine the low-dose effects of bisphenol A (BPA) in a rat two-generation reproduction study. Groups of 25 male and 25 female Crj: CD (SD) IGS (CD-SD) rats were given BPA at 0.2, 2, 20, or 200 microg/kg/day by gastric intubation throughout the study beginning at the onset of a 10- and 2-week premating period, in F0 males and females, respectively, and continuing through the mating, gestation, and lactation periods, for two generations. There were adult (F0, F1, F2) and postnatal day (PND) 22 (F1, F2) necropsies: the oldest F2 males and females being killed at postnatal weeks 7 and 14, respectively. No compound-related clinical signs or effects on body weight or food consumption were observed in any generation. There were no compound-related changes in surface righting reflex, negative geotaxis reflex, mid-air righting reflex, pinna detachment, incisor eruption, eye opening, testes descent, preputial separation, or vaginal opening in F1 and F2 generations, or behavior in the open field or water filled multiple T-maze in the F1 generation. No test compound-related changes in estrous cyclicity, copulation index, fertility index, number of implantations, gestation length, litter size, pup weight, pup sex ratio, pup viability, or other functional reproductive measures were noted in any generation. A few significant changes in the anogenital distance (AGD) per cube root of body weight ratio were found at 0.2 and 20 microg/kg in F1 males, at 2, 20, and 200 microg/kg in F1 females, and at 20 and 200 microg/kg in F2 females. However, the changes in the AGD were consistently small (within 5% of control values), and no continuous changes in the AGD or AGD/cube root of body weight ratio were detected. There were no compound-related changes in epididymal sperm counts or motility in F0 and F1 males. No compound-related necropsy findings or effects on organ weight including the reproductive organs were found in any generation. Histopathologic examinations revealed no evidence of compound-related changes in any organs including the reproductive organs of both sexes. The data indicate that oral doses of BPA of between 0.2 and 200 microg/kg over 2 generations did not cause significant compound-related changes in reproductive or developmental parameters in rats.

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.

There were no significant changes in body weight, liver weight, kidneys weight, testes weight, AGD, the ratio of AGD to BW, or the ratio of AGD to the cube root of BW in BPA exposed CD-SD rat pups compared to the vehicle-exposed control. This suggests that prenatal and postnatal exposure (indirect exposure) to BPA (4-40 mg/kg/day, GD 6-PND 20) does not affect on somatic growth or AGD of F1 generation of male and female rats.

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

Bisphenol A (BPA) is used on a large scale in the manufacture of polycarbonate plastics. BPA has been shown to bind weakly to both estrogen receptor (ER)alpha and ERbeta, and to transactivate reporter genes in vitro. The purpose of the present study was to determine whether exposure of rats to BPA during pre- and postnatal development affects estrogen-mediated end points related to pubertal development and reproductive functions. BPA was administered to pregnant Crl:CD BR Sprague-Dawley (CD-SD) rats by gavage at 0, 3.2, 32, or 320 mg/kg/day from gestation day (GD) 11 through postnatal day (PND) 20. Diethylstilbestrol (DES) at 15 microg/kg/day was used as a reference chemical with known estrogenic effects. Female pubertal development was not affected by indirect BPA exposure of the offspring at any of the dose levels. Treatment with this chemical also did not produce detectable effects on the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA), estrous cyclicity, sexual behavior, or male reproductive organ weights of F(1) offspring. However, DES at 15 microg/kg/day increased the volume of the SDN-POA of female offspring and affected their normal estrous cyclicity following puberty. In this study, pre- and postnatal exposure of rats to BPA at 3.2, 32, or 320 mg/kg/day from GD 11 through PND 20 did not have any apparent adverse effects on female rat pubertal development and reproductive functions.
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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.

Masutomi administered estrogenic chemicals to pregnant and lactating CD-SD (Japan) rats. Bisphenol A was mixed with the feed at concentrations of 60, 600 and 3000 ppm and was provided to maternal CD Sprague-Dawley [Charles River Kanagawa, Japan, Crj: CD(SD)IGS] rats from gestational day (GD) 15 to postnatal day (PND) 10 (gestation doses = 5, 50 and 230, lactation doses = 8, 80 and 380 mg/kg/day). Ethinylestradiol (EE) at 0.5 ppm was used as an estrogenic reference drug (gestation dose 27 and lactation dose 63 µg/kg/day). Among the chemicals MXC alone showed typical estrogenic effects only at the maternally toxic 1200 ppm. The present study was performed to examine the sensitivity of immunohistochemical analysis of pituitary cells of offspring similarly exposed to each chemical for detection of endocrine-disrupting effects. For this purpose, ratios of pituitary cells expressing luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin (PRL), were measured at 3 and 11 weeks of age. Ethinylestradiol (EE) at 0.5 ppm was used as a reference chemical. At week 3, decrease in the relative proportions of LH, FSH, and PRL cells in males and LH cells in females was evident with MXC at 1200 ppm. At week 11, increase was found for PRL cells from 240 ppm MXC, and FSH cells at 1200 ppm in females. On the other hand, EE increased the PRL cell percentage in females at week 3 but no effects were apparent at week 11. The other chemicals were without influence at either time point. The lack of sensitivity of the CD

Sprague-Dawley rat to all of these estrogens is evident. The only significant effect at the lowest doses of BPA (all other doses were in the high-dose range) was s decrease in body weight for males.

Mehmood, Z., A. G. Smith, M. J. Tucker, F. Chuzel and N. G. Carmichael (2000). The development of methods for assessing the in vivo oestrogen-like effects of xenobiotics in CD-1 mice. Food Chem Toxicol 38:493-501.

The increasing awareness and concern about the potential health risks posed to the ecosystem and to man by endocrine disrupting chemicals with oestrogen-like activity in the environment has focused attention on the need for developing sensitive and specific methods for identifying these xenobiotics and to evaluate their degrees of toxic effects. We have conducted dose response studies in immature (21 days old) CD-1 female mice treated with four compounds, diethylstilboestrol (DES) (0.1 microg to 25 mg/kg body weight), alpha-zearalanol (0.5 mg to 25 mg/kg body weight), methoxychlor (0.5 mg to 500 mg/kg body weight) and bisphenol A (10 microg to 100 mg/kg body weight) administered subcutaneously daily for 3 days, and measured a number of uterine markers in treated and control (vehicle treated) mice. These were, in addition to the commonly measured changes in relative uterus weight and histopathological examination of uterine tissue, three other markers indicative of uterotrophic effects, namely, uterine luminal epithelium BrdU labelling index over the last 24 hr, peroxidase activity and lactoferrin expression. All of these markers showed clear dose-related increases in DES- and methoxychlor-treated animals. In the case of alpha-zearalanol treatment, relative uterine weight, peroxidase activity and lactoferrin expression showed dose-related increases at all the doses investigated. BrdU incorporation (an index of cell proliferation) also progressively increased at dose levels ranging from 0.1 mg to 5.0 mg/kg body weight, but apparently decreased at 25 mg/kg body weight. In contrast to these findings, bisphenol-A treatment showed no consistent changes in any of the four markers at the dose levels investigated. Additionally, studies were also conducted on a number of chemicals in CD-1 mice at one dose level. The chemicals investigated were: bisphenol A (1 g/kg body weight/day), naringenin (1 g/kg body weight/day) o,p'-DDT (500 mg/kg body weight/day), genistein (1 g/kg/day), coumestrol (0.5 mg/kg/day) and chlordecone (20 mg/kg/day) administered subcutaneously daily for 3 days. There was some variability in response of the markers perhaps indicating that the chemicals did not all act in the same way. The findings of our exploratory in vivo studies in CD-1 mice suggest that the measurement of a range of uterine markers, in addition to organ weight and histopathology, would provide useful information on the potential oestrogenicity of chemicals.
NOTE: While the magnitude of the increase in uterine weight (expressed as a percent of body weight) was about 4 fold for DES amd methoxychlor, it was only about 20% relative to controls for bisphenol A between 0.01 and 1 mg/kg/dau, after which the response dropped to baseline at 10 and 100 mg/kg/day, forming an inverted-U dose-response curve. If the authors did not conduct a statistical analysis appropriate for these non-monotonic data, the possibility that the results are statistically significant could be missed. Neverrtheless, the magnitude of the uterine response to bisphenol A is a small fraction of the response observed for other chemicals.

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 (this is a very high dose) or 2 mg/kg estradiol benzoate from postnatal Day 1 to 5 was examined after puberty as well as histolopathogic changes in reproductive organs. All male and female rats treated postnatally with estradiol benzoate showed poor reproductive capability, including adverse effects on masculine sexual behavior, and marked histopathologic alterations of the reproductive organs. In addition, estradiol benzoate markedly reduced the volume of the sexually

dimorphic nucleus of the preoptic area (SDN-POA) in males. On the other hand, all male and female rats treated postnatally with bisphenol A showed normal reproductive function and no histopathologic abnormalities of reproductive organs. Bisphenol A did not affect the volume of the SDN-POA. These results indicated that neonatal exposure to estradiol benzoate affects reproductive function in male and female rats, and treatment with bisphenol A at a fairly high dose was ineffective if given postnatally to male and female rats.

Nagao, T., Y. Saito, K. Usumi, S. Yoshimura and H. Ono (2002). Low-dose bisphenol A does not affect reproductive organs in estrogen- sensitive C57BL/6N mice exposed at the sexually mature, juvenile, or embryonic stage. Reprod. Toxicol. **16**:123-30.

In inbred mice (C57BL/6N), bisphenol A was reported to not alter testis, epididymis or seminal vesicle weight at doses of 2, 20 or 200 µg/kg/day administered at different life stages. This finding is interesting in that we have found that C57BL/6J males are 1,000-fold less responsive to the stimulatory effects of fetal DES exposure on prostate size relative to either CF-1 or CD-1 male fetuses, while the effects of DES on the uterus of the female siblings of these males showed an identical response to DES (F. vom Saal, unpublished observation). Our finding, and that of Nagao et al. thus are in contrast to the findings of Spearow (Science 285:1259, 1999), who reported that peripubertal administration of estradiol to C57 mice had a greater suppressing effect on testis relative to CD-1 mice. These findings suggest that the statement by Nagao that C57 mice are "estrogen sensitive" is not accurate for different outcomes at different life stages. Studies to compare the response to different estrogenic chemicals at different life stages in different rat and mouse strains are needed to clarify these diverse findings.

Pickford, D.B., Hetheridge, M.J., Caunter, J.E., Hall, A.T. and Hutchinson, T.H. (2003). Assessing chronic toxicity of bisphenol A to larvae of the African clawed frog (Xenopus laevis) in a flow-through exposure system. Chemisphere 53:223-235.

A number of currently used industrial chemicals are estrogenic, and therefore have potential to disrupt sexual differentiation in vertebrate wildlife during critical developmental windows. We assessed the effect of larval exposure to bisphenol A (BPA) on growth, development and sexual differentiation of the gonad in the African Clawed frog, Xenopus laevis. Larvae were maintained in flow-through conditions at 22 +/- 1 degrees C and exposed to BPA at mean measured concentrations of 0.83, 2.1, 9.5, 23.8, 100, and 497 microg/l, from developmental stages 43/45-66 (completion of metamorphosis). Each test concentration, plus dilution water control (DWC) and positive control (17beta-estradiol (E2), 2.7 microg/l) employed four replicate test vessels with 40 larvae per tank. Individual froglets were removed from test vessels upon reaching stage 66, and the study was terminated at 90 days. Froglets were dissected and sex was determined by inspection of gross gonadal morphology. Test concentrations of BPA had no effect on survival, growth, developmental stage distributions at exposure days 32 and 62, or mean time to completion of metamorphosis, compared to DWC. Analysis of post-metamorphic sex ratio, determined by gross gonadal morphology, indicated no significant deviations from expected (50:50) sex ratio, in DWC or any BPA test concentration. In contrast, exposure of larvae to (E2) resulted in feminisation, with sex ratio deviating significantly (31% male, replicates pooled). Exposure to BPA in the concentration range 0.83-497 microg/l in flow-through conditions had no observable effect on larval growth, development or sexual differentiation (as determined by gross gonadal morphology) in this study.
FUNDED BY THE CHEMICAL INDUSTRY

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.

Bisphenol A and nonylphenol were mixed with diet at concentrations of 60, 600 and 3000 ppm and was provided to maternal (n = 5-6 mothers/group) CD-SD [Charles River Kanagawa, Japan, Crj: CD(SD)IGS] rats from gestational day (GD) 15 to postnatal day (PND) 10 (gestation doses = 5, 50 and 230, lactation doses = 8, 80 and 380 mg/kg/day). Ethinylestradiol (EE) at 0.5 ppm was used as an estrogenic reference drug (gestation dose 27 and lactation dose 63 µg/kg/day). During pregnancy and lactation, including the exposure period, a soy-free rodent diet was provided to eliminate possible modification of the study results by plant-derived phytoestrogens. The animals were housed in polycarbonate cages. Effects on endocrine/reproductive systems were evaluated by examining the anogenital distance, organ weights before puberty, onset of puberty, estrous cyclicity, and organ weights and histopathology of adult endocrine organs (at 11 weeks of age), as well as the volume of the sexually dimorphic nucleus of preoptic area. Both NP and BPA, at high doses, caused decreases in maternal body weights and retardation of offspring growth (reduced body weight), but neither affected any of the endocrine/reproductive endpoints of offspring, whereas EE at about 50 µg/kg/day induced irreversible changes in estrous cyclicity and histopathology of ovaries and uterus of adult females. The results indicated that maternal dietary exposure to NP or BPA at concentrations up to 3000 ppm (230-380 mg/kg/day) from GD 15 through PND 10 do not exert any apparent adverse effects on the endocrine/reproductive systems of offspring.

Tinwell, H., R. Joiner, I. Pate, A. Soames, J. Foster and J. Ashby (2000). Uterotrophic activity of bisphenol A in the immature mouse. Regul Toxicol Pharmacol 32:118-26.

Bisphenol A (BPA) has been evaluated in eight independent immature mouse uterotrophic assays using the subcutaneous route of administration, and in a single study employing oral gavage. The dose range covered was from 0.02 microg to 300 mg/kg BPA and some experiments were supplemented by assessments of uterine hypertrophy and hyperplasia. Pooling of the test data indicates no uterotrophic activity for the chemical. However, in a subset of the subcutaneous injection studies, where control uterine weights were relatively low, significant, but weak, uterotrophic activity was observed over a range of dose levels, but in the complete absence of a dose relationship. In the oral gavage study, no increases in uterine weight were seen, but there were increases in uterine labeling with bromodeoxyuridine at 200-300 mg/kg BPA. The present study illustrated that when a large number of observations are made, a certain level of chance observations may be made, and that surrogates for an increase in uterine weight do not necessarily enhance assay sensitivity, albeit such data may complement uterine weight data. The data indicate that reducing control uterine weights may enhance assay sensitivity, but that animal body weight is an imperfect indicator of control uterine weight. The data also show that it is possible for individual investigators to be unable to confirm their own observations. It is concluded that BPA may be weakly uterotrophic to the mouse under specific conditions of test, and in the complete absence of a dose-response relationship to this activity. However, overall, we have failed to define BPA as reproducibly active in the immature mouse uterotrophic assay, and in that sense, our data are broadly consistent with those reported earlier by Coldham et al. (Environ. Health Perspect. 105, 734-742, 1997) in 1997 using a similar assay.

While there are positive effects reported in this study, the conclusion is that bisphenol A is essentially without effect, which is the consistent message of all studies funded by the chemical industry.

FUNDED BY THE CHEMICAL INDUSTRY

Tinwell, H., J. Haseman, P. A. Lefevre, N. Wallis and J. Ashby (2002). Normal sexual development of two strains of rat exposed in utero to low doses of bisphenol A. Toxicol Sci 68:339-48.

Pregnant Sprague-Dawley (SD) and Alderley Park (Wistar derived) rats were exposed by gavage during gestation days 6-21 to 20 microg/kg, 100 microg/kg, or 50 mg/kg body weight of BPA with ethinylestradiol (EE; 200 microg/kg) acting as a positive control agent. The sexual development of the derived pups was monitored until termination at postnatal day 90-98. The endpoints evaluated were litter size and weight, anogenital distance at birth, days of vaginal opening, first estrus and prepuce separation, weights of the liver, seminal vesicles, epididimydes, testes, ventral prostate, uterus, vagina, cervix and ovaries, and daily sperm production. Males were terminated at postnatal day 90 and females at postnatal day 98. The only statistically significant effects observed for any dose of BPA were a decrease in daily sperm production and an increase in the age of vaginal opening for the Alderley Park animals at the highest dose evaluated (50 mg/kg). The dose of EE evaluated proved to be maternally toxic in our laboratory, but provided gross evidence of endocrine disruption in the treated dams. These results diverge from those of Chahoud and his colleagues who indicated disturbances to the sexual development of both male and female SD rat pups administered the same 3 doses of BPA. This failure to confirm low dose endocrine effects for BPA is discussed within the context of similar divergent conclusions derived from other assessments of the endocrine toxicity of this agent to rats.

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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.

Bisphenol A (BPA) was evaluated at concentrations of 0, 0.015, 0.3, 4.5, 75, 750, and 7500 ppm (approximately 0.001, 0.02, 0.3, 5, 50, and 500 mg/kg/day of BPA) administered in the diet ad libitum to 30 CD((R)) Sprague-Dawley (CD-SD) rats/sex/dose for 3 offspring generations, 1 litter/generation, through F3 adults. Adult systemic toxicity at 750 and 7500 ppm in all generations included: reduced body weights and body weight gains, reduced absolute and increased relative weanling and adult organ weights (liver, kidneys, adrenals, spleen, pituitary, and brain), and female slight/mild renal and hepatic pathology at 7500 ppm. Reproductive organ histopathology and function were unaffected. Ovarian weights as well as total pups and live pups/litter on postnatal day (PND) 0 were decreased at 7500 ppm, which exceeded the adult maximum tolerated dose (MTD). Mating, fertility, gestational indices; ovarian primordial follicle counts; estrous cyclicity; precoital interval; gestational length; offspring sex ratios; postnatal survival; nipple/areolae retention in preweanling males; epididymal sperm number, motility, morphology; daily sperm production (DSP), and efficiency of DSP were all unaffected. At 7500 ppm, vaginal patency (VP) and preputial separation (PPS) were delayed in F1, F2, and F3 offspring, associated with reduced body weights. Anogenital distance (AGD) on PND 0 was unaffected for F2 and F3 males and F3 females (F2 female AGD was increased at some doses, not at 7500 ppm, and was considered not biologically or toxicologically relevant). Adult systemic no observed adverse effect level (NOAEL) = 75 ppm (5 mg/kg/day); reproductive and postnatal NOAELs = 750 ppm (50 mg/kg/day). There were no treatment-related effects in the low-dose region (0.001-5 mg/kg/day) on any parameters and no evidence of nonmonotonic dose-response curves across generations for either sex. BPA should not be considered a selective reproductive toxicant, based on the results of this study.

NOTE: This study also used a type of animal feed (Purina 5002) that contains contaminants that can mask the effects of DES.

FUNDED BY THE CHEMICAL INDUSTRY

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.

This study reports that Charles River Sprague-Dawley derived (CD-SD) strain of rat requires a very high 200 µg/kg/day dose of ethinyl estradiol (the potent estrogenic drug used in birth control pills at a dose of 0.5 µg/kg/day) in order to show a response. This rat is thus extremely insensitive to estrogen relative to women.

Yoshida, M., T. Shimomoto, S. Katashima, G. Watanabe, K. Taya and A. Maekawa (2004). Maternal exposure to low doses of bisphenol a has no effects on development of female reproductive tract and uterine carcinogenesis in Donryu rats. J Reprod Dev 50:349-60.

Effects of maternal exposure to low doses of bisphenol A (BPA), including those comparable with human exposure levels, on growth and development of the female reproductive system and uterine carcinogenesis in Donryu rats were investigated. Dams were administered BPA (0, 0.006 and 6 mg/kg/day) daily by gavage from gestation day 2 up to the day before weaning (postnatal day 21 at offspring). The serum levels of BPA were significantly elevated in the dams receiving 6 mg/kg/day, however, BPA levels in the milk of dams, and those in the serum and liver of offspring were similar between control and treated groups. The treatment did not exert any influences on uterine development including weight, gland genesis and estrogen receptor alpha expression, vaginal opening and gonadotropin secretion in the female offspring up to puberty. After maturation, no effects were evident with regard to estrous cyclicity in female offspring treated with BPA. In addition, the treatment had no effects on age-related morphological changes of the reproductive and endocrine organs and uterine carcinogkenesis until 15 months of age. The results demonstrate that maternal exposure to BPA at levels comparable to human exposure did not have any effects on the female reproductive system of offspring in rats. In addition, BPA was also found in the serum, milk and liver of control dams and pups, and low levels of BPA were detected in drinking water and pellet diet. The present study showed that the experimental animals were also exposed to environmental BPA in the animal room.

Yoshino, H., Ichihara, T., Kawabe, M., Imai, N., Hagiwara, A., Asamoto, M. and Shirai, T. (2002). Lack of significant alteration in the prostate or testis of F344 rat offspring after transplacental and lactational exposure to bisphenol A. J. Toxicol. Sci. 27: 433-439.

Bisphenol A (BPA), a compound of great concern as an estrogenic xenobiotic, was assessed for its ability to cause alteration in the accessory sex organs and spermatogenesis in male offspring exposed preneonatally and neonatally. In a series of experiments focusing on rat sensitivity to gestational and lactational exposure to BPA, we investigated its effects on gestation period and reproductive organs in male offspring. In the first instance, BPA was administered to F344 female rats by gavage at 0, 7.5, 120 mg/kg/day during pregnancy and lactation period. There were no observable adverse effects in pregnant rats and the treatment did not induce any morphological abnormalities in the accessory sex organs of male offspring. However, lowered numbers of sperm in the testis were found with a dose of 120 mg/kg/day. In the second study, the same protocol with a higher number of male offspring was applied, but no reduction in the sperm count was apparent. We conclude that transplacental and lactational exposure to BPA dose not exert any adverse effects on morphogenesis of rat accessory sex organs or spermatogenesis.

Review of the Ema, Tyl and Yamasaki Studies

The male CF-1 mouse used in the vom Saal laboratory studies responds to ethinyl estradiol with significant changes in sperm production and prostate size at a dose of 0.002 µg/kg/day (Thayer, et al. Human Reproduction 16:988, 2001) and is thus 100,000-times more sensitive to ethinyl estradiol than is the CD Sprague Dawley rat used in the studies listed above by: 1. Ema, 2. Tyl, and 3. Yamasaki. Yamasaki also reported that a 600 mg/kg/day dose of bisphenol A was required to see an effect. This dose is approximately 100,000-times higher than the bisphenol A doses used in the studies conducted in the vom Saal laboratory. The insensitivity of the CD Sprague Dawley rat to bisphenol A was predicted by its insensitivity to the positive control drug ethinylestradiol. Only by including in the experiment a positive control estrogenic chemical, such as DES or ethinyl estradiol, can the reason for the failure too find low dose effects of bisphenol A be determined. Since Tyl used this insensitive rat as well as a type of feed (Purina 5002) that Thigpen et al. (2003) reported can mask the effects of estrogenic chemicals, the Tyl study cannot be interpreted as showing that bisphenol is "safe" based on purely negative findings. A positive control (DES or ethinylestradiol) to establish the sensitivity of the animals used in the experiments conducted by Ema and Tyl was not included, but because Yamasaki did include ethinylestradiol as a positive control, the insensitivity of the CD Sprague Dawley rat to any estrogenic chemical, not just bisphenol A, was revealed.

Lack of Antiandrogenic Activity of Bisphenol A in the Hershberger Assay

Kim, H.S., Han, S.Y., Kim, T.S., Kwack, S.J., Lee, R.D., Kim, I.Y., Seok, J., Lee B.M., Yoo, S.D., and Park K.L. (2002) . No androgenic'antiandrogenic effects of bisphenol A in Hershverger assay using immature castrated rats. Toxicology Letters 135: 111-123.

Several studies have demonstrated that bisphenol A (BPA) exhibited weak estrogenic activity in the 3-day uterotrophic assay using ovariectomized (OVX) and immature rats (Toxicol. Lett. 115 (2000) 231; Regul. Toxicol. Pharmacol. 32 (2000) 118; J. Toxicol. Sci. 26 (2001) 111) and BPA also possessed anti-androgenic activity in in vitro yeast based assays (J. Endocrinol. 158 (1998) 327). To investigate anti-androgenic effects of BPA. a rodent Hershberger assay was carried out using immature Sprague-Dawley male rats. An androgen agonist, testosterone (0.4 mg/kg per day), was administered for 7 consecutive days by subcutaneous (s.c.) injection as a positive control. Additionally, a pure androgen antagonist, flutamide (1, 5. 10 mg/kg per day. oral) was co-administered with testosterone (0.4 mg/kg per day s.c.). BPA was also administered orally with or without testosterone (0.4 mg/kg per day, s.c.) for 7 consecutive days. In the testosterone treated groups, glans penis, seminal vesicles, ventral prostate, and levator ani plus bulbocavernosus muscles (LABC) weights were significantly increased compared with control. However. flulamide dose-dependently inhibited the testosterone-induced re-growth of seminal vesicles, ventral prostate, and LABC, with a significant decrease at flutamide 1.0 mg/kg and above ($P<0.05$). Serum LH levels were also significantly increased (5 mg/kg and above, $P<0.05$), but no changes in serum testosterone levels. In contrast, BPA had no effects on the re-growth of seminal vesicles, ventral prostate and LABC induced by testosterone, and no significant differences were observed in serum LH and testosterone levels. In summary, the Hershberger assay could be a sensitive method for detecting androgenic or anti-androgenic chemicals, but BPA did not exhibit any androgenic or anti-androgenic activities in Hershberger assay.

XVI. *IN VITRO* STUDIES OF MOLECULAR MECHANISMS

Ackermann, G.E., Brombacher, E. and Fent, K. (2002). Development of a fish reporter gene system for the assessment of estrogenic compounds and sewage treatment plant effluents. Environ Toxicol Chem 21:1864-1875.

This study reports on the development and application of a fish-specific estrogen-responsive reporter gene assay. The assay is based on the rainbow trout (Oncorhynchus mykiss) gonad cell line RTG-2 in which an acute estrogenic response is created by cotransfecting cultures with an expression vector containing rainbow trout estrogen receptor a complementary DNA (rtERalpha cDNA) in the presence of an estrogen-dependent reporter plasmid and an estrogen receptor (ER) agonist. In a further approach, RTG-2 cells were stably transfected with the rtERalpha cDNA expression vector, and clones responsive to 17beta-estradiol (E2) were selected. The estrogenic activity of E2, 17alpha-ethinylestradiol, 4-nonylphenol, nonylphenoxy acetic acid, 4-tert-octylphenol, bisphenol A, o,p'-DDT, p,p'-DDT, o,p'-2,2-bis(chlorophenyl)-1,1-dichloroethylene (o,p'-DDE), p,p'-DDE, o,p'-2,2-bis(chlorophenyl)-1,1-di-chloroethane (o,p'-DDD), p,p'-DDD, and p,p'-2,2-bis(chlorophenyl)acetic acid (p,p'-DDA) was assessed at increasing concentrations. All compounds except o,p'-DDT, p,p'-DDE, and p,p'-DDA showed logistic dose-response curves, which allowed the calculation of lowest-observed-effect concentrations and the concentrations at which half-maximal reporter gene activities were reached. To check whether estrogen-responsive RTG-2 cells may be used to detect the estrogenic activity of environmental samples, an extract from a sewage treatment plant (STP) effluent was assessed and found to have estrogenic activity corresponding to the transcriptional activity elicited by 0.05 nM of E2. Dose-response curves of nonylphenol, octylphenol, bisphenol A, and o,p'-DDD revealed that the RTG-2 reporter gene assay is more sensitive for these compounds when compared to transfection systems recombinant for mammalian ERs. These differences may have an effect on the calculation of E2 equivalents when estrogenic mixtures of known constitution, or environmental samples, such as STP effluents, are assessed.

Adachi, T., Yasuda, K., Mori, C., Yoshinaga, M., Aoki, N., Tsujimoto, G. and Tsuda, K. (2005). Promoting insulin secretion in pancreatic islets by means of bisphenol A and nonylphenol via intracellular estrogen receptors. Food Chem Toxicol 43:713-719.

In this study, we investigated the effects of endocrine disrupters bisphenol A (BPA) and nonylphenol (NP) on insulin secretion from rat pancreatic islets. Following acute exposure to BPA and NP, neither BPA nor NP (0.1, 1, 10, 100 and 1000 microg/l) affected insulin secretion in concentrations of 16.7 mM glucose. However, insulin secretion following long-term exposure to BPA or NP for 24 h in 16.7 mM glucose was significantly higher than without exposure. To determine whether increased insulin secretion resulting from long-term exposure to BPA and NP is induced via intracellular estrogen receptors, we blocked the cytosolic/nuclear estrogen receptors, using actinomycin-D (Act-D), an inhibitor of RNA synthesis, and ICI 182,780 (ICI), an estrogen receptor inhibitor. Following long-term exposure to BPA (10 microg/l) or NP (10 microg/l), Act-D or ICI treatment eliminated the facilitation of insulin secretion. In conclusion, we have demonstrated for the first time that long-term exposure to endocrine disrupters, such as BPA and NP, promotes in vitro insulin secretion from the pancreatic islets, via cytosolic/nuclear estrogen receptors.

Alonso-Magdalena, P., Laribi, O., Ropero, A.B., Fuentes, E., Ripoll, C., Soria, B. and Nadal, A. (2005). Low doses of bisphenol A and diethylstilbestrol impair Ca2+ signals in pancreatic alpha-cells through a nonclassical membrane estrogen receptor within intact islets of Langerhans. Environ Health Perspect 113:969-977.

Glucagon, secreted from pancreatic alpha-cells integrated within the islets of Langerhans, is involved in the regulation of glucose metabolism by enhancing the synthesis and mobilization of glucose in the liver. In addition, it has other extrahepatic effects ranging from lipolysis in adipose

tissue to the control of satiety in the central nervous system. In this article, we show that the endocrine disruptors bisphenol A (BPA) and diethylstilbestrol (DES), at a concentration of 10(-9) M, suppressed low-glucose-induced intracellular calcium ion ([Ca2+]i) oscillations in alpha-cells, the signal that triggers glucagon secretion. This action has a rapid onset, and it is reproduced by the impermeable molecule estradiol (E2) conjugated to horseradish peroxidase (E-HRP). Competition studies using E-HRP binding in immunocytochemically identified alpha-cells indicate that 17beta-E2, BPA, and DES share a common membrane-binding site whose pharmacologic profile differs from the classical ER. The effects triggered by BPA, DES, and E2 are blocked by the G alpha i- and G alpha o-protein inhibitor pertussis toxin, by the guanylate cyclase-specific inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, and by the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester. The effects are reproduced by 8-bromo-guanosine 3',5'-cyclic monophosphate and suppressed in the presence of the cGMP-dependent protein kinase inhibitor KT-5823. The action of E2, BPA, and DES in pancreatic alpha-cells may explain some of the effects elicited by endocrine disruptors in the metabolism of glucose and lipid.

Alum, A., Y. Yoon, P. Westerhoff and M. Abbaszadegan (2004). Oxidation of bisphenol A, 17beta-estradiol, and 17alpha-ethynyl estradiol and byproduct estrogenicity. Environ. Toxicol. 19:257-64.

This study has implications for experiments in which bisphenol A was added to tap water containing various amounts of chlorine. A human breast cancer cell line (MCF-7) was used to investigate the cumulative estrogenicity profiles elicited during the oxidation of three estrogenic compounds [bisphenol A (BPA), 17beta-estradiol (E2), and 17alpha-ethynyl estradiol (EE2)]. High-performance liquid chromatography (HPLC) with a method detection limit (MDL) of approximately 1 nM was used to measure the initial and final concentrations of test compounds during oxidation. Both chlorination and ozonation removed from 75% to >99% of the test compounds in distilled water. Increasing contact time and chlorination dose improved compound removal. Chlorination byproducts of BPA, E2, and EE2 elicited low levels of estrogenicity over an extended period of time. For equivalent molar oxidant dosages, ozone and chlorine had comparable residual proliferative effect values and >99% loss of the parent compounds. For oxidation studies of estrogenic chemicals, ammonium chloride was found to adequately quench residual chlorine without interfering with cell culture assay. Oxidation of test compounds with chlorine and ozone resulted in a similar estrogenicity trend, with a relative higher level of estrogenicity elicited during the early phases of oxidation, which gradually dissipated over the extended exposure time to a stable point. Oxidation with ozone resulted in the rapid transformation of test compounds, reaching a stabilized estrogenic level in 10 min, whereas for chlorination it took more than 120 min for elicited estrogenicity to stabilize.

Andreescu, S. and O. A. Sadik (2004). Correlation of analyte structures with biosensor responses using the detection of phenolic estrogens as a model. Anal. Chem. 76:552-60.

Bulayeva, N.N. and Watson, C.S. (2004). Xenoestrogen-induced ERK-1 and ERK-2 activation via multiple membrane-initiated signaling pathways. Environ. Health Perspect. 112:1481-1487.

Xenoestrogens can mimic or antagonize the activity of physiological estrogens, and the suggested mechanism of xenoestrogen action involves binding to estrogen receptors (ERs). However, the failure of various in vitro or in vivo assays to show strong genomic activity of xenoestrogens compared with estradiol (E2) makes it difficult to explain their ability to cause abnormalities in animal (and perhaps human) reproductive functions via this pathway of steroid action. E2 has also been shown to initiate rapid intracellular signaling, such as changes in levels of intracellular calcium, cAMP, and nitric oxide, and activations of a variety of kinases, via action at the membrane. In this study, we demonstrate that several xenoestrogens can rapidly activate extracellular-regulated kinases

(ERKs) in the pituitary tumor cell line GH3/B6/F10, which expresses high levels of the membrane receptor for ER-alpha (mER). We tested a phytoestrogen (coumestrol), organochlorine pesticides or their metabolites (endosulfan, dieldrin, and DDE), and detergent by-products of plastics manufacturing (p-nonylphenol and bisphenol A). These xenoestrogens (except bisphenolA) produced rapid (3-30 min after application), concentration (10(-14)-10(-8) M)-dependent ERK-1/2 phosphorylation but with distinctly different activation patterns. To identify signaling pathways involved in ERK activation, we used specific inhibitors of ERs, epidermal growth factor receptors, Ca2+ signaling, Src and phosphoinositide-3 kinases, and a membrane structure disruption agent. Multiple inhibitors blocked ERK activation, suggesting simultaneous use of multiple pathways and complex signaling web interactions. However, inhibitors differentially affected each xenoestrogen response examined. These actions may help to explain the distinct abilities of xenoestrogens to disrupt reproductive functions at low concentrations.

Cappelletti, V., Saturno, G., Miodini, P., Korner, W. and Daidone, M.G. (2003). Selective modulation of ER-beta by estradiol and xenoestrogens in human breast cancer cell lines. 60:567-576.

In the last decades, substances with estrogenic activity have been dispersed into the environment. Xenoestrogens act by binding to estrogen receptors, ligand-regulated transcription factors, for which two subtypes have been described, ER-alpha and ER-beta, which are often coexpressed at variable amounts in different tissues. We investigated variations in the expression of ER-alpha and ER-beta mRNAs following treatment with four xenoestrogens (bisphenol A, 4-tert octylphenol, 2-hydroxybiphenyl, 4-hydroxybiphenyl) and with 17beta-estradiol in estrogen-sensitive (T47D) and estrogen-insensitive (BT20) breast cancer cell lines. Although to a variable extent, both estradiol and the tested xenoestrogens increased the expression of ER-beta mRNA, whereas a slight effect on ER-alpha was observed only in T47D cells. Upregulation of ER-beta expression by estradiol and xenoestrogens was observed only in the presence of detectable ER-alpha protein levels. These findings indicate a regulatory role for ER-beta in ER-alpha-mediated transcription and a role for ER-beta in mediating xenoestrogen toxicity.

Diel, P., Olff, S., Schmidt, S. and Michna, H. (2002). Effects of the environmental estrogens bisphenol A, o,p'-DDT, p-tert-octylphenol and coumestrol on apoptosis induction, cell proliferation and the expression of estrogen sensitive molecular parameters in the human breast cancer cell line MCF-7. J. Steroid Biochem. Mol. Biol. 80:61-70.

In the presented study, we have analysed effects of the environmental estrogens bisphenol A (BPA), p-tert-octylphenol (OCT), o,p'-DDT (DDT) and coumestrol (COU) on cell proliferation, apoptosis induction, progesterone receptor (PR) and androgen receptor (AR) mRNA expression and ER alpha protein expression in comparison to estradiol (E2) and the selective ER modulator (SERM) raloxifene (RAL) and the pure antiestrogen faslodex (ICI 182780) in the human breast cancer cell line MCF-7. A dose dependent analysis of the cell cycle distribution of MCF-7 cells after administration of OCT, DDT and COU revealed a significant induction of cell proliferation and reduced rate of apoptosis. Maximum induction of cell proliferation and the lowest rate of apoptosis could be observed at a dose of 10(-6)M. Interestingly, administration of BPA reduces the rate of apoptosis, but does not enhance proliferation at any dose analysed. PR mRNA expression in MCF-7 cells was up regulated after administration of COU and DDT, whereas treatment with BPA and OCT did not effect PR mRNA expression. AR mRNA expression was down regulated by COU, but not effected by BPA, DDT and OCT. The expression of ER alpha protein in the breast cancer cells was slightly down regulated by COU and DDT, but unaffected by BPA and OCT. In summary and in comparison to the effects observed after administration of E2, RAL and ICI our data indicate that none of the analysed compounds exhibit properties comparable to RAL and ICI. COU and DDT

exhibit properties which are very similar to E2. Administration of BPA and OCT did not effect any of the estrogen sensitive molecular parameters analysed. Nevertheless OCT is a very potent stimulator of cell proliferation in MCF-7 cells. Surprisingly, BPA is not able to induce the proliferation of MCF-7 breast cancer cells, but turns out to be a very potent inhibitor of apoptosis. For this reason and in agreement to the effects of BPA on the molecular parameters analysed, we conclude that BPA does not act in a classical estrogen like manner in MCF-7 breast cancer cells.

Fiorini, C., Tilloy-Ellul, A., Chevalier, S., Charuel, C. and Pointis, G. (2004). Sertoli cell junctional proteins as early targets for different classes of reproductive toxicants. Reprod. Toxicol. 18:413-421.

In the testis, Sertoli cells establish intercellular junctions that are essential for spermatogenesis. The SerW3 Sertoli cell line displays some features of native Sertoli cells. Western blot and immunofluorescence analyses showed that SerW3 Sertoli cells expressed typical components of tight (occludin and zonula occludens-1), anchoring (N-cadherin) and gap (connexin 43) junctions. Testicular toxicants (DDT, pentachlorophenol, dieldrin, dinitrobenzene, cadmium chloride, cisplatin, gossypol, bisphenol A and tert-octylphenol) affected intercellular junctions by either reducing the amount or inducing aberrant intracellular localization of these membranous proteins. Phosphodiesterase inhibitors (isobutyl methylxantine, rolipram, zaprinast, zardaverine) did not alter junctional-complex component levels but caused a rapid and reversible redistribution of these proteins to the cytoplasmic compartment. The present study showed that occludin, ZO-1, N-cadherin and specifically Cx43 could be early targets for testicular toxicants. The SerW3 cell line therefore appears as a useful in vitro model to evaluate molecules with potential anti-reproductive effects.

Goto, M., H. Ono, Y. Takano-Ishikawa, H. Shinmoto and M. Yoshida (2004). Mac1 positive cells are required for enhancement of splenocytes proliferation caused by bisphenol a. Biosci. Biotechnol. Biochem. 68:263-5.

Effects of bisphenol A (BPA) on immune cells, and it was shown that BPA upregulated the proliferation of murine splenocytes stimulated with Concanavalin A (ConA). The upregulating effects of BPA were removed with depleting Mac1+ cells from the splenocytes. This study provides evidence that Mac1+ cells were required for enhancement of splenocytes proliferation caused by bisphenol A.

Gould, J.C., Leonared, L.S., Maness, S.C., Wagner, B.L., Conner, K., Zacharewski, T., Safe, T., McDonnell, D.P., and Gaido, K.W. (1998). Bisphenol A interacts with the estrogen receptor α in a distinct manner from estradiol. Molecular and Cellular Endocrinology 142:203-214.

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

Recently, significant concerns have been placed on the widespread use of chemicals with persistent estrogenic activity for their long-term effects on human health. In this communication, we investigated whether fetal exposure to some of these chemicals at doses consumed by people, has any long-term effect on the reproductive functions of the male offspring. Thus, time-pregnant CD-1 mice were fed diethylstilbestrol (DES), bisphenol A (BPA), and aroclor (aroclor 1016) at an average concentration of 100 ng/kg/day, 50 microg/kg/day, and 50 microg/kg/day, respectively, during Days 16-18 of gestation. A high dose of DES (200 microg/kg/day) was also tested to compare the results of the current study with those of others using the high dose only. The offspring were examined at Day 3, Day 21, and Day 60 following birth. We demonstrated that BPA, aroclor, and the lower dose of DES enhanced anogenital distance, increased prostate size, and decreased epididymal weight. No

effect was found on the testicular weight or size. The chemicals also permanently increased androgen receptor (AR) binding activity of the prostate at this dosage. This is the first demonstration that environmental chemicals program AR function permanently at the dosage consumed by the general population. The higher dosage of DES, on the other hand, produced an opposite effect, decreasing prostate weight, prostate AR binding, and anogenital distance, thus confirming the previous reports. To investigate whether the above mentioned effects of the chemicals represent direct or indirect effects, we also tested the effect of the chemicals on prostate development in vitro. Thus fetal urogenital sinus (UGS), isolated at the 17th day of gestation was cultured with the chemicals in the presence and absence of testosterone (10 ng/ml) for 6 days, and prostate growth was monitored by determining the size and branching of the specimen following histology. Results showed that these chemicals induced prostate growth in the presence and absence of testosterone. They also increased androgen-binding activity. Thus, the results of the in vivo studies were reproduced in the in vitro experiments, suggesting a direct effect of these chemicals on the development of fetal reproductive organs. This is the first demonstration that estrogenic chemicals induce reproductive malformation by direct interference with the fetal reproductive organs and not by interfering with the maternal or fetal endocrine system. The chemicals are able to induce malformation even in the absence of fetal testosterone; however, they are more effective in the presence of testosterone.

Gupta, C. (2000). The role of estrogen receptor, androgen receptor and growth factors in diethylstilbestrol-induced programming of prostate differentiation. Urologic Res. 4:223-229.

Recently, others and we have demonstrated that prenatal exposure to an extremely low dose of diethylstilbestrol (DES) and other estrogenic compounds produces a significant effect on mouse prostate development in vivo and in vitro in the presence and absence of androgen. In this study, we investigated the mechanism by which DES produces this effect and determined the role of its estrogenic activity on the growth and branching, induced by DES in the 17-day-old fetal prostate in culture. Additionally, we investigated whether the androgen receptor (AR) plays a role and whether any of the growth factors, namely, EGF and IGF-1 which are known to modulate the estrogen receptor (ER) and androgen receptor (AR)-dependent process, mediate the DES-induced effects. Using the organ culture bioassay of prostate development, we demonstrate that DES enhanced the growth and branching of the prostate at both 0.1 and 0.5 pg/ml dosages, thus, confirming a previous report of ours. An anti-estrogen, ICI164,387 blocked both of the effect of DES, suggesting that both of these two effects are ER dependent. Anti-androgen, flutamide also blocked both branching and prostatic growth induced by DES, while cyproterone acetate blocked only the branching effect, suggesting a role for AR in the DES-induced effects. Depletion of EGF by anti-EGF antibody blocked the DES-induced effects and this was reversed following EGF replacement in the organ culture system. Anti-IGF-1 antibody, on the other hand, only blocked the branching effect, but produced no effect on the prostatic growth, induced by DES. Estrogenic chemicals, bisphenol A and DES enhanced EGF-mRNA level of the cultured prostates. Taken together, it appears that DES-induced prostatic enlargement involves enhancement of ER-dependent EGF and IGF-1 synthesis, mediating prostatic enlargement and androgen action.

Han, D., Denison, M.S., Tachibana, H. and Yamada, K. (2002). Effects of estrogenic compounds on immunoglobulin production by mouse splenocytes. Biol Pharm Bull 25:1263-1267.

The effects of culture medium and serum components on immunoglobulin (Ig) production by mouse splenocytes were examined. In this study, we showed that culture medium containing charcoal-dextran-treated fetal bovine serum (CDFBS) supported Ig production more efficiently than culture medium containing FBS. In addition, RPMI 1640 medium supported Ig production more efficiently than Dulbecco modified Eagle medium. In addition, an increase in medium IgA

production was observed with the increase in both CDFBS and FBS concentrations, whereas an increase in IgM level was observed in the presence of 5% and 10% FBS and 10% CDFBS. Dose-dependent effects of estrogenic compounds on Ig production were also examined. We found that 17beta-estradiol induced increases in IgM and IgE levels, but diethylstilbestrol enhanced only the IgE level. On the other hand, tamoxifen and bisphenol A enhanced medium IgM level at physiological concentrations. Among flavonoids, daidzein enhanced IgM and IgE levels at concentrations above 10 microM, and genistein induced a decrease in IgM level and an increase in IgE level at concentrations above 10 microm. In addition, quercetin and luteolin enhanced medium IgE level at all concentrations tested, whereas IgA, IgG, and IgM levels were not affected. These results suggest that environmental estrogens affect Ig production of mouse Splenocytes in a complex and class-specific manner.

Iida, H., Maehara, K., Doiguchi, M., Mori, T. and Yamada, F. (2003). Bisphenol A-induced apoptosis of cultured rat Sertoli cells. Reprod. Toxicol. 17:457-464.

Bisphenol A (BPA) was examined for its effects on cultured Sertoli cells established from 18-day-old rat testes. We demonstrated that exposure of cultured Sertoli cells to BPA decreased the cell viability in a dose- and a time-dependent manner and that exposure to BPA brought about morphologic changes of the cells, such as membrane blebs, cell rounding, cytoskeletal collapse, and chromatin condensation or fragmentation, all of which conform to the morphologic criteria for apoptosis. Immunocytochemistry showed that active caspase-3, a major execution caspase, was expressed in round Sertoli cells positively labeled by the TUNEL method. Co-localization of active caspase-3 and aggregated actin fragments was also observed in the round Sertoli cells. Theses results suggest that BPA induces cell death of Sertoli cells by promoting apoptosis. Apoptosis-inducing cell death was observed in cells exposed to 150-200 microM BPA, while BPA at <100 microM had only slight cytotoxic effects on the cells.

Inoue, A., Hayashi, S., Aoyagi, K., Nishigaki, M., Sasaki, H. and Kiyama, R. (2002). A reporter gene assay for evaluation of tissue-specific responses to estrogens based on the differential use of promoters A to F of the human estrogen receptor alpha gene. J Pharmacol Toxicol Methods 47:129-135.

INTRODUCTION: Reporter gene assays are useful means for monitoring cellular responses. We report here a reporter gene assay for evaluating and monitoring estrogen activities by estrogen-like compounds and xenoestrogens, which is based on the promoters from the human estrogen receptor alpha (ERalpha) gene. METHODS: The reporter gene constructs contained a proximal promoter region (containing promoters A and B: ProAB) or either of promoters C to F (ProC, ProD, ProE, and ProF) or fused minor promoters (ProCDEF). These constructs were first used to evaluate promoter activity in cell lines derived from breast (MCF-7 and T-47D cells), ovary (SK-OV-3 cells), endometrium (Ishikawa cells), and stomach (MKN-28 cells). RESULTS: Besides very high levels of activity by ProAB in all of the cell lines tested, moderate levels were detected for ProD in the breast and endometrium cell lines and for ProF in the ovary and endometrium cell lines. A moderate level of activity by ProE was detected only in the stomach cells. Differences in estrogen-like activity between ProAB and ProD were observed for tamoxifen and bisphenol A (BPA) in MCF-7 cells. DISCUSSION: The assay proposed here might provide expression profiles of cancer cells of various origins for evaluating the estrogen responsiveness and for identifying tissue- or cancer cell-specific transcription factors.

Ishihara, A., N. Nishiyama, S. Sugiyama and K. Yamauchi (2003). The effect of endocrine disrupting chemicals on thyroid hormone binding to Japanese quail transthyretin and thyroid hormone receptor. Gen. Comp. Endocrinol. 134:36-43.

A number of chemicals were examined for effects on 3,3('),5-L-[125I]triiodothyronine ([125I]T(3)) binding to thyroid binding proteins in human plasma and to purified Japanese quail transthyretin (qTTR), a major thyroid hormone-binding protein in plasma, and to the ligand-binding domain of thyroid hormone receptor beta (qTR LBD). Bisphenol A was a more potent inhibitor of T3 binding in human serum than DES (doses tested were about 100 µM). Scatchard plots of T(3) binding to qTTR and qTR LBD revealed two classes of binding sites, with K(d) values of 6.9 and 185nM, and a single class of binding sites, with K(d) value of 0.31nM, respectively. Among the test chemicals, DES was the most powerful inhibitor of [125I]T(3) binding to qTTR (IC(50)<0.4nM). DES, ioxynil (IC(50)=1.1+/-0.5nM) and pentachlorophenol (IC(50)=6.3+/-3.8nM) displaced [125I]T(3) from qTTR more effectively than unlabeled T(3) (IC(50)= 9.7+/-0.9nM) did. Although malathion, 4-nonylphenol, bisphenol A and n-butylbenzyl phthalate were effective inhibitors of [125I]T(3) binding to qTTR, their potency was two orders of magnitude less than that of T(3). All test chemicals except for diethylstilbestrol had either a weak or no effect on [125I]T(3) binding to qTR LBD. These results show that several EDCs tested in this study target the plasma thyroid hormone binding protein qTTR rather than the nuclear receptor qTR LBD.

James, S.Y., Lin, F., Kolluri, S.K., Dawson, M.I. and Zhang, X.K. (2003). Regulation of retinoic acid receptor beta expression by peroxisome proliferator-activated receptor gamma ligands in cancer cells. Cancer Res. 63:3531-3538.

The peroxisome proliferator-activated receptor gamma (PPAR gamma) is a nuclear receptor family member that can form a heterodimeric complex with retinoid X receptor (RXR) and initiate transcription of target genes. In this study, we have examined the effects of the PPAR gamma ligand ciglitazone and the RXR ligand SR11237 on growth and induction of retinoic acid receptor (RAR) beta expression in breast and lung cancer cells. Our results demonstrated that ciglitazone and SR11237 cooperatively inhibited the growth of ZR-75-1 and T-47D breast cancer and Calu-6 lung cancer cells. Gel shift analysis indicated that PPAR gamma, in the presence of RXR, formed a strong complex with a retinoic acid response element (beta retinoic acid response element) in the RAR beta promoter. In reporter gene assays, RXR ligands and ciglitazone, but not the PPAR gamma ligand 15d-PGJ(2), cooperatively promoted the transcriptional activity of the beta retinoic acid response element. Ciglitazone, but not 15d-PGJ(2), strongly induced RAR beta expression in human breast and lung cancer cell lines when used together with SR11237. The induction of RAR beta expression by the ciglitazone and SR11237 combination was diminished by a PPAR gamma-selective antagonist, bisphenol A diglycidyl ether. All-trans-retinoic acid or the combination of ciglitazone and SR11237 was able to induce RAR beta in all-trans-retinoic acid-resistant MDA-MB-231 breast cancer cells only when the orphan receptor chick ovalbumin upstream promoter transcription factor was expressed, or in the presence of the histone deacetylase inhibitor trichostatin A. These studies indicate the existence of a novel RAR beta-mediated signaling pathway of PPAR gamma action, which may provide a molecular basis for developing novel therapies involving RXR and PPAR gamma ligands in potentiating antitumor responses.

Jin, H. and Audus, K.L. (2005). Effect of bisphenol A on drug efflux in BeWo, a human trophoblast-like cell line. Placenta 26 Suppl A:S96-S103.

Bisphenol A (BPA) is a monomer of polycarbonate plastics that has estrogenic activities and has been shown to be a substrate for multidrug resistant efflux mechanisms, specifically, P-glycoprotein. Since the natural hormone estrogen reverses multidrug resistance in some cell types, we

hypothesized that BPA might have a similar activity in trophoblasts. We have used BeWo cells as an in vitro model for human trophoblasts and calcein AM as a substrate for drug efflux mechanism to characterize BPA interactions with placental P-glycoprotein. We found that chronic exposure of BeWo cells to BPA did not alter intracellular calcein accumulation in a fashion that would be reflective of changes in P-glycoprotein expression. Immunoblots affirmed that BPA had small effects on P-glycoprotein expression. However, BeWo cells acutely exposed to BPA pretreatment were observed to have a significantly decreased calcein accumulation. Addition of cyclosporin A, a P-glycoprotein inhibitor and substrate, completely reversed BPA's effects on calcein accumulation and resulted in a net increase, relative to controls, in calcein accumulation by the BeWo cells. BPA was found not to stimulate P-gp ATPase or alter intracellular esterases mediating calcein release from calcein AM. Therefore, our results suggested that BPA stimulated drug efflux by BeWo cells probably by direct effects on P-glycoprotein.

Kanno, S., Hirano, S. and Kayama, F. (2004). Effects of phytoestrogens and environmental estrogens on osteoblastic differentiation in MC3T3-E1 cells. Toxicol. 196:137-145.

Phytoestrogens and environmental estrogens, which have in part some structural similarity to 17beta-estradiol, are reported to act as agonists/antagonists of estrogen in animals and humans. Estrogen is known to play an important role in maintaining bone mass, since the concentration of serum estrogen decreases after menopause and the estrogen deficiency results in bone loss. In this study, we report the effects of phytoestrogens (genistein, daidzein, and coumestrol) and environmental estrogens (bisphenol A (BPA), p-n-nonylphenol (NP) and bis(2-ethylhexyl)phthalate (DEHP)) on osteoblast differentiation using MC3T3-E1 cells, a mouse calvaria osteoblast-like cell line. Coumestrol (10(-10) to 10(-6)M) slightly enhanced cell proliferation, while neither the other phytoestrogens (daidzein, genistein) nor environmental estrogens increased cell proliferation. Alkaline phosphatase (ALP) activity and cellular calcium (Ca) and phosphorus (P) contents were increased by phytoestrogens and BPA; however, neither NP nor DEHP affected those osteoblastic indicators. The effects of estrogenic potency, using the cell proliferation of MCF-7 cells, an estrogen receptor (ER)-positive human breast cancer cell line, indicate that coumestrol has the highest estrogenic potency among those phytoestrogens and environmental estrogens. The estrogenic potency of NP and DEHP were lower than the others. In conclusion, phytoestrogens, such as coumestrol, genistein and daidzein, and BPA increased ALP activity and enhanced bone mineralization in MC3T3-E1 cells, suggesting that not only phytoestrogen but also BPA, an environmental estrogen, is implicated in bone metabolism.

Kester, M.H.A., van Toor, S.B.H., Tibboel, D., Meinl, W., Glatt. H., Falany, C..N., Coughtrie, W.H., Schuur. A.G., Brouwer, A., Visser, T.J. (2002). Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters. J. Clin. Endocrinol. Metab. 87:1142-1150.

Estrogen sulfotransferase (SULT1E1) hydroxylates E2 and thus deactivates it. Marked inhibition of SULT1E1 takes place at subnanomolar concentrations by a number of polyhalogenated aromatic hydrocarbons including BPA at uMs and tetrabrominated and tetrachlorinated congeners (3,3'5,5') at 60nM. These researchers offer the hypothesis that inhibition of E2 hydroxylation contributes to the toxicity of these chemicals.

Kim, J.Y. and Jeong, H.G. (2003). Down-regulation of inducible nitric oxide synthase and tumor necrosis factor-alpha expression by bisphenol A via nuclear factor-kappaB inactivation in macrophages. Cancer Lett 196:69-76.

Bisphenol A [BPA, 2,2bis(4hydroxyphenyl)propane] is reported to have estrogenic activity; however, its influence on cytokine production or immune system function remains unclear. In this study, we investigated the effects of BPA on the production of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-alpha), and on the level of inducible nitric oxide synthase (iNOS) and TNF-alpha gene expression in mouse macrophages. BPA alone did not affect NO or TNF-alpha production. In contrast, BPA inhibited lipopolysaccharide (LPS)-induced NO and TNF-alpha production, and the levels of iNOS and TNF-alpha mRNA in a dose-dependent manner. Treatment with ICI 182.780, an estrogenreceptor antagonist, inhibited the suppressive effects of BPA. Transient expression and electrophoretic mobility shift assays with NF-kappaB binding sites revealed that BPA reduced the levels of the LPS-induced NF-kappaB transcription factor complex. These results demonstrate that BPA may affect the regulation of the immune system function by reducing NO and TNF-alpha production via the inhibition of NF-kappaB transactivation mediated through the estradiol receptor.

Kitamura, S., Suzuki, T., Sanoh, S., Kohta, R., Jinno, N., Sugihara, K., Yoshihara, S., Fujimoto, N., Watanabe, H. and Ohta, S. (2005). Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. Toxicol Sci 84:249-59.

The endocrine-disrupting activities of bisphenol A (BPA) and 19 related compounds were comparatively examined by means of different in vitro and in vivo reporter assays. BPA and some related compounds exhibited estrogenic activity in human breast cancer cell line MCF-7, but there were remarkable differences in activity. Tetrachlorobisphenol A (TCBPA) showed the highest activity, followed by bisphenol B, BPA, and tetramethylbisphenol A (TMBPA); 2,2-bis(4-hydroxyphenyl)-1-propanol, 1,1-bis(4-hydroxyphenyl)propionic acid and 2,2-diphenylpropane showed little or no activity. Anti-estrogenic activity against 17beta-estradiol was observed with TMBPA and tetrabromobisphenol A (TBBPA). TCBPA, TBBPA, and BPA gave positive responses in the in vivo uterotrophic assay using ovariectomized mice. In contrast, BPA and some related compounds showed significant inhibitory effects on the androgenic activity of 5alpha-dihydrotestosterone in mouse fibroblast cell line NIH3T3. TMBPA showed the highest antagonistic activity, followed by bisphenol AF, bisphenol AD, bisphenol B, and BPA. However, TBBPA, TCBPA, and 2,2-diphenylpropane were inactive. TBBPA, TCBPA, TMBPA, and 3,3'-dimethylbisphenol A exhibited significant thyroid hormonal activity towards rat pituitary cell line GH3, which releases growth hormone in a thyroid hormone-dependent manner. However, BPA and other derivatives did not show such activity. The results suggest that the 4-hydroxyl group of the A-phenyl ring and the B-phenyl ring of BPA derivatives are required for these hormonal activities, and substituents at the 3,5-positions of the phenyl rings and the bridging alkyl moiety markedly influence the activities.

Kubo, T.; Maezawa, N.; Osada, M.; Katsumura, S.; Funae, Y., and Imaoka, S. (2004). Bisphenol A, an environmental endocrine-disrupting chemical, inhibits hypoxic response via degradation of hypoxia-inducible factor 1alpha (HIF-1alpha): structural requirement of bisphenol A for degradation of HIF-1alpha. Biochem Biophys Res Commun. 318(4):1006-11.

Bisphenol A (BpA), an endocrine-disrupting chemical, is known to be a xenoestrogen and to affect the reproductive functions of animals. Recent reports have documented BpA-induced developmental abnormalities in the neuronal systems of humans and animals, and these effects appear to be non-estrogenic. In this study, we found that BpA inhibited the hypoxic response of human hepatoma cells. The expression of hypoxic response genes such as the erythropoietin (EPO) gene is done via a hypoxia inducible factor 1 (HIF-1)-dependent signaling pathway. To investigate possible structural requirements for this inhibitory effect, several BpA analogs were synthesized and added to this system. The blocking of two phenol groups in BpA did not change the effect, but the

inhibition completely disappeared by the removal of two central methyl groups in BpA (the resulting compound is designated BpF). BpA, but not BpF, promoted degradation of the HIF-1alpha protein, which is a component of HIF-1, followed by inhibition of EPO induction. An immunoprecipitation assay indicated that BpA dissociated heat shock protein 90 (Hsp90) from HIF-1alpha and destabilized HIF-1alpha protein. HIF-1alpha is usually degraded first by ubiquitination and then by the proteasome pathway. Cobalt ion inhibits ubiquitination of HIF-1alpha and stabilizes it. In the present study, BpA promoted HIF-1alpha degradation in the presence of cobalt and in the presence of proteasome inhibitor. These results suggest that BpA degraded HIF-1alpha via a currently unknown pathway, and that this phenomenon required two methyl groups in BpA.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S. and Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. 138, 863-870.

The rat estrogen receptor (ER) exists as two subtypes, ER alpha and ER beta, which differ in the C-terminal ligand binding domain and in the N-terminal transactivation domain. In this study we investigated the messenger RNA expression of both ER subtypes in rat tissues by RT-PCR and compared the ligand binding specificity of the ER subtypes. Saturation ligand binding analysis of in vitro synthesized human ER alpha and rat ER beta protein revealed a single binding component for 16 alpha-iodo-17 beta-estradiol with high affinity [dissociation constant (Kd) = 0.1 nM for ER alpha protein and 0.4 nM for ER beta protein]. Most estrogenic substances or estrogenic antagonists compete with 16 alpha-[125I]iodo-17 beta-estradiol for binding to both ER subtypes in a very similar preference and degree; that is, diethylstilbestrol > hexestrol > dienestrol > 4-OH-tamoxifen > 17 beta-estradiol > coumestrol, ICI-164384 > estrone, 17 alpha-estradiol > nafoxidine, moxestrol > clomifene > estriol, 4-OH-estradiol > tamoxifen, 2-OH-estradiol, 5-androstene-3 beta, 17 beta-diol, genistein for the ER alpha protein and dienestrol > 4-OH-tamoxifen > diethylstilbestrol > hexestrol > coumestrol, ICI-164384 > 17 beta-estradiol > estrone, genistein > estriol > nafoxidine, 5-androstene-3 beta, 17 beta-diol > 17 alpha-estradiol, clomifene, 2-OH-estradiol > 4-OH-estradiol, tamoxifen, moxestrol for the ER beta protein. The rat tissue distribution and/or the relative level of ER alpha and ER beta expression seems to be quite different, i.e. moderate to high expression in uterus, testis, pituitary, ovary, kidney, epididymis, and adrenal for ER alpha and prostate, ovary, lung, bladder, brain, uterus, and testis for ER beta. The described differences between the ER subtypes in relative ligand binding affinity and tissue distribution could contribute to the selective action of ER agonists and antagonists in different tissues.

Kurosawa, T., H. Hiroi, O. Tsutsumi, T. Ishikawa, Y. Osuga, T. Fujiwara, S. Inoue, M. Muramatsu, M. Momoeda and Y. Taketani (2002). The activity of bisphenol A depends on both the estrogen receptor subtype and the cell type. 49: 465-71.

Bisphenol A (BPA), a monomer of plastic used in consumer products, is abundant in the environment and enters the body by ingestion or adsorption. In order to characterize the estrogenic effect of BPA, we performed luciferase assay on three independent cell lines derived from different tissues transfected with either human ERalpha cDNA or ERbeta cDNA. The estrogenic activities of BPA were detectable in all cell lines via both ERalpha and ERbeta. In 293T cells and Hec-1 cells, the estrogenic activities were significantly decreased when cells expressing ERalpha were incubated with 10(-6) M BPA in the presence of 10(-8) M 17beta-estradiol (E2) while the activities via ERbeta were essentially unchanged in the same conditions. Interestingly, no reduction of estrogenic activity was detected in HOS-TE85 cells via either ERalpha or ERbeta. Our results indicate that BPA only acts as an agonist of estrogen via ERbeta whereas it has dual actions as an agonist and antagonist in some

types of cells via ERalpha. Thus, the activity of BPA may depend on the ER subtype and the tissue involved.

Lee, B.-C., Kamata, M., Akatsuka, Y., Takeda, M., Ohno, K., Kamei, T. and Magara, Y. (2004). Effects of chlorine on the decrease of estrogenic chemicals. Water Research 38:733-739.

The effects of chlorination on the elimination of three estrogenic chemicals such as 17beta-estradiol, nonylphenol and bis-phenol A were investigated using yeast two-hybrid assay (YTA), estrogen receptor (ER) competition assay (ER-CA), and high-performance liquid chromatography/mass spectrometry (LC/MS). The results of YTA, ER-CA and the analysis of LC/MS indicated that the estrogenic activity of the above-mentioned three endocrine disruptors were significantly reduced as a result of chlorination. The decrease in estrogenic activity paralleled a decrease in estrogenic chemicals under the influence of free chlorine. One common characteristic of estrogenic chemicals is the presence of a phenolic ring. Considering that a phenolic ring is likely to undergo some sort of transformation in an aqueous chlorination solution, the above-mentioned results may be applied to the rest of the estrogenic chemicals in natural waters.

Lee, H. J., S. Chattopadhyay, E. Y. Gong, R. S. Ahn and K. Lee (2003). Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. 75:40-6.

This study used the ARhLBD-activating signal cointegrator 1 (ASC1) yeast two-hybrid system, which reflects the androgen-dependent interaction between androgen receptor (AR) and its coactivator, ASC1. Both bisphenol A and nonylphenol acted as potent AR antagonists comparable to a known strong antagonist, cyproterone acetate. Ligand competition assays revealed that [3H]5alpha-dihydroxytestosterone (DHT) binding to AR is inhibited a maximum of 30 and 40% at approximately 5 nM of nonylphenol and 50 nM 11.5 ng/ml; ppb) of bisphenol A, respectively (50% competition, or the IC50, was 80 nM bisphenol A). In addition, the nuclear translocation of green fluorescent protein (GFP)-AR fusion protein in the presence of testosterone was affected by the addition of bisphenol A and nonylphenol, which cause rather dispersed distribution of GFP-AR between the nuclear and the cytoplasmic compartments. Furthermore, in transient transfection assays, both chemicals inhibited androgen-induced AR transcriptional activity. Taken together, the results suggest that bisphenol A and nonylphenol affect multiple steps of the activation and function of AR, thereby inhibiting the binding of native androgens to AR, AR nuclear localization, AR interaction with its coregulator, and its subsequent transactivation. These effects of bisphenol A are occurring in the low ng/ml (ppb) range and are thus of concern based on the ppb levels being reported in human blood. Bisphenol A also has anti-thyroid hormone activity (Moriyama et al., 2002; Ishihara et al., 2003). Thus, in addition to acting as an estrogem-mimicking chemical, bisphenol A can block the action of testosterone and thyroid hormone in cells. Paris et al (22002) also showed that BPA had antiandrogenic activity.

Lee, M.S., Hyun, S.H., Lee, C.K., Im, K.S., Hwang, I.T. and Lee, H.J. (2003). Impact of xenoestrogens on the growth of human endometrial epithelial cells in a primary culture system. Fertil Steril 79:1464-1465.

Lee, M.H., Chung, S.W., Kang, B.Y., Park, J., Lee, C.H., Hwang, S.Y. and Kim, T.S. (2003). Enhanced interleukin-4 production in CD4+ T cells and elevated immunoglobulin E levels in antigen-primed mice by bisphenol A and nonylphenol, endocrine disruptors: involvement of nuclear factor-AT and Ca2+. Immunol. 109:76-86.

Bisphenol A (BPA) and p-nonylphenol (NP) are representative endocrine disruptors (EDs) that may have adverse effects on human health. The influence of these compounds on allergic immune responses remains unclear. In this study, we have examined the effects of BPA and NP on

production of interleukin-4 (IL-4), a pro-inflammatory cytokine closely associated with allergic immune responses. Both BPA and NP significantly enhanced IL-4 production in keyhole limpet haemocyanin (KLH)-primed CD4+ T cells in a concentration-dependent manner. Treatment with BPA or NP in vivo resulted in significant increase of IL-4 production in CD4+ T cells and of antigen-specific immunoglobulin E (IgE) levels in the sera of KLH-primed mice. Furthermore, BPA and NP enhanced the activation of IL-4 gene promoter in EL4 T cells transiently transfected with IL-4 promoter/reporter constructs, and the enhancing effect mapped to a region in the IL-4 promoter containing binding sites for nuclear factor (NF)-AT. Activation of T lymphocytes by phorbol 12-myristate 13-acetate/ionomycin resulted in markedly enhanced binding activities to the NF-AT site, which significantly increased upon addition of BPA or NP, as demonstrated by the electrophoretic mobility shift assay, indicating that the transcription factor NF-AT was involved in the enhancing effect of BPA and NP on IL-4 production. The enhancement of IL-4 production by BPA or NP was significantly reduced by nitrendipine, a blocker of Ca2+ influx, and by FK506, a calcineurin inhibitor. FK506 inhibited the NF-AT-DNA binding activity and IL-4 gene promoter activity enhanced by BPA or NP. These results represent the first report describing possible enhancement of allergic response by EDs through increasing IL-4 production in CD4+ T cells and antigen-specific IgE levels in the sera via the stimulation of Ca2+/calcineurin-dependent NF-AT activation.

Letcher, R.J., Sanderson, J.T., Bokkers, A., Giesy, J.P. and van den Berg, M. (2005). Effects of bisphenol A-related diphenylalkanes on vitellogenin production in male carp (Cyprinus carpio) hepatocytes and aromatase (CYP19) activity in human H295r adrenocortical carcinoma cells. Toxicol. Appl. Pharmacol. Online: May 2005.

The present study investigated the effects of the known xenoestrogen bisphenol A (BPA) relative to eight BPA-related diphenylalkanes on estrogen receptor (ER)-mediated vitellogenin (vtg) production in hepatocytes from male carp (Cyprinus carpio), and on aromatase (CYP19) activity in the human adrenocortical H295R carcinoma cell line. Of the eight diphenylalkanes, only 4,4'-(hexafluoropropylidene)diphenol (BHF) and 2,2'-bis(4-hydroxy-3-methylphenyl)propane (BPRO) induced vtg, i.e., to a maximum of 3% to 4% (at 100 muM) compared with 8% for BPA relative to the maximum induction by 17beta-estradiol (E2, 1 muM). Bisphenol A diglycidyl ether (BADGE) was a potent antagonist of vtg production with an IC50 of 5.5 muM, virtually 100% inhibition of vtg at 20 muM, and an inhibitive (IC50) potency about one-tenth that of the known ER antagonist tamoxifen (IC50, 0.6 muM). 2,2'-Diallyl bisphenol A, 4,4'-(1,4-phenylene-diisopropylidene)bisphenol, BPRO, and BHF were much less inhibitory with IC50 concentrations of 20-70 muM, and relative potencies of 0.03 and 0.009 with tamoxifen. Bisphenol ethoxylate showed no anti-estrogenicity (up to 100 muM), and 4,4'-isopropylidene-diphenol diacetate was only antagonistic at 100 muM. When comparing the (anti)estrogenic potencies of these bisphenol A analogues/diphenylalkanes, anti-estrogenicity occurred at lower concentrations than estrogenicity. 4,4'-Isopropylidenebis(2,6-dimethylphenol) (IC50, 2.0 muM) reduced E2-induced (EC50, 100 nM) vtg production due to concentration-dependent cytotoxicity as indicated by a parallel decrease in MTT activity and vtg, whereas the remaining diphenylalkanes did not cause any cytotoxicity relative to controls. None of the diphenylalkanes (up to 100 muM) induced EROD activity indicating that concentration-dependent, CYP1A enzyme-mediated metabolism of E2, or any Ah-receptor-mediated interaction with the ER, was not a likely explanation for the observed anti-estrogenic effects. At concentrations as great as 100 muM, none of the diphenylalkanes directly inhibited aromatase (CYP19) activity in H295R cells. Environmental exposure of fish to BPA and related diphenylalkanes, depending on the structure, may pose anti-estrogenic, and to a lesser extent estrogenic, risks to development and reproduction.

Masuno, H., T. Kidani, K. Sekiya, K. Sakayama, T. Shiosaka, H. Yamamoto and K. Honda (2002). Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. J. Lipid Res. **43**: 676-684.

Bisphenol A induced fibroblasts (3T3-L1 cells) to differentiate into adipocytes in cell culture when tested at a dose of 20 µg/ml. In combination with insulin (5 µg/ml), bisphenol A dramatically increased lipoprotein lipase activity and increased triacylglycerol (TG) content in cells (uptake of TG is regulated by lipoprotein lipase). These findings suggest that bisphenol A can increase body fat mass, which is also suggested by the findings of Howdeshell et al. (1999) in mice exposed prenatally to a very low dose of bisphenol A, as well as findings by others: Ashby et al., 1999; Takai et al., 2000; Rubin et al. 2001; Akingbemi et al., 2004.

Masuno, H., Iwanami, J., Kidani, T., Sakayama, K. and Honda, K. (2005). Bisphenol A accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway. Toxicol Sci 84:319-27.

In order to identify whether bisphenol A (BPA) acts as an adipogenic agent, following the hormonal induction of differentiation into adipocytes, 3T3-L1 cells were treated for six days with BPA alone. Treatment with BPA increased the triacylglycerol (TG) content of the cultures, increased the percentage of Oil Red O-staining cells in the cultures, and increased the levels of lipoprotein lipase (LPL) and adipocyte-specific fatty acid binding protein (aP2) mRNAs. These findings indicate that BPA was able to accelerate terminal differentiation of 3T3-L1 cells into adipocytes. LY294002, a chemical inhibitor of phosphatidylinositol 3-kinase (PI 3-kinase), blocked completely the increasing effect of BPA on TG accumulation and expression of LPL and aP2 mRNAs. Western blot analysis revealed that BPA increased the level of phosphorylated Akt kinase. Based on these findings, we concluded that BPA acted through the PI 3-kinase and Akt kinase pathway, resulting in increased TG accumulation and expression of adipocyte genes. The structure-activity relationship for BPA-related chemicals was examined. Eight derivatives of BPA (three diphenylalkanes with different substituents at the central carbon atom, three diphenylalkanes with ester bonds on hydroxyl groups in the phenolic rings, one bisphenol consisting of a sulphur atom at the central position, one chemical with cyanic groups, instead of hydroxyl groups, in the phenolic rings) accelerated terminal adipocyte differentiation and their potencies to increase TG accumulation were 73-97% of that of BPA. Two diphenylalkanes with ether bonds on hydroxyl groups and two alkylphenols (4-nonylphenol and 4-tert-octylphenol) did not have the ability to accelerate terminal adipocyte differentiation.

Masuyama, H. and Hiramatsu, Y. (2004). Involvement of suppressor for Gal 1 in the ubiquitin/proteasome-mediated degradation of estrogen receptors. J Biol Chem 279:12020-12026.

The proteasome-mediated pathway involves the degradation of several nuclear receptors. Previously we demonstrated that the interaction between the suppressor for Gal 1 (SUG1) and nuclear receptors, the vitamin D receptor, or the pregnane X receptor was involved in proteasome-mediated degradation. In our recent experiments, we examined the potential role of SUG1 in the proteasome-mediated degradation of estrogen receptors (ER)alpha and -beta. Both ERs interacted with SUG1 in a ligand-dependent manner. Functionally, the overexpression of SUG1 inhibited both ERalpha- and ERbeta-mediated transcription in the presence of ligands. Transient expression studies demonstrated that the overexpression of wild-type SUG1 generated proteolytic fragments of both ERs and that these products were blocked by a proteasome inhibitor. The overexpression of SUG1 also enhanced the formation of ubiquitinated proteins of both ERs in the presence of ligand. On the other hand, bisphenol A (BSA), which activated ER-mediated transcription, did not enhance the interaction between ERbeta and SUG1. Furthermore, the degradation of ERbeta was much slower in the presence of BSA than in the presence of estradiol or phthalate, which is another endocrine-

disrupting chemical. Also, BSA had no effect on the formation of proteolytic fragments of ERbeta, and neither did it have any effect on the ubiquitination of ERbeta. These findings indicate that the ubiquitin/proteasome-mediated degradation of both ER proteins may involve the interaction of SUG1 with both ERs. Moreover, BSA strongly blocked the ubiquitination and degradation of ERbeta compared with estradiol, suggesting that BSA may affect the ERbeta-mediated transcription of target genes by inhibiting ERbeta degradation.

Matthews, J. B., K. Twomey and T. R. Zacharewski (2001). In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. Chemical Research in Toxicology **14**(2): 149-157.

Bisphenol A ERß binding affinity 38-fold greater than ERα.

Meerts, I. A. T. M., R. J. Letcher, S. Hoving, G. Marsh, A. Bergman, J. G. Lemmen, B. van der Burg and A. Brouwer (2001). In vitro estrogenicity of polybrominated diphenyl ethers, hydroxylated PBDEs and polybrominated bisphenol A compounds. Environ. Health Perspect. **109**:399-407.

Moriyama, K., T. Tagami, T. Akamizu, T. Usui, M. Saljo, N. Kanamoto, Y. Hataya, A. Shimatsu, H. Kuzuya and K. Nakao (2002). Thyroid hormone action is disrupted by bisphenol A as an antagonist. Jounral of Clinical Endocrinology and Metabolism 87:5185-5190.

Bisphenol A (BPA), a monomer of polycarbonate plastics, has been shown to possess estrogenic properties and act as an agonist for the estrogen receptors. Although an epidemiologically based investigation has suggested that some chemicals could disrupt thyroid function in animals, the effects on thyroid hormone receptors (TRs) are unknown. We show here that BPA inhibits TR-mediated transcription by acting as an antagonist. In the transient gene expression experiments, BPA suppressed transcriptional activity that is stimulated by thyroid hormone (T(3)) in a dose-dependent manner. The inhibitory effects were observed in the presence of physiological concentrations of T(3). In contrast, in the case of negatively regulated TSHalpha promoter, BPA activated the gene transcription that is suppressed by T(3). To elucidate possible mechanisms of the antagonistic action of BPA, the effects on T(3) binding and cofactor interaction with TR were examined. The K(i) value for BPA was 200 micro M when assessed by inhibition of [(125)I]T(3) binding to rat hepatic nuclear TRs. In a mammalian two-hybrid assay, BPA recruited the nuclear corepressor to the TR. These results suggest that BPA could displace T(3) from the TR and recruit a transcriptional repressor, resulting in gene suppression. This is the first report that BPA can antagonize T(3) action at the transcriptional level. BPA may disrupt the function of various types of nuclear hormone receptors and their cofactors to disturb our internal hormonal environment.

Mueller, S.O., Kling, M., Arifin Firzani, P., Mecky, A., Duranti, E., Shields-Botella, J., Delansorne, R., Broschard, T. and Kramer, P.J. (2003). Activation of estrogen receptor alpha and ERbeta by 4-methylbenzylidene-camphor in human and rat cells: comparison with phyto- and xenoestrogens. Toxicol Lett 142:89-101.

4-Methylbenzylidene-camphor (4-MBC) is an organic sunscreen that protects against UV radiation and may therefore help in the prevention of skin cancer. Recent results on the estrogenicity of 4-MBC have raised concerns about a potential of 4-MBC to act as an endocrine disruptor. Here, we investigated the direct interaction of 4-MBC with estrogen receptor (ER) alpha and ERbeta in a series of studies including receptor binding, ER transactivation and functional tests in human and rat cells. 4-MBC induced alkaline phosphatase activity, a surrogate marker for estrogenic activity, in human endometrial Ishikawa cells. Interestingly, 4-MBC induced weakly ERalpha and with a higher potency ERbeta mediated transactivation in Ishikawa cells at doses more than 1 microM, but showed

no distinct binding affinity to ERalpha or ERbeta. In addition, 4-MBC was an effective antagonist for ERalpha and ERbeta. In an attempt to put 4-MBC's estrogenic activity into perspective we compared binding affinity and potency to activate ER with phyto- and xenoestrogens. 4-MBC showed lower estrogenic potency than genistein, coumestrol, resveratrol, bisphenol A and also camphor. Analysis of a potential metabolic activation of 4-MBC that could account for 4-MBC's more distinct estrogenic effects observed in vivo revealed that no estrogenic metabolites of 4-MBC are formed in primary rat or human hepatocytes. In conclusion, we were able to show that 4-MBC is able to induce ERalpha and ERbeta activity. However, for a hazard assessment of 4-MBC's estrogenic effects, the very high doses of 4-MBC required to elicit the reported effects, its anti-estrogenic properties as well as its low estrogenic potency compared to phytoestrogens and camphor has to be taken into account.

Nakagawa, Y., Suzuki, T., Nakagawa, Y. and Suzuki, T. (2001). Metabolism of bisphenol A in isolated rat hepatocytes and oestrogenic activity of a hydroxylated metabolite in MCF-7 human breast cancer cells. Xenobiotica 31:113-123.

1. The metabolites of bisphenol A (BPA; 2, 2-bis(4-hydroxyphenyl)propane) in freshly isolated rat hepatocytes and the oestrogenic activities of BPA and its metabolites, particularly 3-hydroxybisphenol A (3-OH-BPA), in MCF-7 cells and competitive binding assays have been studied, respectively.2. During a 2-h incubation, almost all of the BPA (0.25 mM) added to the hepatocyte suspensions was rapidly converted to a major conjugate, monoglucuronide (approximately 75% of total metabolites), and two minor conjugates, which were tentatively identified as monosulphates of BPA and a hydroxylated intermediate, 3-OH-BPA, as determined by mass spectroscopy coupled with HPLC or GC/MS. On the other hand, free 3-OH-BPA was identified as a trace metabolite, whose level was approximately 1 or 2 muM at 1 h in hepatocyte suspensions treated with 0.25 or 0.5 mM BPA, respectively.3. In another experiment, 3-OH-BPA as well as BPA displaced competitively 17 beta -oestradiol bound to the recombinant human oestrogen receptor alpha in a concentration dependent-manner: IC50 of diethylstilbestrol, BPA and 3-OH-BPA were approximately 2.5 x 10(-8), 10(-5) and 5 x 10(-5) M, respectively. Further, BPA and 3-OH-BPA at intermediate concentrations (10(-7)-10(-6) M) caused proliferation of MCF-7 human breast cancer cells, whereas the effect of BPA was more potent than that of 3-OH-BPA. At higher concentrations, both BPA (> 10(-4) M) and 3-OH-BPA (> 10(-5) M) were cytotoxic.4. Based on the proliferative potency in MCF-7 cells and the IC50 for the competitive binding, the oestrogenic activity of 3-OH-BPA was less than that of BPA. These results indicate that BPA itself rather than its metabolite acts as a xeno-oestrogen and that 3-OH-BPA is cytotoxic, possibly acting via reactive semiquinone and/or quinone metabolites, rather than a xeno-oestrogenic mechanism, in MCF-7 cells.

Nagel, S.C., vom Saal, F.S., Thayer, K.A., Dhar, M.G., Boechler, M. And Welshons, W.V. (1997). Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol. Environ. Health Perspect. 105:70-76.

The lower binding of bisphenol A in plasma relative to estradiol predicted a greater potency of bisphenol A in vivo than predicted, particularly in fetuses when plasma binding restricts estrogen entry into tissues. As predicted, administration to pregnant CF-1 mice of low doses (2 and 20 µg/kg/day) of bisphenol A resulted in prostate enlargement in male offspring.

Nikula, H., T. Talonpoika, M. Kaleva and J. Toppari (1999). Inhibition of hCG stimulated steroidogenesis in cultured mouse leydig cells by bisphenol A and octylphenols. Toxicol. Appl. Pharmacol. 157: 166-173.

Niwa, T., M. Fujimoto, K. Kishimoto, Y. Yabusaki, F. Ishibashi and M. Katagiri (2001). Metabolism and interaction of bisphenol A in human hepatic cytochrome P450 and steroidogenic CYP17. Biol. Pharm. Bull. 24(9): 1064-7.

The metabolism of bisphenol A (BPA) was determined for 11 forms of human hepatic cytochromes P450 (CYPs) expressed in the yeast Saccharomyces cerevisiae and for human steroidogenic CYP17 expressed in Escherichia coli. Additionally, the effect of BPA on the progesterone 17alpha-chydroxylase activity of CYP17 was investigated. CYP2C18 catalyzed BPA metabolism most efficiently, followed by CYP2C19 and CYP2C9. CYP2C9 and CYP2C18 exhibited the highest affinity (Km=3.9 microM) for BPA metabolism. The Vmax of CYP2C18 (8.10 nmol x min(-1) x nmol CYP(-1)) was 5 times higher than that of CYP2C9. Although the Vmax of CYP2C19 was 1.5 times higher than that of CYP2C18, the affinity of CYP2C19 was 12 times lower than that of CYP2C9 and CYP2C18. Therefore the intrinsic clearance (Vmax/Km) of CYP2C18 was more than 5 times higher than that of CYP2C9 and CYP2C19. On the other hand, BPA exhibited a competitive-type inhibition of the progesterone 17alpha-hydroxylase activity of CYP17 with a Ki value of 71 microM, whereas no metabolism of BPA by CYP17 was detected. These results suggest that BPA is mainly metabolized by the CYP2C subfamily in human liver, and that BPA inhibits human steroidogenic CYP17 activities.

Niwa, T., M. Tsutsui, K. Kishimoto, Y. Yabusaki, F. Ishibashi and M. Katagiri (2000). Inhibition of drug-metabolizing enzyme activity in human hepatic cytochrome P450s by bisphenol A. Biol. Pharm. Bull. 23:498-501.

Effect of bisphenol A on drug-metabolizing enzyme activities by human hepatic cytochrome P450s (CYP) was investigated. We measured aminopyrine N-demethylation by eleven kinds of cDNA-expressed CYPs. CYP2C19 and CYP2B6 catalyzed most efficiently the aminopyrine N-demethylation, followed by CYP2C8 and CYP2D6. Bisphenol A (1 mM) most efficiently inhibited aminopyrine N-demethylation by CYP2C8 and CYP2C19 by 82% and 85%, respectively, whereas inhibition of the activities by CYP 2B6 and 2D6 was less than 40%. Bisphenol A exhibited a noncompetitive-type inhibition of aminopyrine N-demethylase activity by CYP2C8 with Ki value of 97 microM. Additionally, we investigated the inhibitory effect of bisphenol A on CYP2C19-mediated S-mephenytoin 4-hydroxylation. Bisphenol A exhibited a mixed-type inhibition with Ki value of 113 microM. These results suggest that bisphenol A inhibits human hepatic CYP activities, especially CYP2C8 and CYP2C19.

Paris, F., P. Balaguer, B. Terouanne, N. Servant, C. Lacoste, J. P. Cravedi, J. C. Nicolas and C. Sultan (2002). Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines. Mol. Cell Endocrinol. 193(1-2): 43-49.

We previously demonstrated the interactions of different chemical compounds with estrogen receptors ERalpha and ERbeta and the androgen receptor (AR) using different reporter cell lines. In this study, we characterize the ERalpha, ERbeta and AR activity of different biphenyls using the same tools. We provide evidence that several phenyl derivatives present both estrogenic and antiandrogenic activity. The extent of hydroxylation and the position of the hydroxyl function were important in determining their estrogenicity and antiandrogenicity. Of the tested compounds, bisphenol-A and 4,4' biphenol had very high estrogenic activity, although it was lower than that of the strong estrogenic alkylphenol, 4-tert-octylphenol. Bisphenol-A and 4,4' biphenol were able to activate ERs at concentrations lower than 1 microM, whereas the other compounds only activated at concentrations above 1 microM. Interestingly, 4,4' biphenol was a better agonist for ERbeta than for ERalpha. No androgenic activity was detected for any of these compounds. Bisphenol-A, 3-OH

phenylphenol, 4-OH phenylphenol and 4,4' biphenol exhibited antiandrogenic activity close to that of 4-tert-octylphenol (IC(50) approximately 5 microM). In whole cell binding assays, these compounds displaced [3H] R1881 with Ki = 10 microM. Although these Ki values seem high in comparison with that of hydroxyflutamide (0.4 microM), one must keep in mind that environmental chemicals can accumulate in adipose tissues for several years. In conclusion, these environmental chemicals may have a negative impact on androgen action during fetal and post-natal life.

Quesada, I., E. Fuentes, M. C. Viso-Leon, B. Soria, C. Ripoll and A. Nadal (2002). Low doses of the endocrine disruptor bisphenol-A and the native hormone 17beta-estradiol rapidly activate transcription factor CREB. FASEB. J. 16(12): 1671-3.

Pancreatic ß cells were examined using 228 pg/ml (1nM) bisphenol A and estradiol (282 pg/ml, 1nM) administered for 5 min for effects on phosphorylation of the transcription factor CREB following depolarization of the cell membrane and opening of Ca++ channels and a rapid influx into the cell of calcium. Bisphenol A and estradiol had the same stimulating effect on phosphorylation of CREB and calcium uptake, in that they potentiated the effect of glucose as a stimulator of CREB phosphorylation. The estrogen receptor antagonist ICI 182,780 had no effect on this response while estradiol conjugated to peroxidase (with limited capacity to enter cells) caused the response, suggesting a membrane-receptor mediates the response rather than the classical genomic ER.

Recchia AG, Vivacqua A, Gabriele S, Carpino A, Fasanella G, Rago V, Bonofiglio D, Maggiolini M. 2004. Xenoestrogens and the induction of proliferative effects in breast cancer cells via direct activation of oestrogen receptor alpha. Food Additives & Contaminants 21:134-144.

Environmental contamination with a variety of industrial products has been associated with developmental and reproductive abnormalities in wildlife species. Increasing evidence has suggested that bisphenol A (BPA) and 4-nonylphenol (NPH), two major endocrine-disrupting chemicals, might be responsible for adverse effects on humans as a consequence of ubiquitous use together with potential oestrogen-like activity. To provide insight into the oestrogen-like nature of BPA and NPH, their ability to activate a reporter gene construct via an oestrogen response element in the hormone-dependent breast cancer cell lines MCF7 and T47D was ascertained. Both compounds transactivated the endogenous oestrogen receptor (ER) alpha in a direct fashion since the anti-oestrogen 4-hydroxytamoxifen abolished the response. In addition, using steroid-receptor-negative HeLa cells engineered to express ERalpha and ER beta and the hormone-binding domains of both ERalpha and ER beta , BPA and NPH confirmed the direct transcriptional activity. Interestingly these properties were supported in MCF7 cells by the ability to autoregulate ERalpha expression as well as to induce its nuclear compartmentalization. We therefore evaluated by reverse transcriptase polymerase chain reaction the expression of oestrogen-controlled genes such as cathepsin D and TFF1 (formerly pS2), which were increased by both chemicals tested. The agonistic effects exhibited in all assays performed prompted the evaluation of a more complex biological response such as the proliferation of MCF7 and T47D cells. The same concentration of xenoestrogens eliciting substantial transcriptional activity significantly stimulated the proliferation of both breast cancer cell lines, although with a reduced effectiveness with respect to the natural hormone 17beta-oestradiol. The results indicate that the biological action of environmental oestrogen such as BPA and NPH should be taken into account for the potential impact on human disease-like hormone-dependent breast cancer. However, further studies are needed to clarify their bioavailability and metabolism as well as whether compound mixtures could produce noticeable effects by synergistic activity.

Routhledge, E.J., White, R., Parker, M.G. and Sumpter, J.P. (2000). Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) and ERß. J. Biol. Chem. 46:35986-35993.

It has been proposed that tissue-specific estrogenic and/or antiestrogenic actions of certain xenoestrogens may be associated with alterations in the tertiary structure of estrogen receptor (ER) alpha and/or ERbeta following ligand binding; changes which are sensed by cellular factors (coactivators) required for normal gene expression. However, it is still unclear whether xenoestrogens affect the normal behavior of ERalpha and/or ERbeta subsequent to receptor binding. In view of the wide range of structural forms now recognized to mimic the actions of the natural estrogens, we have assessed the ability of ERalpha and ERbeta to recruit TIF2 and SRC-1a in the presence of 17beta-estradiol, genistein, diethylstilbestrol, 4-tert-octylphenol, 2',3',4', 5'-tetrachlorobiphenyl-ol, and bisphenol A. We show that ligand-dependent differences exist in the ability of ERalpha and ERbeta to bind coactivator proteins in vitro, despite the similarity in binding affinity of the various ligands for both ER subtypes. The enhanced ability of ERbeta (over ERalpha) to recruit coactivators in the presence of xenoestrogens was consistent with a greater ability of ERbeta to potentiate reporter gene activity in transiently transfected HeLa cells expressing SRC-1e and TIF2. We conclude that ligand-dependent differences in the ability of ERalpha and ERbeta to recruit coactivator proteins may contribute to the complex tissue-dependent agonistic/antagonistic responses observed with certain xenoestrogens. In summary, HeLa cells were transfected with ERα and ERß, and bisphenol A showed a 10-fold greater binding affinity (relative to estradiol) for ERß than ERα. Binding of bisphenol A to ERß but not ERα also resulted in recruitment of the ER coactivator TIF2 with a potency relative to estradiol of 0.05.

Roy, P., H. Salminen, P. Koskimies, J. Simola, A. Smeds, P. Saukko and I. T. Huhtaniemi (2004). Screening of some anti-androgenic endocrine disruptors using a recombinant cell-based in vitro bioassay. J. Steroid Biochem. Mol. Biol. 88:157-66.

The Chinese hamster ovarian cell line (CHO K1) in the 96-well format were cotransfected with plasmids encoding mouse mammary tumour virus-neomycin-luciferase and human androgen receptor (hAR), and a stable cell line was established which stably expressed both the hAR and the androgen-responsive luciferase reporter. Stimulation of the cells with androgens for 24h resulted in about 15-fold stimulation of luciferase activity, with the minimum effective dose of testosterone being 0.1nmol/l. Potent steroidal and non-steroidal anti-androgens, such as hydroxyflutamide and cyproterone acetate, significantly inhibited the androgen-induced transactivation. Non-androgenic steroids like estradiol, progesterone, dexamethasone and cortisol showed weak activity at high concentrations. About 60 different chemicals (mostly pesticides or their metabolites, and common industrial chemicals) were screened with the cell line for their ability to stimulate luciferase activity or inhibit that evoked by 0.1nmol/l R1881, used as a positive androgenic control. About 10 highly potent anti-androgenic chemicals were identified. The most potent anti-androgenic compounds identified included bisphenol A (IC-50 = 19.6 µmol/l), alpha-hexachlorocyclohexane (7.7 µmol/l), vinclozolin (3.9 µmol/l) and 4,4-DDE (20.9 µmol/l). These compounds had alone either no effect or were weak agonists (with cytotoxic effects at very high concentrations), but none showed any significant agonistic activity.

Sakurai, K., M. Kawazuma, T. Adachi, T. Harigaya, Y. Saito, N. Hashimoto and C. Mori (2004). Bisphenol A affects glucose transport in mouse 3T3-F442A adipocytes. Br. J. Pharmacol. 141(2): 209-214.

At doses between 1-100 µM bisphenol A stimulated an increase in the glucose transporter (GLUT-4) and glucose uptake into 3T3-F442A adiposities in cell culture. Interestingly, this effect of

bisphenol A was not inhibited by the estrogen receptor antagonist ICI 182,780, revealing that this effect is not mediated by nuclear estrogen receptors.

Sato, K., Matsuki, N., Ohno, Y. and Nakazzawa, K. (2002). Effects of 17beta-estradiol and xenoestrogens on the neuronal survival in an organotypic hippocampal culture. Neuroendocrinol. 76:223-234.

Bisphenol A a showed a markedly lower affinity to estrogen receptors relative to estradiol, ethinylestradiol or DES, but all chemicals showed the same maximal effects on neuronal survival at 1 nM (230 ppt bisphenol A), suggesting that effects are not mediated through the classical estrogen receptors. This hypothesis is supported by the finding that at 1 nM estradiol and bisphenol A equally increased expression of N-methyl-D-aspartate receptors and increased dendritic spine density in the hippocampal CA3 neurons.

Schrader, T.J., Langlois, I., Soper, K. and Cherry, W. (2002). Mutagenicity of bisphenol A (4,4'-isopropylidenediphenol) in vitro: effects of nitrosylation. Teratog. Carcinog. Mutagen. 22:425-441.

Bisphenol A (4,4'-isopropylidenediphenol) is a common component of polycarbonate plastics and epoxy resins. Since bisphenol A-containing plastics and resins have found uses in food-contact items, its potential migration into foodstuffs and possible health consequences have been the focus of many recent studies. However, the potential mutagenic activation of bisphenol A by nitrosylation has received little attention. Incubation of bisphenol A with sodium nitrite under acidic conditions produced a yellow-brown product. When nitrosylated bisphenol A was tested in the Ames Salmonella/microsome assay at 100 ng to 1 mg/plate, dose-dependent increases in mutagenicity were found in both TA98 and TA100 Salmonella strains. These results indicated the presence of a direct-acting mutagenic activity causing both frameshift and base pair mutations, respectively. When compared to colony formation in untreated controls, the addition of rat liver S9 for metabolic activation had little influence on revertant colony formation. Unreacted bisphenol A dissolved in DMSO, acidic buffer, or inactivated nitrosylation solution showed negligible mutagenicity. When the nature of the mutagenic changes was examined using the Ames II trade mark Assay, a variety of base pair changes was found including T:A to A:T - S9, G:C to A:T +/- S9,C:G to A:T +/- S9 and C:G to G:C +/- S9. Bisphenol A also induced frameshift mutations at G:C sites. In addition, the presence of electrophiles was shown by the production of an intensely coloured orange-red product upon incubation of nitrosylated bisphenol A with the nucleophile 4-(4'-nitrobenzyl)pyridine. These findings suggest that migration of bisphenol A into nitrite containing foodstuffs, or its ingestion in the presence of nitrite, could lead to the formation of mutagenic compounds.

Seidlova-Wuttke, D., H. Jarry and W. Wuttke (2004). Pure estrogenic effect of benzophenone-2 (BP2) but not of bisphenol A (BPA) and dibutylphtalate (DBP) in uterus, vagina and bone. Toxicology 205:103-12.

Contradictory results whether the endocrine disrupters (ED) benzophenone-2 (BP2), bisphenol A (BPA) and dibutylphtalate (DBP) exert estrogenic effects have been published. Selective estrogen receptor modulators (SERMs) exert estrogenic effects in some but not in all organs and ED may be SERMs. Therefore, we studied their binding properties to recombinant ERalpha and ERbeta protein and their effects in the uterus, vagina and bone of ovariectomized rats. BP2 bound to both receptor subtypes, while BPA had a relatively high ERbeta selectivity. DBP did not bind to ERalpha but with a low affinity to ERbeta. In the uterus, only E(2) and BP2 increased uterine weight and the complement C3 but decreased ERbeta gene expression. Discrete effects of BPA and DBP in the uterus were found upon histological examination. In the vagina, BP2 but not BPA and DBP had clear estrogenic effects. E(2) and BP2 had antiosteoporotic effects in the metaphysis of the tibia. The

serum surrogate parameters of bone metabolism, i.e. osteocalcin and the cross (rat) laps were significantly reduced by E(2), an effect shared with BP2 but not by the two other EDs. The conclusion: BP2 acts as ERalpha and ERbeta agonist mimicking effects of E(2), while the effects of BPA and DBP are not pure estrogenic.

Seiwa, C., J. Nakahara, T. Komiyama, Y. Katsu, T. Iguchi and H. Asou (2004). Bisphenol A exerts thyroid-hormone-like effects on mouse oligodendrocyte precursor cells. Neuroendocrinology 80:21-30.

We report studies on the mechanism of action of bisphenol A (BPA) on the differentiation of oligodendrocyte precursor cells (OPCs). Our results show that: (1) BPA inhibits the differentiation of OPCs induced by exposure to thyroid hormone (T3). (2) The effect is mediated through various mechanisms via the thyroid hormone receptor (TRbeta1) which is considered to be responsible for OPC differentiation. (3) The action of BPA on OPC differentiation does not involve the FcRgamma-Fyn-myelin basic protein (MBP) cascade as an inducer of OPC differentiation nor does it suppress CREB phosphorylation, which is considered to be induced by the T3-TR complex. (4) The presence of MBP isoforms (21.5, 18.5, 17.0 and 14.0 kDa) was detected in OPCs, and the expression of exon 2-containing isoforms (i.e. 17.0 and 21.5 kDa) was upregulated upon treatment with T3. In contrast, expression of MBP was inhibited by BPA.

Singleton, D. W., Y. Feng, Y. Chen, S. J. Busch, A. V. Lee, A. Puga and S. A. Khan (2004). Bisphenol-A and estradiol exert novel gene regulation in human MCF-7 derived breast cancer cells. Mol Cell Endocrinol 221:47-55.

Xenoestrogens such as bisphenol-A (BPA) can mimic endogenous 17beta-estradiol (E2) in vitro and in vivo through binding the estrogen receptor (ER), and modulating target gene expression. In the present study, we compared global gene regulation by BPA and E2 in estrogen responsive (ERalpha-HA) human breast cancer cells derived from the MCF-7 cell line. The ERalpha-HA cells (stably over-expressing ERalpha) were exposed to E2 (10(-8)M) or BPA (10(-6)M), for 3h followed by analysis of global gene expression. More than 40 transcripts were significantly changed in ERalpha-HA cells, with many being unique to BPA. At least 15 genes were modulated by BPA in the ER-null C4-12 cell line, indicating ER independent activity. Utilizing quantitative reverse transcription-polymerase chain reaction (RT-PCR), we confirmed BPA and E2 mediated regulation of four selected genes. A consensus Alu-type estrogen responsive element (ERE) was found in the Wiskott-Aldrich syndrome protein (WASP) gene, which conferred responsiveness to BPA and E2 in a reporter gene assay. Significant stimulation was seen only in ERalpha expressing cells, thus indicating a functional ERE. Taken together these data illustrate novel gene regulation by BPA and E2, which has implications for in vivo actions and previous reports of additive and synergistic effects on breast cancer cell growth.

Singleton, D.W., Feng, Y., Yang, J., Puga, A., Lee, A.V. and Khan, S.A. (2005). Gene expression profiling reveals novel regulation by bisphenol-A in estrogen receptor-alpha-positive human cells. Environ Res. Online: Nov 2005 www.sciencedirect.com.

Bisphenol-A (BPA) shows proliferative actions in uterus and mammary glands and may influence the development of male and female reproductive tracts in utero or during early postnatal life. Because of its ability to function as an estrogen receptor (ER) agonist, BPA has the potential to disrupt normal endocrine signaling through regulation of ER target genes. Some genes are regulated by both estradiol (E2) and BPA, but those exclusive to either agent have not been described. Using a yeast strain incorporating a vitellogenin A2 ERE-LacZ reporter gene into the genome, we found that BPA (BPA was examined at 1 μM and E2 at 10 nM concentrations) induced expression of the

reporter in colonies transformed with the ERalpha expression plasmid, illustrating BPA-mediated regulation within a chromatin context. Additionally, a reporter gene transiently transfected into the endometrial cancer (Ishikawa) cell line also showed BPA activity, although at 100-fold less potency than E2. To compare global gene expression in response to BPA and E2, we used a variant of the MCF-7 breast cancer cell line stably expressing HA-tagged ERalpha. Cultures were treated for 3h with an ethanol vehicle, E2 (10(-8)M), or BPA (10(-6)M), followed by isolation of RNA and microarray analysis with the human U95A probe array (Affymetrix, Santa Clara, CA, USA). More than 300 genes were changed 2-fold or more by either or both agents, with roughly half being up-regulated and half down-regulated. A number of growth- and development-related genes, such as HOXC1 and C6, Wnt5A, Frizzled, TGFbeta-2, and STAT inhibitor 2, were found to be affected exclusively by BPA. We used quantitative real-time PCR to verify regulation of the HOXC6 gene, which showed decreased expression of approximately 2.5-fold by BPA. These results reveal novel effects by BPA and E2, raising interesting possibilities regarding the role of endocrine disruptors in sexual development.

Sohoni, P. and Sumpter, J..P. (1998). Several environmental oestrogens are also anti-androgens. Journal of Endocrinology. 158:327-339.

Bisphenol A has an efficacy similar to the antiandrogenic drug Flutamide in inhibiting binding of DHT to androgen receptors in a yeast reporter assay.

Takemura, H., Ma, J., Sayama, K., Terao, Y., Zhu, B.T. and Shimoi, K. (2005). In vitro and in vivo estrogenic activity of chlorinated derivatives of bisphenol A. Toxicology 207:215-221.

The estrogenic activity of bisphenol A (BPA) and its chlorinated derivatives, 2-(3-chloro-4-hydroxyphenyl)-2-(4-hydroxyphenyl)propane (3-ClBPA) and 2,2-bis(3-chloro-4-hydroxyphenyl)propane (3,3'-diClBPA) was assessed by determining their relative binding affinity for the human estrogen receptor-alpha and -beta (ERalpha and ERbeta) and also their uterotrophic activity in ovariectomized female rats. BPA and its chlorinated derivatives were active in competing with [3H]17beta-estradiol for their binding to the human ERalpha and ERbeta proteins. While 3-ClBPA and 3,3'-diClBPA competed more effectively for ERalpha binding than BPA (IC50 values of 2.48x10(-5), 1.28x10(-5), and 1.08x10(-4)M, respectively), they had similar activity as BPA for competing the binding to ERbeta (IC50 values of 1.43x10(-5), 1.87x10(-5), and 2.59x10(-5)M, respectively). To determine the uterotropic activity, three doses (10, 50 and 100 mg/kg/day) of BPA and its derivatives were given to mature ovariectomized Sprague-Dawley rats for 3 consecutive days. Treatment of animals with 50 and 100 mg/kg/day of BPA or its chlorinated derivatives caused a significant increase in the uterine wet weight and the endometrial area. The results of our present study demonstrated that the affinities of 3-ClBPA and 3,3'-diClBPA for ERalpha were higher than the affinity of BPA, although the in vivo estrogenic activity of the two chlorinated BPAs in ovariectomized female Sprague-Dawley rats appeared to be comparable to that of BPA.

Tarumi, H., Imazato, S., Narimatsu, M., Matsuo, M. and Ebisu, S. (2000). Estrogenicity of fissure sealants and adhesive resins determined by reporter gene assay. J Dent Res 79:1838-1843.

It is controversial whether the dental resinous materials containing 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)phenyl]propane (Bis-GMA), which is synthesized from the estrogenic compound bisphenol A (BPA), include unreacted BPA and/or can mimic the effects of natural steroid hormones. In the present study, the estrogenic activities of 3 fissure sealants and 5 adhesive resins, which were all unpolymerized, were determined by means of a reporter gene assay, and the relevance of the components to the estrogenicity was investigated. Two commercially available sealants were confirmed to have estrogenic activity, although none of the tested materials contained BPA. In

contrast, hydrophobic monomer bisphenol A dimethacrylate (BPA-DMA), which is also estrogenic, was found to be included in these estrogenic sealants in an amount greater than the minimum concentration to show estrogenicity. This suggests that the estrogenicity of the two proprietary sealants was associated with BPA-DMA rather than with BPA.

Toyohira, Y., K. Utsunomiya, S. Ueno, K. Minami, Y. Uezono, R. Yoshimura, M. Tsutsui, F. Izumi and N. Yanagihara (2003). Inhibition of the norepinephrine transporter function in cultured bovine adrenal medullary cells by bisphenol A. Biochem. Pharmacol. 65:2049-54.

Bisphenol A and estradiol altered norepinephrine (NE) transporter function in cultured bovine adrenal medullary cells. Bisphenol A significantly inhibited [3H]NE uptake by the cells in a concentration-dependent manner (1-100 microM). Kinetic analysis revealed that bisphenol A, as well as 17beta-estradiol, noncompetitively inhibited [3H]NE uptake. Bisphenol A and 17beta-estradiol inhibited the specific binding of [3H]desipramine to plasma membranes isolated from bovine adrenal medulla. As shown by Scatchard analysis of [3H]desipramine binding, bisphenol A increased the dissociation constant (K(d)) and decreased the maximal binding (B(max)), indicating a mixed type of inhibition. 17beta-Estradiol increased the K(d) without altering the B(max), thereby indicating competitive inhibition. The present findings suggest that bisphenol A inhibits the function of the NE transporter by acting on a site different from that of 17beta-estradiol in the adrenal medulla and probably in the brain noradrenergic neurons.

Tsutsui, T., Tamura, Y., Yagi, E., Hasegawa, K., Takahashi, M., Maizumi, N., Yamaguchi, F. and Barrett, J.C. (1998). Bisphenol-A induces cellular transformation, aneuploidy and DNA adduct formation in cultured Syrian hamster embryo cells. Int, J, Cancer 75:290-294.

Bisphenol-A (BP-A) is a major component of epoxy, polycarbonate and other resins. For an assessment of in vitro carcinogenicity and related activity of BP-A, the abilities of this compound to induce cellular transformation and genetic effects were examined simultaneously using the Syrian hamster embryo (SHE) cell model. Cellular growth was reduced by continuous treatment with BP-A at doses > or = 100 microM. However, colony-forming efficiencies were not decreased significantly following treatment with up to 200 microM BP-A for 48 hr. Morphological transformation of SHE cells was induced by treatment of cells with BP-A at 50 to 200 microM for 48 hr. BP-A exhibited transforming activity at doses > or = 50 microM but was less active than the benzo[alpha]pyrene used as a positive control. Over the dose range that resulted in cellular transformation, treatment of SHE cells with BP-A failed to induce gene mutations at the Na+/K+ ATPase locus or the hprt locus. No statistically significant numbers of chromosomal aberrations were detected in SHE cells treated with BP-A. However, treatment of cells with BP-A induced numerical chromosomal changes in the near diploid range at doses that induced cellular transformation. 32P-Postlabeling analysis revealed that exposure of cells to BP-A also elicited DNA adduct formation in a dose-dependent fashion. Our results indicate that BP-A has cell-transforming and genotoxic activities in cultured mammalian cells and potential carcinogenic activity.

Tsutsui, T., Tamura, Y., Suzuki, A., Hirose, Y., Kobayashi, M., Nishimura, H., Metzler, M. and Barrett, J.C. (2000). Mammalian cell transformation and aneuploidy induced by five bisphenols. Int. J. Cancer 86:151-154.

Bisphenol-A (BP-A), a monomer of plastics used in numerous consumer products and a xenoestrogen, induces cellular transformation and aneuploidy in Syrian hamster embryo (SHE) cells. In this study, the abilities of 4 other bisphenols to induce cellular transformation and genetic effects in SHE cells were examined and compared to BP-A. Cellular growth was inhibited by all bisphenols in a concentration-related manner. The growth inhibitory effect of the bisphenols ranked: BP-5 > BP-

4 > BP-3 > BP-2 or BP-A. Morphological transformation of SHE cells was induced by BP-A, BP-3, BP-4 and BP-5, and the induced-transformation frequencies were highest with BP-4. None of the bisphenols induced gene mutations at the Na(+)/K(+) ATPase locus or the hprt locus, or chromosomal aberrations in SHE cells. By contrast, aneuploidy induction in the near-diploid range was exhibited by BP-A, BP-3, BP-4 or BP-5, corresponding to the transforming activity of each compound. The results indicate that BP-A, BP-3, BP-4 and BP-5 exhibit transforming activity in SHE cells, while BP-2 does not, and that aneuploidy induction may be a causal mechanism of the transforming activity.

Wada, H., Tarumi, H., Imazato, S., Narimatsu, M. and Ebisu, S. (2004). In vitro estrogenicity of resin composites. J. Dent. Res. 83:222-226.

Previously, we have reported that sealants incorporating bisphenol A dimethacrylate showed estrogenicity by a reporter gene assay. This study tested the hypothesis that commercial composites, which contain various monomers and additives, exhibit estrogenic activity in vitro. The estrogenic activities of eluates obtained from 24 composites and 18 chemicals identified from the composites tested were examined with the use of the reporter gene assay. Among the 24 composites, 6 products were estrogenic, and among the 18 constituents, 1 photostabilizer, 2-hydroxy-4-methoxy-benzophenone (HMBP), 1 photoinitiator, 2,2-dimethoxy-2-phenyl-acetophenone (DMPA), and 1 inhibitor, 2,6-di-tert-butyl-p-cresol (BHT) had significant estrogenic activity. The concentration of HMBP in 4 estrogenic eluates was greater than the minimum concentration required for estrogenicity, and DMPA was found at a higher level than the minimum estrogenic concentration in the remaining 2 estrogenic specimens. These results suggest that the observed estrogenic activity of 6 composites is associated with the elution of either HMBP or DMPA.

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

The aim of this study was to identify and characterize an alternative pathway through which environmental estrogenic compounds may mediate their intracellular effects. Three human breast cancer cell lines were employed including MCF-7 cells, which express both ERalpha and ERbeta; MDA-MB-231 cells, which express ERbeta but not ERalpha; and SKBR-3 cells, which express neither ERalpha nor ERbeta. The effect of environmental estrogenic compounds on intracellular calcium ion concentration ([Ca(2+)](i)) was measured and compared to that of 17beta-estradiol (E2). A rapid and maintained increase in [Ca(2+)](i) was observed following the application of nanomolar concentrations of environmental estrogens and E2 regardless of the expression of ERalpha and ERbeta. Removal of extracellular Ca(2+) completely abolished the steroid-induced [Ca(2+)](i) increase. Pre-treatment of cells with the estrogen receptor (ER) antagonist ICI 182,780 had no effect on either basal [Ca(2+)](i) or the steroid-triggered [Ca(2+)](i) response. In summary, we have demonstrated ER independent rapid non-genomic effects of environmental estrogenic compounds, at nanomolar concentrations, on [Ca(2+)](i). The results of this study demonstrate an alternative pathway to explain potent intracellular effects of endocrine disrupting chemicals. Regarding bisphenol A, rapid (within 1.5 min) influx of calcium was observed in human MCF-7 breast cancer cells in response to both oestradiol and bisphenol A that was significant at the lowest dose tested, which was 0.1 nM (23 ppt bisphenol A); for oestradiol, the EC50 was 0.11 nM) and for bisphenol A the EC50 was 0.15 nM or 342 ppt.

Wetherill, Y. B., Petra, C. E., Monk, K. R., Puga, A. and Knudsen, K. E. (2002). The xenoestrogen bisphenol A induces inappropriate androgen receptor activation and mitogenesis in prostate adenocarcinoma cells. Molecular Cancer Therapeutics 7:515-524.

Bisphenol A stimulated proliferation of human prostate cancer (LNCaP) cells. There was an inverted-U dose-response curve, with maximum stimulation at 230 ppt, lower stimulation at 23 ppt and 2.3 ppb, and no stimulation at either 2.3 ppt (NOAEL) and 23 ppb (which also would have been erroneously thought to be the NOAEL if this was the lowest dose tested.

Wetherill, Y. B., N. I. Fisher, A. Staubach, M. Danielsen, R. W. de Vere White and K. E. Knudsen (2005) Xenoestrogen action in prostate cancer: Pleiotropic effeccts dependent of androgen receeptor status. Cancer Res. 65, 54-65.

Androgen is critical for prostate development, growth, and survival. Therapies for advanced prostate cancer aim to block androgen receptor (AR) action. However, recurrent tumors ultimately arise, which harbor restored AR activity. One mechanism of such reactivation occurs through AR mutations, rendering the receptor responsive to noncanonical ligands. We have shown previously that a known xenoestrogen, bisphenol A (BPA), activates a tumor-derived AR mutant (T877A), leading to androgen-independent prostate cancer cell proliferation. Here, we show that BPA cooperates with androgen to activate AR-T877A as shown by both reporter assays and increased levels of prostate-specific antigen expression. Further investigations using both yeast and mammalian model systems revealed that multiple AR alleles are responsive to BPA, thus expanding the potential influence of xenoestrogens on prostate cancer. Moreover, in vitro radioligand binding assay revealed that BPA alters 5a-dihydrotestosterone binding to AR-T877A likely through noncompetitive inhibition. We also show that higher concentrations of BPA block proliferation of AR-positive, androgen-dependent prostate adenocarcinoma cells (LNCaP and LAPC-4), with a more modest inhibitory effect on androgen-independent cells (22Rv-1). By contrast, AR-negative prostate cancer cells failed to show growth inhibition after exposure to high BPA dose. Together, these data show that BPA can serve as a potential ‘‘hormone sensitizer’’ of the mutant ARs present in advanced prostate adenocarcinomas, thereby possibly contributing toward therapeutic relapse in advanced prostate cancer patients and supporting the notion that nonsteroidal environmental compounds can alter the function of nuclear receptor complexes

Wozniak, A. L., N. N. Bulayeva and C. S. Watson (2005) Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-a mediated Ca++ fluxes and prolactin release in GH3/B6 pituitary tumor cells. Environ. Health Perspect. 113:431-439.

Xenoestrogens (XEs) are widespread in our environment, and known to have deleterious effects in animal (and perhaps human) populations. Acting as inappropriate estrogens, XEs are thought to interfere with endogenous estrogens such as estradiol (E2) to disrupt normal estrogenic signaling. We investigated the effects of E2 vs. several XEs representing organochlorine pesticides (dieldrin, endosulfan, DDE), plastics manufacturing byproducts/detergents (nonylphenol, bisphenol A), a phytoestrogen (coumestrol), and a synthetic estrogen (DES) on the pituitary tumor cell subline GH3/B6/F10, previously selected for expression of high levels of membrane estrogen receptor-alpha. Picomolar to nanomolar concentrations of both E2 and XEs caused intracellular Ca++ changes within 30 sec of administration. Each XE produced a unique temporal pattern of Ca++ elevation. Removing Ca++ from the extracellular solution abolished both spontaneous and XE-induced intracellular Ca++ changes, as did 10µM nifedipine. This suggests that XEs mediate their actions via voltage dependent L-type Ca++ channels in the plasma membrane. None of the Ca++ fluxes came from intracellular Ca++ stores. E2 and each XE also caused unique time- and concentration dependent patterns of prolactin (PRL) secretion that were largely complete within 3 minutes of administration. PRL

secretion was also blocked by nifedipine, demonstrating a correlation between Ca++ influx and PRL secretion. These data indicate that at very low concentrations, XEs mediate membrane-initiated intracellular Ca++ increases resulting in PRL secretion via a mechanism similar to that for E2, but with distinct patterns and potencies which could explain their abilities to disrupt endocrine functions. With regard to bisphenol A, it significantly stimulated a rapid (within 30 sec) influx of calcium at the lowest dose that was examined (0.23 ppt or 10^{-12} M); the greatest response occurred at 230 ppt, while the magnitude of the response decreased at 2.3 ppb, forming an inverted-U dose-response curve. The calcium influx response to bisphenol A at 230 ppt was actually greater than that for oestradiol or DES. Prolactin release, which is triggered by calcium influx in these cells, was detected within 1 min at 0.23 ppt bisphenol A, similar to the response to oestradiol.

Yanagihara N, Toyohira Y, Ueno S, Tsutsui M, Utsunomiya K, Liu M, Tanaka K. 2004 Oct 14. Stimulation of catecholamine synthesis by environmental estrogenic pollutants. Endocrinology, on line, 10/14.

Environmental estrogenic pollutants are compounds that have been shown to have estrogenic effects on fetal development and reproductive systems. Less attention, however, has been paid to their influence on neuronal functions. We report here the effects of estrogenic pollutants on catecholamine synthesis in bovine adrenal medullary cells used as a model system of noradrenergic neurons. Treatment of cultured bovine adrenal medullary cells with p-nonylphenol and bisphenol A at 10 nM for 3 days stimulated (14)C-catecholamine synthesis from [(14)C]tyrosine and tyrosine hydroxylase activity, an effect that was not inhibited by ICI 182,780, an antagonist of estrogen receptors. Significant effects of p-nonylphenol on (14)C-catecholamine synthesis were observed at 0.1 nM that is 45 times lower than that of the international regulatory standard (4.5 nM) and the maximum effects were around at 10 - 100 nM. The concentrations (0.1 - 10 nM) used in the present study are similar to the range observed in rivers in the U.S. or Europe. On the other hand, short-term treatment of cells with 10 nM p-nonylphenol for 10 min also activated tyrosine hydroxylase, which was suppressed by U0126, an inhibitor of mitogen-activated protein kinase (MAPK) kinase. Furthermore, treatment of cells with p-nonylphenol for 5 min increased the phospho-p44/42MAPK in a concentration (1 - 1000 nM)-dependent manner while p-nonylphenol (100 nM, 2 days) enhanced both levels of nonphospho- and phospho-p44/42MAPK. These findings suggest that short-term and long-term treatment of cells with estrogenic pollutants at environmental concentrations stimulates catecholamine synthesis and MAPK through an estrogen receptor-independent pathway.

Yoneda, T.; Hiroi, T.; Osada, M.; Asada, A., and Funae, Y. (2003). Non-genomic modulation of dopamine release by bisphenol-A in PC12 cells. Journal of Neurochemistry. 87(6):1499-1508.

An endocrine disruptor chemical, bisphenol-A (BPA), is reported to have several short-term actions in various tissues and/or cells; however, the mechanisms of these actions have not been fully elucidated. We investigated short-term actions evoked by BPA in pheochromocytoma PC12 cells. BPA elicited dopamine release in PC12 cells in a dose-dependent manner. A selective N-type calcium channel antagonist (omega-conotoxin GVIA) and a ryanodine receptor blocker (ryanodine) inhibited the BPA-induced dopamine release. The expression of ryanodine receptor mRNA was detected by RT-PCR in PC12 cells. Subsequently, in order to prove whether membrane receptors participate in BPA-evoked dopamine release, a guanine nucleotide-binding protein inhibitor [guanosine 5'-(beta-thio) diphosphate], cyclic AMP antagonist (Rp-cAMPS) or protein kinase A inhibitor (H7 or H89) was added to PC12 cells prior to BPA-treatment. All of these agents suppressed BPA-evoked dopamine release, indicating that multiple signaling pathways may be involved in BPA-evoked dopamine release in PC12 cells. In conclusion, we demonstrated that BPA induced dopamine release in a non-genomic manner through guanine nucleotide-binding protein and N-type calcium

channels. These findings illustrate a novel function of BPA and suggest that exposure to BPA influences the function of dopaminergic neurons.

Yu Z, Zhang L, Wu D. 2004 Jul. [Effects of three environmental estrogens on expression of proliferation and apoptosis-associated genes in PEO4 cells]. Wei Sheng Yan Jiu 33:404-6.

This study was designed to investigate the molecular mechanisms of proliferation and apoptosis by environmental estrogens (n-4-noniphenol, NP; Bisphenol A, BisA; Dibutylphthalate, DBP) in ovarian cancer PEO4 cells. METHODS: PEO4 cells were maintained in DMEM medium with 10% neonatal bovine serum. Five days before the beginning of experiments, the cells were seeded in phenol red-free DMEM medium containing 5% charcoal dextran-treated FBS. The cells were harvested and seeded in 6-well culture plates or in 75ml flacks. After various concentration of NP, BisA and DBP treatment for 72h, the cells were harvested and detected mRNA and protein expression of PCNA, bcl-2 and bax by reverse transcription-polymerase chain reaction and immunohistochemistry, respectively. RESULTS: 32 x 10(-7) mol/L NP and 32 x 10(-7) mol/L BisA could significantly up-regulate PCNA and bcl-2 mRNA expression and down-regulate the bax mRNA expression, and 32 x 10(-6) mol/L DBP could up-regulate PCNA mRNA expression, but had no effect on bax and bcl-2 mRNA expression. These results were further confirmed by following immunohistochemistry. CONCLUSION: PCNA, bcl-2 and bax pathway might involve in cell proliferation and apoptosis events by environmental estrogens in ovarian cancer PEO4 cells.

XVII. BISPHENOL A BINDING AND RESPONSES WITH ER-alpha AND ER-beta

There is now considerable evidence that bisphenol A acts as a SERM, and relative to estradiol: 1) interacts differently within the ligand-binding domain of estrogen receptors (Gould, Leonard et al. 1998), 2) shows a different binding affinity for and regulation of ERα and ERß in target cells (Kuiper, Carlsson et al. 1997; Routhledge, White et al. 2000), and 3) interacts differently with transcriptional co-regulators (Routhledge, White et al. 2000). Additional studies concerning this issue are also listed below.

Cappelletti, V., Saturno, G., Miodini, P., Korner, W. and Daidone, M.G. (2003). Selective modulation of ER-beta by estradiol and xenoestrogens in human breast cancer cell lines. 60:567-576.

In the last decades, substances with estrogenic activity have been dispersed into the environment. Xenoestrogens act by binding to estrogen receptors, ligand-regulated transcription factors, for which two subtypes have been described, ER-alpha and ER-beta, which are often coexpressed at variable amounts in different tissues. We investigated variations in the expression of ER-alpha and ER-beta mRNAs following treatment with four xenoestrogens (bisphenol A, 4-tert octylphenol, 2-hydroxybiphenyl, 4-hydroxybiphenyl) and with 17beta-estradiol in estrogen-sensitive (T47D) and estrogen-insensitive (BT20) breast cancer cell lines. Although to a variable extent, both estradiol and the tested xenoestrogens increased the expression of ER-beta mRNA, whereas a slight effect on ER-alpha was observed only in T47D cells. Upregulation of ER-beta expression by estradiol and xenoestrogens was observed only in the presence of detectable ER-alpha protein levels. These findings indicate a regulatory role for ER-beta in ER-alpha-mediated transcription and a role for ER-beta in mediating xenoestrogen toxicity.

Kurosawa, T., H. Hiroi, O. Tsutsumi, T. Ishikawa, Y. Osuga, T. Fujiwara, S. Inoue, M. Muramatsu, M. Momoeda and Y. Taketani (2002). The activity of bisphenol A depends on both the estrogen receptor subtype and the cell type. 49: 465-71.

Bisphenol A (BPA), a monomer of plastic used in consumer products, is abundant in the environment and enters the body by ingestion or adsorption. In order to characterize the estrogenic effect of BPA, we performed luciferase assay on three independent cell lines derived from different tissues transfected with either human ERalpha cDNA or ERbeta cDNA. The estrogenic activities of BPA were detectable in all cell lines via both ERalpha and ERbeta. In 293T cells and Hec-1 cells, the estrogenic activities were significantly decreased when cells expressing ERalpha were incubated with 10(-6) M BPA in the presence of 10(-8) M 17beta-estradiol (E2) while the activities via ERbeta were essentially unchanged in the same conditions. Interestingly, no reduction of estrogenic activity was detected in HOS-TE85 cells via either ERalpha or ERbeta. Our results indicate that BPA only acts as an agonist of estrogen via ERbeta whereas it has dual actions as an agonist and antagonist in some types of cells via ERalpha. Thus, the activity of BPA may depend on the ER subtype and the tissue involved.

Masuyama, H. and Hiramatsu, Y. (2004). Involvement of suppressor for Gal 1 in the ubiquitin/proteasome-mediated degradation of estrogen receptors. J Biol Chem 279:12020-12026.

The proteasome-mediated pathway involves the degradation of several nuclear receptors. Previously we demonstrated that the interaction between the suppressor for Gal 1 (SUG1) and nuclear receptors, the vitamin D receptor, or the pregnane X receptor was involved in proteasome-mediated degradation. In our recent experiments, we examined the potential role of SUG1 in the proteasome-mediated degradation of estrogen receptors (ER)alpha and -beta. Both ERs interacted with SUG1 in a ligand-dependent manner. Functionally, the overexpression of SUG1 inhibited both ERalpha- and ERbeta-mediated transcription in the presence of ligands. Transient expression studies demonstrated that the overexpression of wild-type SUG1 generated proteolytic fragments of both ERs and that these products were blocked by a proteasome inhibitor. The overexpression of SUG1 also enhanced the formation of ubiquitinated proteins of both ERs in the presence of ligand. On the other hand, bisphenol A (BSA), which activated ER-mediated transcription, did not enhance the interaction between ERbeta and SUG1. Furthermore, the degradation of ERbeta was much slower in the presence of BSA than in the presence of estradiol or phthalate, which is another endocrine-disrupting chemical. Also, BSA had no effect on the formation of proteolytic fragments of ERbeta, and neither did it have any effect on the ubiquitination of ERbeta. These findings indicate that the ubiquitin/proteasome-mediated degradation of both ER proteins may involve the interaction of SUG1 with both ERs. Moreover, BSA strongly blocked the ubiquitination and degradation of ERbeta compared with estradiol, suggesting that BSA may affect the ERbeta-mediated transcription of target genes by inhibiting ERbeta degradation.

Matthews, J. B., K. Twomey and T. R. Zacharewski (2001). In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptors alpha and beta. Chemical Research in Toxicology **14**(2): 149-157.

The estrogenic activities of bisphenol A (BPA) and its major metabolite BPA glucuronide (BPA-G) were assessed in a number of in vitro and in vivo assays. BPA competed with [H-3]-17 beta -estradiol (E2) for binding to mouse uterine cytosol ER, a glutathione S-transferase (GST)-human ER D, E, and F domain fusion protein (GST-hER alpha def) and full-length recombinant hER beta. The IC50 values for E2 were similar for all three receptor preparations, whereas BPA competed more effectively for binding to hER beta (0.96 muM) than to either mouse uterine cytosol ER (26 muM) or GST-hERadef (36 CIM) In contrast, BPA-G did not competitively displace [H-3]E2 from any of the ER preparations. In MCF-7 cells transiently transfected with Gal4-hER alpha def or Gal4-hER beta def, BPA induced reporter gene activity with comparable EC50 values (71 and 39 muM, respectively). No significant induction of reporter gene activity was seen for BPA-G. Cotreatment

studies showed that concentrations of (10 muM) BPA and BPA-G did not antagonize EB-induced luciferase mediated through either Gal4-hER alpha def or Gal4-hER beta def. In vivo, the uterotropic effect of gavage or subcutaneous (sc) administration of 0.002-800 mg of BPA/kg of body weight/day for three consecutive days was examined in immature rats. Dose-related estrogenic effects on the rat uterus were observed at oral doses of 200 and 800 mg/kg and at sc doses of 10, 100, and 800 mg/kg. These results demonstrate that BPA competes more effectively for binding to ER beta, but induces ER alpha- and ER beta -mediated gene expression with comparable efficacy. In contrast, BPA-G did not exhibit any in vitro estrogenic activity. In addition, there was a clear route dependency on the ability of BPA to induce estrogenic responses in vivo. In summary, bisphenol A ERß binding affinity was 38-fold greater than for ERα.

Paris, F., P. Balaguer, B. Terouanne, N. Servant, C. Lacoste, J. P. Cravedi, J. C. Nicolas and C. Sultan (2002). Phenylphenols, biphenols, bisphenol-A and 4-tert-octylphenol exhibit alpha and beta estrogen activities and antiandrogen activity in reporter cell lines. Mol. Cell Endocrinol. **193**(1-2): 43-9.

This study provides evidence that several phenyl derivatives present both estrogenic and antiandrogenic activity. The extent of hydroxylation and the position of the hydroxyl function were important in determining their estrogenicity and antiandrogenicity. Of the tested compounds, bisphenol-A and 4,4' biphenol had very high estrogenic activity, although it was lower than that of the strong estrogenic alkylphenol, 4-tert-octylphenol. Bisphenol-A and 4,4' biphenol were able to activate ERs at concentrations lower than 1 microM, whereas the other compounds only activated at concentrations above 1 microM. Interestingly, 4,4' biphenol was a better agonist for ERbeta than for ERalpha. No androgenic activity was detected for any of these compounds. Bisphenol-A, 3-OH phenylphenol, 4-OH phenylphenol and 4,4' biphenol exhibited antiandrogenic activity close to that of 4-tert-octylphenol (IC(50) approximately 5 microM). In whole cell binding assays, these compounds displaced [3H] R1881 with Ki = 10 microM. Although these Ki values seem high in comparison with that of hydroxyflutamide (0.4 microM), one must keep in mind that environmental chemicals can accumulate in adipose tissues for several years. In conclusion, these environmental chemicals may have a negative impact on androgen action during fetal and post-natal life.

Pennie, W. D., T. C. Aldridge and A. N. Brooks (1998). Differential activation by xenoestrogens of ER alpha and ER beta when linked to different response elements. J. Endocrinol. 158: R11-R14.

Routhledge, E.J., White, R., Parker, M.G. and Sumpter, J.P. (2000). Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) and ERß. J. Biol. Chem. 46:35986-35993.

HeLa cells were transfected with ERα and ERß, and bisphenol A showed a 10-fold greater binding affinity (relative to estradiol) for ERß than ERα. Binding of bisphenol A to ERß but not ERα also resulted in recruitment of the ER coactivator TIF2 with a potency relative to estradiol of 0.05.

Seidlova-Wuttke, D., H. Jarry and W. Wuttke (2004). Pure estrogenic effect of benzophenone-2 (BP2) but not of bisphenol A (BPA) and dibutylphtalate (DBP) in uterus, vagina and bone. Toxicology 205:103-12.

Contradictory results whether the endocrine disrupters (ED) benzophenone-2 (BP2), bisphenol A (BPA) and dibutylphtalate (DBP) exert estrogenic effects have been published. Selective estrogen receptor modulators (SERMs) exert estrogenic effects in some but not in all organs and ED may be SERMs. Therefore, we studied their binding properties to recombinant ERalpha and ERbeta

protein and their effects in the uterus, vagina and bone of ovariectomized rats. BP2 bound to both receptor subtypes, while BPA had a relatively high ERbeta selectivity. DBP did not bind to ERalpha but with a low affinity to ERbeta. In the uterus, only E(2) and BP2 increased uterine weight and the complement C3 but decreased ERbeta gene expression. Discrete effects of BPA and DBP in the uterus were found upon histological examination. In the vagina, BP2 but not BPA and DBP had clear estrogenic effects. E(2) and BP2 had antiosteoporotic effects in the metaphysis of the tibia. The serum surrogate parameters of bone metabolism, i.e. osteocalcin and the cross (rat) laps were significantly reduced by E(2), an effect shared with BP2 but not by the two other EDs. The conclusion: BP2 acts as ERalpha and ERbeta agonist mimicking effects of E(2), while the effects of BPA and DBP are not pure estrogenic.

Takao, T., W. Nanamiya, H. P. Nazarloo, R. Matsumoto, K. Asaba and K. Hashimoto (2003). Exposure to the environmental estrogen bisphenol A differentially modulated estrogen receptor-alpha and -beta immunoreactivity and mRNA in male mouse testis. 72(10): 1159-1169.

Bisphenol A at concentrations of 0.5 or 50 µg/ml in the drinking water was fed to young male mice (doses are likely in the range of 0.2 and 20 mg/kg/day). Effects on estrogen receptor (ER) alpha and beta proteins and mRNA in the testis following 8-weeks of oral administration of bisphenol A. ERß was localized in the nuclei of spermatogonia and/or spermatocytes, and the number of ERß containing cells (and mRNA) per testis were significantly decreased in the 50 microg/ml bisphenol A-treated group compared with controls. In contrast, ERα immunopositive cells (and mRNA) per testis were markedly increased in the 50 microg/ml bisphenol A-treated group compared with the controls. The existence of ER alpha and beta in the testis suggests that estrogens directly affect germ cells during testicular development and spermatogenesis, and differential modulation of ER alpha and beta in the testis could be involved in the effects of bisphenol A.

XVIII. EXPOSURE LEVELS IN HUMAN ADULTS AND FETUSES

Calafat, A.M., Kuklenyik, Z., Reidy, J.A., Caudill, S.P, Ekong, J. and Needham, L.L. (2005). Urinary concentrations of bisphenol A and 4-nonyl phenol in a human reference population. Environ. Health Perspect. 113:391-395.

Bisphenol A (BPA) is used to manufacture polycarbonate plastic and epoxy resins, which are used in baby bottles, as protective coatings on food containers, and for composites and sealants in dentistry. 4-Nonyl phenol (NP) is used to make nonylphenol ethoxylates, nonionic surfactants applied as emulsifying, wetting, dispersing, or stabilizing agents in industrial, agricultural, and domestic consumer products. The potential for human exposure to BPA and NP is high because of their widespread use. We measured BPA and NP in archived urine samples from a reference population of 394 adults in the United States using isotope-dilution gas chromatography mass spectrometry. The concentration ranges of BPA and NP were similar to those observed in other human populations. BPA was detected in 95% of the samples examined at concentrations at or above 0.1 micrograms per liter of urine (µg/L); the geometric mean and median concentrations were 1.33 µg/L (1.36 µg per gram of creatinine [µg/g creatinine]) and 1.28 µg/L (1.32 µg/g creatinine), respectively; the 95th percentile concentration was 5.18 µg/L (7.95 µg/g creatinine). NP was detected in 51% of the samples examined at or above 0.1 µg/L. The median and 95th percentile concentrations were less than 0.1 µg/L and 1.57 µg/L (1.39 µg/g creatinine), respectively. The frequent detection of BPA suggests widespread exposure to this compound in residents of the United States. The lower frequency of detection of NP than of BPA could be explained by a lower exposure of humans to NP, by different pharmacokinetic factors (i.e., absorption, distribution, metabolism, elimination), by the

fact that 4-n-nonyl phenol —the measured NP isomer— represents a small percentage of the NP used in commercial mixtures, or a combination of all of the above. Additional research is needed to determine the best urinary biomarker(s) to assess exposure to NP. Despite the sample population's non-representativeness of the U.S. population (although sample weights were used to improve the extent to which the results represent the U.S. population) and relatively small size, this study provides the first reference range of human internal dose levels of BPA and NP in a demographically diverse human population.

Engel, S.M., Levy, B., Liu, Z., Kaplan, D. and Wolff, M.S. (2005). Xenobiotic phenols in early pregnancy amniotic fluid. Reprod Toxicol Online August 17, 2005. Accession # 16112541.

We found detectable levels of three phytoestrogens (enterolactone, daidzein and genistein) and bisphenol A (BPA) in 21 residual amniotic fluid specimens that were collected before 20 weeks gestation. Samples were obtained by amniocentesis from women who were referred to the Mount Sinai Medical center because of advanced maternal age. Phytoestrogens were present in higher concentrations than BPA. Enterolactone was detected at the highest concentration (median 95.9mug/L), followed by daidzein and genistein (9.5 and 1.4 mug/L, respectively). BPA was present at very low concentrations (10%>LOD of 0.5mug/L). The relative concentration of the chemicals measured in amniotic fluid were identical to those in urine reported by other studies, i.e. enterolactone>daidzein>genistein>>BPA. Amniotic fluid is a source of fetal exposure to polar xenobiotics that come from the mother.

Hiroi, H., Tsutsumi, O., Takeuchi, T., Momoeda, M., Ikezuki, Y., Okamura, A., Yokota, H. and Taketani, Y. (2004). Differences in serum bisphenol a concentrations in premenopausal normal women and women with endometrial hyperplasia. Endocr J 51:595-600.

Exposure to endocrine disrupting chemicals (EDCs) has been raised in relation to its potential for adverse health outcomes. Bisphenol A (BPA) is an estrogenic EDC widely found in plastic products. We determined BPA concentrations in premenopausal women by an enzyme-linked immunosorbent assay and evaluated possible linkage between its contamination levels and endometrial hyperplasia, an estrogen-related disorder of the uterus. It has been implied that higher levels of BPA, which binds to estrogen receptor and plays estrogenic roles may, enhance endometrial hyperplasia. Serum BPA was detectable in all subjects and its concentrations in healthy controls with normal endometrium were 2.5 +/- 1.5 ng/ml (mean +/- SD). BPA levels in patients with simple endometrial hyperplasia with benign nature were 2.9 +/- 2.0 ng/ml and were not significantly different from the controls. Unexpectedly, BPA levels in patients with complex endometrial hyperplasia with malignant potential were 1.4 +/- 0.4 ng/ml and significantly lower compared to both control and simple endometrial hyperplasia groups. In addition, we measured the serum BPA levels in postmenopausal endometrial cancer patient (1.4 +/- 0.5 ng/ml), which were also significantly lower than control and simple endometrial hyperplasia groups. These findings suggest the presence of associations between BPA exposure and complex endometrial hyperplasia and endometrial cancer. The mode of action of BPA may be more complex than expected and the contradictory results may serve as a clue to addressing the mechanisms of linkage between occurrence of estrogen-dependent diseases and endocrine disruption.

Ikezuki, Y., O. Tsutsumi, Y. Takai, Y. Kamei and Y. Taketani (2002). Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. Human Reprod. **17**:2839-2841.

Using an enzyme-linked immunosorbent assay, Ikezuki et al. determined bisphenol A in serum of non-pregnant women, ovarian follicular fluid obtained during *in vitro* fertilization

procedures, serum from early and late pregnancy women, fetal umbilical cord blood at delivery, and amniotic fluid at 15-18 weeks gestation and at late pregnancy. The human sera showed average bisphenol A at 1.4 to 2.4 ng/ml levels, while the 15-18 week fetal amniotic fluid showed higher levels averaging 8.3 ng/ml, the highest level of exposure in the study coinciding with a period of great fetal sensitivity. The authors concluded that there was accumulation of bisphenol A in early fetuses, and significant prenatal exposure. Human ovarian follicular fluid contained bisphenol A at an average concentration of 2.4 ng/ml, which is of particular concern following the publication of effects of the chemical on ovarian meiotic congression failure and aneuploidy in mice at levels of bisphenol A that are presumably well below this value (Hunt, Koehler et al. 2003) based on the findings of Zalko et al. (2003).

Inoue, K., K. Kato, Y. Yoshimura, T. Makino and H. Nakazawa (2000). Determination of bisphenol A in human serum by high-performance liquid chromatography with multi-electrode electrochemical detection. J. Chromat. B **749**:17-23.

Inoue et al. described HPLC linked to multielectrode electrochemical detection that yielded highly sensitive detection of bisphenol A at 0.01 ng/ml in solvent and 0.05 ng/ml from serum. The authors also described the preparation and importance of water free of bisphenol A at this level of detection. Bisphenol A was detected in five healthy human volunteers at a mean value of 0.32 ng/ml serum.

Kuroda, N., Y. Kinoshita, Y. Sun, M. Wada, N. Kishikawa, K. Nakashima, T. Makino and H. Nakazawa (2003). "Measurement of bisphenol A levels in human blood serum and ascitic fluid by HPLC using a fluorescent labeling reagent." Journal of Pharmaceutical and Biomedical Analysis 30(6): 1743-1749.

Matsumoto, A., N. Kunugita, K. Kitagawa, T. Isse, T. Oyama, G. L. Foureman, M. Morita and T. Kawamoto (2003). "Bisphenol A levels in human urine." Environmental Health Perspectives 111(1): 101-104.

Ouchi, K. and S. Watanabe (2002). "Measurement of bisphenol A in human urine using liquid chromatography with multi-channel coulometric electrochemical detection." Journal of Chromatography B: Analytical Technologies in the Biomedical & Life Sciences 780(2): 365-370.

Sajiki, J., K. Takahashi and J. Yonekubo (1999). Sensitive method for the determination of bisphenol-A in serum using two systems of high-performance liquid chromatography. J. Chromat. B 736:255-261.

Sajiki et al. described HPLC linked to either electrochemical detection or mass spectrometry/electrospray ionization, with detection limits for bisphenol A of 0.2 and 0.1 ng/ml, respectively. UV-linked detection may be limited to 10 to 100 ng/ml. These authors reported bisphenol A at levels of 0 to 1.6 ng/ml serum in 20 healthy human samples.

Sasaki, N., Okuda, K., Kato, T., Kakishima, H., Okuma, H., Abe, K., Tachino, H., Tuchida, K. and Kubono, K. (2005). Salivary bisphenol-A levels detected by ELISA after restoration with composite resin. J Mater Sci Mater Med 16:297-300.

Bisphenol-A diglycidylether methacrylate (Bis-GMA), which is synthesized from bisphenol-A (BPA), a compound with exogenous endocrine disrupter action, is widely used as a dental material. During clinical filling with sealants and composite resins, the compounds are solidified by polymerization and then used. However, it has been noted that unpolymerized monomers may

become dissolved in saliva. In this study using a competitive ELISA system, we investigated the changes in the BPA concentration in saliva after restoration with composite resins. Commercial composite resins from nine companies were tested. Mixed saliva was collected from 21 subjects. Based on the dynamics of salivary BPA detected by this ELISA system, we concluded that several tens to 100 ng/ml of BPA were contained in saliva after filling teeth with composite resin but that sufficient gargling can remove it from the oral cavity. Our data suggest that sufficient gargling after treatment is important for risk management.

Schonfelder, G. Wittfoht, W. Hopp, H., Talsness, C., Paul, I and Chahoud, I. (2002). Parent bisphenol A accumulation in human maternal-fetal-placental unit. Environ. Health Perspect. 110:A703-A707.

Parent (unconjugated) bisphenol A was measured at parturition in blood from women, fetuses and placentae in Berlin, Germany. Bisphenol A in maternal serum ranged from 0.3 - 18.9 ng/ml (median = 3.1), in fetal serum from 0.2 - 9.2 ng/ml (median = 2.3), and in placentae from 1.0 - 104.9 ng/ml (median = 12.7). Male fetuses had higher levels of bisphenol A than did females.

Sugiura-Ogasawara, M., Ozaki, Y., Sonta, S., Makino, T. and Suzumori, K. (2005). Exposure to bisphenol A is associated with recurrent miscarriage. Human Reproduction 20:2325-2329.

BACKGROUND: Little is known about the influence of high exposure to bisphenol A on recurrent miscarriage and immunoendocrine abnormalities. METHODS: Serum bisphenol A, antiphospholipid antibodies (aPLs), antinuclear antibodies (ANAs), natural killer cell (NK) activity, prolactin, progesterone, thyroid-stimulating hormone (TSH) and free T4 were examined in 45 patients with a history of three or more (3-11) consecutive first-trimester miscarriages and 32 healthy women with no history of live birth and infertility. Subsequent pregnancy outcome and embryonic karyotype of abortuses were examined prospectively. RESULTS: The mean 6 SD values for bisphenol A in patients were 2.59 6 5.23 ng/ml, significantly higher than the 0.77 6 0.38 ng/ml found for control women (P 5 0.024). High exposure to bisphenol A was associated with the presence of ANAs but not hypothyroidism, hyperprolactinaemia, luteal phase defects, NK cell activity or aPLs. A high level of bisphenol A in itself did not predict subsequent miscarriage. CONCLUSION: Exposure to bisphenol A is associated with recurrent miscarriage.

Sun Y, Irie M, Kishikawa N, Wada M, Kuroda N, Nakashima K. 2004 Oct. Determination of bisphenol A in human breast milk by HPLC with column-switching and fluorescence detection. Biomed Chromatogr 18:501-7.

A highly sensitive HPLC method was developed for the determination of xenoestrogenic compound, bisphenol A (BPA) in human breast milk samples. After a two-step liquid-liquid extraction, BPA was derivatized with fluorescent labeling reagent, 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIB-Cl). The excess fluorescent reagent could be removed effectively using a column-switching system. The separation of DIB-BPA from endogenous materials in milk was carried out on two C(18) columns and fluorescence intensity was monitored at 475 nm with the excitation of 350 nm. A good linearity (r = 0.994) was observed of BPA in the concentration range of 0.2-5.0 ng mL(-1) in breast milk, and the detection limit was 0.11 ng mL(-1) at a signal-to-noise ratio of 3. Intra- and inter-day precision (RSD, %) were less than 8.7 and 10.4, respectively. Twenty-three breast milk samples of healthy lactating women were analyzed for the BPA concentration; the mean value was 0.61 +/- 0.20 ng mL(-1), with no correlation to the lipid content of milk samples.

Takeuchi, T. and O. Tsutsumi (2002). Serum bisphenol A concentrations showed gender differences, possibly linked to androgen levels. Biochem. Biophys. Res. Communic. 291:76-78.

Takeuchi described an antibody-linked detection with sensitivity of approximately 0.3 ng/ml serum. This assay detected bisphenol A at 1.49 ng/ml in human male serum, and 0.64 in serum from women during the mid-follicular stage of the cycle and 1.04 ng/ml in human females with polycystic ovary syndrome. A positive correlation between serum BPA and both total and free testosterone was discovered in all subjects. The authors point out that these levels were above the level that affected preimplantation development.

Takeuchi T, Tsutsumi O, Ikezuki Y, Takai Y, Taketani Y. 2004. Positive relationship between androgen and the endocrine disruptor, bisphenol A, in normal women and women with ovarian dysfunction. Endocrin. J. 51:165-169.

This study was performed to investigate the serum levels of bisphenol A (BPA), an endocrine disruptor, in women with ovarian dysfunction and obesity. Fasting serum samples were obtained from 19 non-obese and 7 obese women with normal menstrual cycles: 7 patients with hyperprolactinemia, 21 patients with hypothalamic amenorrhea, and 13 non-obese and 6 obese patients with polycystic ovary syndrome (PCOS). BPA was measured by an enzyme-linked immunosorbent assay. BPA was detected in all human sera. Serum BPA concentrations were significantly higher in both non-obese and obese women with polycystic ovary syndrome (1.05 +/- 0.10 ng/ml, 1.17 +/- 0.16 ng/ml; $p<0.05$, respectively) and obese normal women (1.04 +/- 0.09 ng/ml, $p<0.05$) compared with those in non-obese normal women (0.71 +/- 0.09 ng/ml). There was no difference among women with hyperprolactinemia, women with hypothalamic amenorrhea, and non-obese normal women. There were significant positive correlations between serum BPA and total testosterone ($r = 0.391$, $p<0.001$), free testosterone ($r = 0.504$, $p<0.001$), androstenedione ($r = 0.684$, $p<0.001$), and DHEAS ($r = 0.514$, $p<0.001$) concentrations in all subjects. These findings show that there is a strong relationship between serum BPA and androgen concentrations, speculatively due to the effect of androgen on the metabolism of BPA.

Tan, B.L.L. and Mohd, M.A. (2003). Analysis of selected pesticides and alkylphenols in human cord blood by gas chromatograph-mass spectrometer. Talanta 61:385-391.

Thomson, B. M., P. J. Cressey and I. C. Shaw (2003). Dietary exposure to xenoestrogens in New Zealand. J. Environ. Monit. **5**(2): 229-235.

Todaka, E. and C. Mori (2002). Necessity to establish new risk assessment and risk communication for human fetal exposure to multiple endocrine disruptors in Japan. Congenital Anomalies **42**: 87-93.

Bisphenol A, as well as many other endocrine disrupting chemicals, was measured by high resolution GC-MS in umbilical cords obtained at birth in Japan. From 20 umbilical cords examined, bisphenol A was detected in 11 samples (55%). The mean (SEM) was 4.4±1.5 ng/g wet weight (4.4 parts per billion), and the range was 0.35 – 15.2 ppb for samples with detectable bisphenol A.

Volkel, W., T. Colnot, G. A. Csanady, J. G. Filser and W. Dekant (2002) Metabolism and kinetics of bisphenol a in humans at low doses following oral administration. 15, 1281-1287.

Bisphenol A is a widely used industrial chemical with many potential sources of human exposure. Bisphenol A is a weak estrogen and has been implicated as an "endocrine disruptor". This term is used for a variety of chemicals encountered in the environment which have estrogenic activity. It has been postulated that human exposure to these chemicals may elicit unwanted estrogenic effects in humans such as reduced fertility, altered development and cancer. Up to now the body burden of bisphenol A in humans is unknown. Therefore, we investigated the metabolism and toxicokinetics of bisphenol A in humans exposed to low doses since systemic bioavailability has a

major influence on possible estrogenic effects in vivo. Human subjects (three males and three females, and four males for detailed description of blood kinetics) were administered d(16)-bisphenol A (5 mg). Blood and urine samples were taken in intervals (up to 96 h), metabolites formed were identified by GC/MS and LC-MS/MS and quantified by GC/MS-NCI and LC-MS/MS. d(16)-Bisphenol A glucuronide was the only metabolite of d(16)-bisphenol A detected in urine and blood samples, and concentrations of free d(16)-bisphenol A were below the limit of detection both in urine (6 nM) and blood samples (10 nM). d(16)-Bisphenol A glucuronide was cleared from human blood and excreted with urine with terminal half-lives of less than 6 h; the applied doses were completely recovered in urine as d(16)-bisphenol A glucuronide. Maximum blood levels of d(16)-bisphenol A glucuronide (approximately 800 nM) were measured 80 min after oral administration of d(16)-bisphenol A (5 mg). The obtained data indicate major species differences in the disposition of bisphenol A. Enterohepatic circulation of bisphenol A glucuronide in rats results in a slow rate of excretion, whereas bisphenol A is rapidly conjugated and excreted by humans due to the absence of enterohepatic circulation. The efficient glucuronidation of bisphenol A and the rapid excretion of the formed glucuronide result in a low body burden of the estrogenic bisphenol A in humans following oral absorption of low doses.

Wilson, N.K., Chuang, J.C., Lyu, C., Menton, R. and Morgan, M.K. (2003). Aggregate exposures of nine preschool children to persistent organic pollutants at day care and at home. J. Expo. Anal. Environ. Epidemiol. 13:187-202.

In the summer of 1997, we measured the aggregate exposures of nine preschool children, aged 2-5 years, to a suite of organic pesticides and other persistent organic pollutants that are commonly found in the home and school environment. The children attended either of two child day care centers in the Raleigh-Durham-Chapel Hill area of North Carolina and were in day care at least 25 h/week. Over a 48-h period, we sampled indoor and outdoor air, play area soil and floor dust, as well as duplicate diets, hand surface wipes, and urine for each child at day care and at home. Our target analytes were several polycyclic aromatic hydrocarbons (PAH), organochlorine pesticides, and polychlorinated biphenyls (PCB); two organophosphate pesticides (chlorpyrifos and diazinon), the lawn herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), three phenols (pentachlorophenol (PCP), nonyl phenols, and bisphenol-A), 3,5,6-trichloro-2-pyridinol (TCP), and two phthalate esters (benzylbutyl and dibutyl phthalate). In urine, our target analytes were hydroxy-PAH, TCP, 2,4-D, and PCP. To allow estimation of each child's aggregate exposures over the 48-h sampling period, we also used time-activity diaries, which were filled out by each child's teacher at day care and the parent or other primary caregiver at home. In addition, we collected detailed household information that related to potential sources of exposure, such as pesticide use or smoking habits, through questionnaires and field observation. We found that the indoor exposures were greater than those outdoors, that exposures at day care and at home were of similar magnitudes, and that diet contributed greatly to the exposures. The children's potential aggregate doses, calculated from our data, were generally well below established reference doses (RfDs) for those compounds for which RfDs are available. Journal of Exposure Analysis and Environmental Epidemiology (2003) 13, 187-202. doi:10.1038/sj.jea.7500270

Yamada, H., I. Furuta, E. H. Kato, S. Kataoka, Y. Usuki, G. Kobashi, F. Sata, R. Kishi and S. Fujimoto (2002). Maternal serum and amniotic fluid bisphenol A concentrations in the early second trimester. Reproductive Toxicology 16(6): 735-739.

To assess human exposure to bisphenol A (BPA) over a 10-year period, BPA concentrations in maternal serum (MS) and amniotic fluid (AF) obtained at early second trimester were determined. ELISA was used to measure BPA in 200 MS/AF pairs in women carrying fetuses with normal

karyotypes (Group 1) and in 48 pairs with abnormal karyotypes (Group II). In Group 1, BPA concentrations in AF (median: 0.26 ng/ml) were lower ($P < 0.01$) than in MS (2.24 ng/ml). Over a 10-year period, yearly BPA concentrations in MS decreased from 5.62 to 0.99 ng/ml ($P < 0.001$). Eight of the Group I AF samples had relatively high concentrations of BPA (2.80-5.62 ng/ml). In Group II, BPA concentrations in AF (0 ng/ml) were lower ($P < 0.01$) than in MS (2.97 ng/ml). MS BPA concentrations in Group II were higher ($P < 0.01$) than in Group I.

XIX. BISPHENOL A METABOLISM STUDIES IN MICE AND RATS, INCLUDING EXPOSURE DURING POSTNATAL LIFE AND OF FETUSES AFTER MATERNAL EXPOSURE

Domoradzki, J.Y., Pottenger, L.H., Thornton, C.M., Hansen, S.C., Card, T.L., Markham, D.A., Dryzga, M.D., Shiotsuka, R.N. and Waechter, J.M., Jr. (2003). Metabolism and Pharmacokinetics of Bisphenol A (BPA) and the Embryo-Fetal Distribution of BPA and BPA-monoglucuronide in CD Sprague-Dawley Rats at Three Gestational Stages. Toxicol Sci. 76:21-34.

The pharmacokinetics of bisphenol A (BPA), including the quantification of the major BPA metabolite BPA-monoglucuronide conjugate (BPA-glucuronide) was studied in Sprague-Dawley rats at different stages of gestation. (14)C-BPA was administered orally at 10 mg BPA/kg body weight (0.2 mCi/rat) to non-gravid rats and to other groups on gestation days (gd) 6, 14, and 17. GD 0 was defined as day when vaginal smear was sperm positive or a copulatory plug was observed. Radioactivity derived from (14)C-BPA was quantified in the maternal blood, selected tissues and the embryo or fetus. BPA and BPA-glucuronide were quantified in maternal plasma and excreta. Additional rats were dosed orally at 10 mg (14)C-BPA/kg (0.2 mCi/rat or 0.5 mCi/rat) on gd 11, 13 and 16 to further study the distribution of BPA and BPA-glucuronide to the embryo/fetal tissue. The tissue distribution, metabolism, or the rates or routes of excretion of BPA, or the plasma concentration-time profiles of BPA-glucuronide did not appear to be altered at any stage of gestation as compared to non-pregnant rats. In the gd 11 group, neither BPA nor BPA-glucuronide were detected in the yolk sacs or embryos, except for trace concentrations of BPA-glucuronide in the yolk sacs at 15 min post-dosing. In the gd 13 group, both BPA and BPA-glucuronide were detected in the yolk sacs of the conceptus but not the embryo/fetuses, except for BPA at 15 min. For the animals dosed with 0.2 mCi/rat on gd 16, both analytes were detected in the placentae at 15 min and 12 hr, but not at 96 h. Traces of both analytes were detected in fetal tissue in two of five specimens at 15 min only. In rats dosed on gd 16 with 0.5 mCi/rat, the BPA-glucuronide and BPA concentrations in maternal plasma at 15 min were 1.7 and 0.06 micro g equivalents (eq)/g plasma, respectively. At the same time post-dosing in these animals, the placental BPA-glucuronide concentrations were lower (0.34 micro g eq BPA (as glucuronide)/g), and the BPA concentrations were about equivalent (0.095 micro g/g). Fetal BPA-glucuronide and BPA concentrations were markedly lower, 0.013 and 0.018 micro g eq/g, respectively. Therefore, no selective affinity of either yolk sac/placenta or embryo/fetus for BPA or BPA metabolites relative to maternal plasma or tissues was observed in this study. FUNDED BY THE CHEMICAL INDUSTRY

Domoradzki, J. Y.; Thornton, C. M.; Pottenger, L. H.; Hansen, S. C.; Card, T. L.; Markham, D. A.; Dryzga, M. D.; Shiotsuka, R. N., and Waechter, J. M. Jr. (2004). Age and dose dependency of the pharmacokinetics and metabolism of bisphenol A in neonatal Sprague-Dawley rats following oral administration. Toxicol Sci. 77(2):230-42.

Previous studies demonstrated the rapid clearance of bisphenol A (BPA) from blood following oral administration to adult rats with the principal metabolite being BPA-monoglucuronide

(BPA-glucuronide). Since the ontogeny of glucuronyl transferases (GT) differs with age, the pharmacokinetics of BPA were studied in neonatal animals. (14)C-BPA was administered via gavage at 1 or 10 mg/kg body weight to rats at postnatal day (pnd) 4, pnd 7, pnd 21, or to 11 week old adult rats (10 mg/kg dose only). Blood (neonates and adults) and selected tissues (neonates) were collected at 0.25, 0.75, 1.5, 3, 6, 12, 18, and 24 h postdosing. BPA and BPA-glucuronide in the plasma were quantified by high-performance liquid chromatography; radioactivity in the plasma and tissues was quantified by liquid scintillation spectrometry. The data indicate that neonatal rats at all three ages metabolized BPA to BPA-glucuronide, although an age dependency in the number and concentration of plasma metabolites was observed, consistent with the ontogeny of GT. BPA-glucuronide and BPA concentrations in the plasma were greater in neonates than in adults, except at 24 h postdosing, suggesting an immaturity in the development of hepatic excretory function in neonatal rats. Nevertheless, the half-lives for the elimination of BPA-glucuronide in plasma were more rapid in neonatal animals than in adults, likely due to reduced microflora beta-glucuronidase activity and an absence of enterohepatic recirculation. A dose dependency in the metabolism and pharmacokinetics of BPA administered to neonates was also observed with nearly complete metabolism of BPA to BPA-glucuronide (94-100% of the plasma radioactivity) at a dose of 1 mg/kg. This was in contrast to finding up to 13 different plasma metabolites observed at the 10 mg/kg dose. These data indicate that, from early in neonatal life through pnd 21, there is sufficient GT activity in rats to efficiently metabolize BPA to its nonestrogenic metabolite at low doses.
FUNDED BY THE CHEMICAL INDUSTRY

Inoue, H., Yuki, G., Yokota, H. and Kato, S. (2003). Bisphenol A glucuronidation and absorption in rat intestine. Drug Metab Dispos 31:140-144.

Bisphenol A, an environmental estrogen, can be leached from plastic tableware and from the coating of food and drink cans, orally exposing human beings to the compound. The present study focuses on the absorption and metabolism of bisphenol A in the rat intestine, as elucidated experimentally by segmented everted intestine. One hour after the application of 2 micromol of bisphenol A to the mucosal fluid, the absorption of bisphenol A was slightly greater in the colon (48.6%) than in the proximal jejunum (37.5%). In the serosal side, unconjugated bisphenol A appeared in small amounts, increasing distally (maximal 1.6 nmol, colon). Large amounts of the bisphenol A glucuronide were then transported into the serosal side, also increasing distally (proximal, 80.4 nmol; distal, 478.4 nmol). The greatest amount of the glucuronide (approximately 573 nmol) was excreted into the mucosal side of the small intestine, whereas in the colon, mucosal excretion was minimal (67.2 nmol). On high-dose application of bisphenol A to the mucosal fluid, the transported unconjugated bisphenol A increased markedly throughout the intestine and colon. These results suggest that bisphenol A in the intestinal lumen is glucuronidated almost exclusively during its passage through the intestinal wall.

Inoue, H., A. Tsuruta, S. Kudo, T. Ishii, Y. Fukushima, H. Iwano, H. Yokota and S. Kato (2004). Bisphenol A Glucuronidation and Excretion in Liver of Pregnant and Nonpregnant Female Rats. Drug Metab Dispos. On line, 10/4.

In male rats challenged with the environmental estrogen bisphenol A, the compound is highly glucuronidated in the liver and is excreted largely into the bile. Given that in pregnancy the microsomal glucuronidation toward bisphenol A is attenuated, we hypothesized that elimination of bisphenol A from the liver may be reduced in pregnancy. This study was conducted to trace the elimination of bisphenol A in female rats, especially in pregnancy. In Sprague-Dawley rats, 1.5 micromol bisphenol A was perfused into the liver via the portal vein. In both the male and the nonpregnant female the infused bisphenol A was glucuronidated, then the resultant glucuronide was

excreted mainly into the bile. In pregnant rats, however, bilious excretion of bisphenol A glucuronide was 60% of that observed in nonpregnant rats, and venous excretion increased reciprocally. During 1 h perfusion, total excretion of the glucuronide from the liver of male, nonpregnant female and pregnant rats were 889.5 +/- 69.6, 1256.7 +/- 54.8 and 1038.8 +/- 33.3 nmoles, respectively. In Eisai hyperbilirubinemic rats (EHBR), perfusion of the liver with bisphenol A enabled us to determine that multidrug resistance-associated protein (MRP) 2-mediating transport is the mechanism behind excretion of the glucuronide into the bile. The expression of MRP2 has been reported to be noticeably reduced in pregnancy. These results suggest that bisphenol A elimination by hepatic glucuronidation is slightly less in pregnancy than in nonpregnancy and that in pregnancy more bisphenol A glucuronide is eliminated to the vein because of reduced MRP2 expression.

Inoue, H., Tsuruta, A., Kudo, S., Ishii, T., Fukushima, Y., Iwano, H., Yokota, H. and Kato, S. (2005). Bisphenol a glucuronidation and excretion in liver of pregnant and nonpregnant female rats. Drug Metab. Dispos. 33:55-59.

In male rats challenged with the environmental estrogen bisphenol A, the compound is highly glucuronidated in the liver and is excreted largely into the bile. Given that in pregnancy the microsomal glucuronidation toward bisphenol A is attenuated, we hypothesized that elimination of bisphenol A from the liver may be reduced in pregnancy. This study was conducted to trace the elimination of bisphenol A in female rats, especially in pregnancy. In Sprague-Dawley rats, 1.5 mumol of bisphenol A was perfused into the liver via the portal vein. In both the male and the nonpregnant female, the infused bisphenol A was glucuronidated, then the resultant glucuronide was excreted mainly into the bile. In pregnant rats, however, bilious excretion of bisphenol A glucuronide was 60% of that observed in nonpregnant rats, and venous excretion increased reciprocally. During 1-h perfusion, total excretion of the glucuronide from the liver of male, nonpregnant female, and pregnant rats was 889.5 +/- 69.6, 1256.7 +/- 54.8, and 1038.8 +/- 33.3 nmoles, respectively. In Eisai hyperbilirubinemic rats (EHBR), perfusion of the liver with bisphenol A enabled us to determine that multidrug resistance-associated protein (MRP)2-mediating transport is the mechanism behind excretion of the glucuronide into the bile. The expression of MRP2 has been reported to be noticeably reduced in pregnancy. These results suggest that bisphenol A elimination by hepatic glucuronidation is slightly less in pregnancy than in non-pregnancy and that in pregnancy, more bisphenol A glucuronide is eliminated to the vein because of reduced MRP2 expression.

Kabuto, H., S. Hasuike, N. Minagawa and T. Shishibori (2003). Effects of bisphenol A on the metabolisms of active oxygen species in mouse tissues. Environ. Res. **93**(1): 31-35.

Adult male ICR mice were injected ip for 5 days with 25 or 50 mg/kg/day bisphenol A and tissues were examined 6 hours after the last injection. Thiobarbituric acid-reaactive substance (TBARS) was measured as a peroxidation indicator. Also measured were the antioxidant (free radical scavenger), glutathione, as well as enzymes: superoxide dismutase (that catalyzes the dismutation of superoxide to hydrogen peroxide), and both glutathione peroxidase and catalyse (that convert hydrogen peroxide into water (hydrogen oxide). Oxidative stress reduces tissue levels of glutathione. Bisphenol A levels 6 hours after the last 50 mg/kg injection ranged from 0.19 (plasma) to 1.2 (fat) to 2 (kidney) ppb (ng/mg wet weight). There was a dose-related increase in liver superoxide dismutase activity, while liver catalyse decreased at 50 mg/kg/day bisphenol A, suggesting that bisphenol A produces an overproduction of hydrogen peroxide in the liver. The interesting aspect of this findings is that while bisphenol A, similar to other phenols, has antioxidant properties, it acts as a peroxidant via its other biological pathways. The 50 mg/kg/day dose of bisphenol A is the current LOAEL used by the EPA to calculate the reference dose of 50 µg/kg/day, which would not be affected by the results of this study. Significant effects were observed at the 25 µg/kg/day dose.

Kubo, T., N. Maezawa, M. Osada, S. Katsumura, Y. Funae and S. Imaoka (2004). Bisphenol A, an environmental endocrine-disrupting chemical, inhibits hypoxic response via degradation of hypoxia-inducible factor 1alpha (HIF-1alpha): structural requirement of bisphenol A for degradation of HIF-1alpha. Biochem Biophys Res Commun 318:1006-11.

Bisphenol A (BpA), an endocrine-disrupting chemical, is known to be a xenoestrogen and to affect the reproductive functions of animals. Recent reports have documented BpA-induced developmental abnormalities in the neuronal systems of humans and animals, and these effects appear to be non-estrogenic. In this study, we found that BpA inhibited the hypoxic response of human hepatoma cells. The expression of hypoxic response genes such as the erythropoietin (EPO) gene is done via a hypoxia inducible factor 1 (HIF-1)-dependent signaling pathway. To investigate possible structural requirements for this inhibitory effect, several BpA analogs were synthesized and added to this system. The blocking of two phenol groups in BpA did not change the effect, but the inhibition completely disappeared by the removal of two central methyl groups in BpA (the resulting compound is designated BpF). BpA, but not BpF, promoted degradation of the HIF-1alpha protein, which is a component of HIF-1, followed by inhibition of EPO induction. An immunoprecipitation assay indicated that BpA dissociated heat shock protein 90 (Hsp90) from HIF-1alpha and destabilized HIF-1alpha protein. HIF-1alpha is usually degraded first by ubiquitination and then by the proteasome pathway. Cobalt ion inhibits ubiquitination of HIF-1alpha and stabilizes it. In the present study, BpA promoted HIF-1alpha degradation in the presence of cobalt and in the presence of proteasome inhibitor. These results suggest that BpA degraded HIF-1alpha via a currently unknown pathway, and that this phenomenon required two methyl groups in BpA.

Kurebayashi, H., Betsui, H. and Ohno, Y. (2003). Disposition of a Low Dose of 14C-Bisphenol A in Male Rats and Its Main Biliary Excretion as BPA Glucuronide. Toxicol Sci 73:17-25.

Bisphenol A (BPA) is a weak xenoestrogen mass-produced with potential human exposure. The disposition of bisphenol A in male Fischer-344 (F344) rats dosed orally (100 or 0.10 mg/kg) or intravenously (0.10 mg/kg) was determined. Smaller amounts of the dose appeared in the urine. The main excretion route was feces in rats irrespective of dose and administration route. The biliary excretion during 6 h was 58-66% after iv dosing and 45-50% after oral dosing at 0.10 mg 14C-BPA/kg. Toxicokinetic parameters obtained from 14C-BPA-derived radioactivity in blood were the terminal elimination half-life, t1/2beta = 39.5 h, and total body clearance, CLtot = 0.52 l/h/kg after iv dosing of 0.10 mg 14C-BPA/kg to male rats. The blood concentration reached its maximum of 5.5 ng-eq/ml at 0.38 h after oral dose. AUC(0-6 h), AUC(0-48 h), and AUCinf of 14C-BPA-derived radioactivity, were 34, 118, and 192 ng-eqh/ml for the iv dose and 18, 102, and 185 ng-eqh/ml for the oral dose, respectively. The oral bioavailability of F(0-6 h), F(0-48 h), and Finf were 0.54, 0.86, and 0.97, respectively. The 14C-BPA-derived radioactivity was strongly bound to plasma protein (free fraction, fu = 0.046) and preferentially distributed to the plasma with a blood/plasma ratio of 0.67. From the bile of male rats orally dosed at 100 mg/kg, we have isolated and characterized BPA glucuronide (BPA-gluc) by ESI/MS, 1H and 13C NMR spectroscopy. HPLC analysis showed that BPA-gluc was the predominant metabolite in bile and urine. Unchanged BPA was mostly detected in feces. These results suggest that BPA is mainly metabolized to BPA-gluc and excreted into feces through the bile and subject to enterohepatic circulation in rats irrespective of dose and administration route.

Kurebayashi, H., Nagatsuka, S., Nemoto, H., Noguchi, H. and Ohno, Y. (2005). Disposition of low doses of 14C-bisphenol A in male, female, pregnant, fetal, and neonatal rats. Arch Toxicol 79:243-52.

Bisphenol A (BPA) is a weak xenoestrogen mass-produced with potential human exposure. The disposition of bisphenol A in male Fischer-344 (F344) rats dosed orally (100 or 0.10 mg/kg) or intravenously (0.10 mg/kg) was determined. Smaller amounts of the dose appeared in the urine. The main excretion route was feces in rats irrespective of dose and administration route. The biliary excretion during 6 h was 58-66% after iv dosing and 45-50% after oral dosing at 0.10 mg 14C-BPA/kg. Toxicokinetic parameters obtained from 14C-BPA-derived radioactivity in blood were the terminal elimination half-life, t1/2beta = 39.5 h, and total body clearance, CLtot = 0.52 l/h/kg after iv dosing of 0.10 mg 14C-BPA/kg to male rats. The blood concentration reached its maximum of 5.5 ng-eq/ml at 0.38 h after oral dose. AUC(0-6 h), AUC(0-48 h), and AUCinf of 14C-BPA-derived radioactivity, were 34, 118, and 192 ng-eqh/ml for the iv dose and 18, 102, and 185 ng-eqh/ml for the oral dose, respectively. The oral bioavailability of F(0-6 h), F(0-48 h), and Finf were 0.54, 0.86, and 0.97, respectively. The 14C-BPA-derived radioactivity was strongly bound to plasma protein (free fraction, fu = 0.046) and preferentially distributed to the plasma with a blood/plasma ratio of 0.67. From the bile of male rats orally dosed at 100 mg/kg, we have isolated and characterized BPA glucuronide (BPA-gluc) by ESI/MS, 1H and 13C NMR spectroscopy. HPLC analysis showed that BPA-gluc was the predominant metabolite in bile and urine. Unchanged BPA was mostly detected in feces. These results suggest that BPA is mainly metabolized to BPA-gluc and excreted into feces through the bile and subject to enterohepatic circulation in rats irrespective of dose and administration route.

Lee, B.-C., Kamata, M., Akatsuka, Y., Takeda, M., Ohno, K., Kamei, T. and Magara, Y. (2004). Effects of chlorine on the decrease of estrogenic chemicals. Water Research 38:733-739.

The effects of chlorination on the elimination of three estrogenic chemicals such as 17β-estradiol, nonylphenol and bis-phenol A were investigated using yeast two-hybrid assay (YTA), estrogen receptor (ER) competition assay (ER-CA), and high-performance liquid chromatography/mass spectrometry (LC/MS). The results of YTA, ER-CA and the analysis of LC/MS indicated that the estrogenic activity of the above-mentioned three endocrine disruptors were significantly reduced as a result of chlorination. The decrease in estrogenic activity paralleled a decrease in estrogenic chemicals under the influence of free chlorine. One common characteristic of estrogenic chemicals is the presence of a phenolic ring. Considering that a phenolic ring is likely to undergo some sort of transformation in an aqueous chlorination solution, the above-mentioned results may be applied to the rest of the estrogenic chemicals in natural waters.

Matsumoto, J., H. Yokota and A. Yuasa, 2002. Developmental increases in rat hepatic microsomal UDP-glucuronosyltransferase activities toward xenoestrogens and decreases during pregnancy. Environ. Health Perspect. 110: 193-196.

Glucuronidation enzymes are hepatic UDP-glucuronosyltransferase activities, specifically the bisphenol A-active isoform UGT2B1. Interestingly, Matsumoto et al. have reported that this UGT2B1 enzymatic activity with bisphenol A as the substrate is reduced in the pregnant mother compared to the non-pregnant female. The enzyme is absent in the fetus, and activity appears slowly after birth, with non-pregnant adult levels attained by three weeks. Therefore, the pregnant mother, the fetus and the newborn pup may show heightened sensitivity to bisphenol A due to reduced rate of clearance and increased half-life of unconjugated, estrogenically active bisphenol A.

Miyakoda, H., M. Tabata, S. Onodera and K. Takeda (1999). "Passage of bisphenol A into the fetus of the pregnant rat." J. Health Science 45(6): 318-323.

Sakamoto, H., H. Yokota, R. Kibe, Y. Sayama and A. Yuasa (2002). Excretion of bisphenol A-glucuronide into the small intestine and deconjugation in the cecum of the rat. Biochim. Biophys. Acta 1573:171-6.

In rats, BPA is metabolized to glucuronide by UDP-glucuronosyltransferase UGT2B1 in the liver and excreted into the bile. In the present study, we found that most of the bisphenol A-glucuronide (BPA-GA) excreted into the small intestine was deconjugated in the contents of the cecum. After BPA administration, BPA-GA was (immediately should be 15 min) found in the contents of the upper part of the small intestine, and then it moved to the lower part of the small intestine. However, only free BPA was found in the content of the cecum, and there was smaller amount of free BPA in the colon contents, indicating that BPA had been reabsorbed in the colon. BPA-GA was deconjugated by extract prepared from the cecum content which included highest beta-glucuronidase (beta-Gase) observed in Western blot analysis using antibodies against bacterial beta-Gase.These results indicate enterohepatic circulation of BPA and suggest that the adverse effects of BPA are enhanced by repeated exposure.

Shibata, N., J. Matsumoto, K. Nakada, A. Yuasa and H. Yokota (2002). Male-specific suppression of hepatic microsomal UDP-glucuronosyl transferase activities toward sex hormones in the adult male rat administered bisphenol A. 368:783-788.

This study reported that UDP-glucuronosyltransferase (UGT) activities towards bisphenol A, testosterone and oestradiol were significantly decreased in liver microsomes prepared from adult male Wistar rats fed the endocrine disruptor bisphenol A or DES (1 mg/2 days, 0.5 mg/day/300 g animal, 1.5 mg/kg/day; duration of treatment was 2 or 4 weeks). However, suppression of the transferase activities was not observed in female rats, even after bisphenol A treatment for 4 weeks. DES had the same effects, but p-cumylphenol had no effect on UGT activities towards sex hormones. Co-administration of an anti-oestrogen, tamoxifen (1 mg), inhibited the suppression of the transferase activities by bisphenol A. Western blotting analysis showed that the amount of UGT2B1, an isoform of UGT which glucuronidates bisphenol A, was decreased in the rat liver microsomes by the treatment. Northern blotting analysis also indicated that UGT2B1 mRNA in the liver was decreased by bisphenol A treatment. The suppression of UGT activities, UGT2B1 protein and UGT2B1 mRNA expression did not occur in female rats. The results indicate that bisphenol A treatment reduces the mRNA expression of UGT2B1 and other UGT isoforms that mediate the glucuronidation of sex hormones in adult male rats, and this suggests that the endocrine balance may be disrupted by suppression of glucuronidation.

Yuji Takao, Y., Miki Shimazua, Shinya Kohraa, Masaki Nagaea, Yasuhiro Ishibashib, Nobuaki Tominagac, Hiroshi Ishibashid, Shinich Yoshiharae, Koji Arizono. (2005). Photodecomposition and bioconcentration of a bisphenol A metabolite in medaka, *Oryzias latipes*. J. Health Sci.

Exposure experiments in medaka and photodecomposition tests were performed using a metabolite of bisphenol A (4-methyl-2,4-bis(p-hydroxyphenyl)-pent-1-ene; MBP), the solubility limit of which is 42 mg/L of water. Three adult medaka were kept in a 2 L glass beaker at 25±1°C for 4 days. The LC50 for 96 h was >1000 ppb. The measured average MBP concentration in the breeding water (nominal concentration of 100 ppb) was 49.2 ppb. The average concentration in the whole bodies of medaka after 4 days was 1.92 mg/g-wet body, and the bioconcentration factor (BCF) of MBP was calculated to be 39.0. MBP in water and acetone was decomposed very easily, with about 98% of the MBP being decomposed after several hours under sunlight. MBP was also decomposed after 48 hours of illumination under a white fluorescent lamp.

Takeuchi, T. and O. Tsutsumi (2002). "Serum bisphenol A concentrations showed gender differences, possibly linked to androgen levels." Biochemical & Biophysical Research Communications 291(1): 76-78.

Teeguarden, J.G., Waechter, J.M., Jr., Clewell, H.J., 3rd, Covington, T.R. and Barton, H.A. (2005). Evaluation of oral and intravenous route pharmacokinetics, plasma protein binding, and uterine tissue dose metrics of bisphenol A: a physiologically based pharmacokinetic approach. Toxicol Sci 85:823-838.

Bisphenol A (BPA) is a weakly estrogenic monomer used in the production of polycarbonate plastic and epoxy resins, both of which are used in food contact and other applications. A physiologically based pharmacokinetic (PBPK) model of BPA pharmacokinetics in rats and humans was developed to provide a physiological context in which the processes controlling BPA pharmacokinetics (e.g., plasma protein binding, enterohepatic recirculation of the glucuronide [BPAG]) could be incorporated. A uterine tissue compartment was included to allow the correlation of simulated estrogen receptor (ER) binding of BPA with increases in uterine wet weight (UWW) in rats. Intravenous- and oral-route blood kinetics of BPA in rats and oral-route plasma and urinary elimination kinetics in humans were well described by the model. Simulations of rat oral-route BPAG pharmacokinetics were less exact, most likely the result of oversimplification of the GI tract compartment. Comparison of metabolic clearance rates derived from fitting rat i.v. and oral-route data implied that intestinal glucuronidation of BPA is significant. In rats, but not humans, terminal elimination rates were strongly influenced by enterohepatic recirculation. In the absence of BPA binding to plasma proteins, simulations showed high ER occupancy at doses without uterine effects. Restricting free BPA to the measured unbound amount demonstrated the importance of including plasma binding in BPA kinetic models: the modeled relationship between ER occupancy and UWW increases was consistent with expectations for a receptor-mediated response with low ER occupancy at doses with no response and increasing occupancy with larger increases in UWW.
FUNDED BY THE CHEMICAL INDUSTRY

Yoo, S. D., B. S. Shin, Lee, B. M., Lee, K. C., Han, S. Y., Kim, H. S., Kwack, S. J. and Park, K. L. (2001). "Bioavailability and mammary excretion of bisphenol a in Sprague-Dawley rats." J. Toxicol. Enviro.n Health 64(5): 417-26.

Yoshihara, S., T. Mizutare, M. Makishima, N. Suzuki, N. Fujimoto, K. Igarashi and S. Ohta (2004). Potent estrogenic metabolites of bisphenol A and bisphenol B formed by rat liver S9 fraction: their structures and estrogenic potency. Toxicol Sci 78:50-9.

We previously demonstrated that the estrogenicity of either bisphenol A [BPA; 2,2-bis(4-hydroxyphenyl)propane] or bisphenol B [BPB; 2,2-bis(4-hydroxyphenyl)butane] was increased several times after incubation with rat liver S9 fraction (Yoshihara et al., 2001). This metabolic activation, requiring both microsomal and cytosolic fractions, was observed with not only rat liver, but also human, monkey, and mouse liver S9 fractions. To characterize the active metabolites of BPA and BPB, we investigated the structures of the isolated active metabolites by negative mode LC/MS/MS and GC/MS. The active metabolite of BPA gave a negative mass peak at [M-H](-) 267 on LC/MS and a single daughter ion at m/z 133 on MS/MS analysis, suggesting an isopropenylphenol dimer structure. Finally, this active metabolite was confirmed to be identical with authentic 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBP) by means of various instrumental analyses. The corresponding peaks of the BPB metabolite were [M-H](-) 295 and m/z 147, respectively, suggesting an isobutenylphenol dimer structure. Further, coincubation of BPA and BPB with rat liver S9 afforded an additional active metabolite(s), which gave a negative mass peak at [M-H](-) 281 and two daughter ion peaks at m/z 133 and m/z 147 on MS/MS analysis. These results strongly suggest that the active metabolite of either BPA or BPB might be formed by recombination of a radical fragment, a one-electron oxidation product of carbon-phenyl bond cleavage. It is

noteworthy that the estrogenic activity of MBP, the active metabolite of BPA, is much more potent than that of the parent BPA in several assays, including two reporter assays using a recombinant yeast expressing human estrogen receptor alpha and an MCF-7-transfected firefly luciferase plasmid.

Zalko, D., A. M. Soto, L. Dolo, C. Dorio, E. Rathahao, L. Debrauwer, R. Faure and J. P. Cravedi (2003). Biotransformations of bisphenol A in a mammalian model: answers and new questions raised by low-dose metabolic fate studies in pregnant CD-1 mice. Environ. Health Perspect. **111**:309-319.

The authors report that after maternal administration of a 20-23 µg/kg dose of bisphenol A, 2-3% of parent bisphenol A (about 50 ppt = 50 pg/ml) is bioavailable in the blood of mouse fetuses, and this dose of bisphenol A administered to mice and rats caused developmental effects in several prior studies, for example: (Nagel, vom Saal et al. 1997; Elswick, Welsch et al. 2000; Gupta 2000; Markey, Luque et al. 2001; Ramos, Varayoud et al. 2001. This is important since it has been reported that the levels of free bisphenol A in human fetal serum is in the range of 100-10,000 pg/ml (0.1 - 10 ppb), and the mean bisphenol A concentration in human male fetuses is 3.5 ppb [Schonfelder, 2002 #8). The effects being reported in fetal mice and rats thus appear to occur at doses below those found in human fetuses. Approximately 4% of bisphenol A appears in the mouse fetus after maternal exposure, indicating substantial trans-placental fetal exposure to low doses (Zalko, Soto et al. 2003). The maternal level of unconjugated, bioavailable bisphenol A was measured directly at these experiments. Circulating levels were 1,060 and 150 pg/ml at 0.5 and 2 hours after exposure, respectively, and the longer term residual exposure to 24 hours was lower. In a second experiment the circulating level in blood was 27 pg/g after the 25 µg/kg dose.

XX. EXPOSURE AND METABOLISM OF BISPHENOL A IN OTHER SPECIES

Biggers, W.J. and Laufer, H. (2004). Identification of juvenile hormone-active alkylphenols in the lobster Homarus americanus and in marine sediments. Biol Bull 206:13-24.

We have identified, by gas chromatography/mass spectrometry, four alkylphenols that are present in the hemolymph and tissues of the American lobster Homarus americanus and in marine sediments. These alkylphenols are used industrially in antioxidant formulations for plastic and rubber polymer manufacturing, and are similar in structure to a known endocrine disruptor, bisphenol A. The compound 2-t-butyl-4-(dimethylbenzyl)phenol was present at concentrations of 0.02 to 1.15 microg/ml in hemolymph and 8.95 to 21.58 microg/g in sediments. A second compound, 2,4-bis-(dimethylbenzyl)phenol, was present at concentrations between 0.07 and 19.78 microg/ml in hemolymph and 138.94 to 224.89 microg/g in sediment, while a third compound, 2,6-bis-(t-butyl)-4-(dimethylbenzyl)phenol, was found at concentrations between 0.01 and 13.00 microg/ml in hemolymph, 2.55 and 6.11 microg/g in hepatopancreas, and 47.85 and 74.66 microg/g in sediment. A fourth compound, 2,4-bis-(dimethylbenzyl)-6-t-butylphenol, was found at concentrations of 0.20 to 70.71 microg/ml in hemolymph, 23.56 to 26.89 microg/g in hepatopancreas, and 90.68 to 125.58 microg/g in sediment. These compounds, along with bisphenol A, 4-dimethylbenzylphenol, and nonylphenol, display high juvenile hormone activity in bioassays. Alkylphenols at high concentrations are toxic to crustaceans and may contribute significantly to lobster mortality; at lower concentrations, they are likely to have endocrine-disrupting effects. Bisphenol A showed a very low EC50 of 0.05 µM (11.4 parts per billion) in a bioassay that detects an effect of juvenile hormone on larval metamorphosis, which is stimulated by juvenile hormone. This indicates that bisphenol A has very high juvenile hormone activity in lobsters.

Lindholst C, Wynne PM, Marriott P, Pedersen SN, Bjerregaard P. 2003. Metabolism of bisphenol A

in zebrafish (Danio rerio) and rainbow trout (Oncorhynchus mykiss) in relation to estrogenic response. Comparative Biochemistry & Physiology C Toxicol Pharmacol 135:169-177.

The kinetics of bisphenol A (BPA) were investigated in zebrafish (Danio rerio) exposed to 100 mug BPA/1. BPA uptake was measured during a 7-day period followed by an elimination phase of similar duration. After 2, 6, 12, 24, 48, 72, 120 and 168 h of uptake/elimination, fish were analysed for their content of BPA, bisphenol A glucuronic acid (BPAGA) and bisphenol A sulfate (BPAS). Within the first 24 h steady state levels of BPA, BPAGA and BPAS were reached and the total body concentrations were calculated to be 569, 12 600 and 39.9 ng/g fish, respectively. Elimination rates of the three compounds in zebrafish were estimated by fitting the data to a compartment model. An initial rapid elimination phase was observed for BPA and BPAS with total body half lives (T-1/2) of <1.1 h and 30 min, followed by a slower second elimination phase with T-1/2 values of 139 and 71 h, respectively. Excretion of BPAGA occurred from a single compartment with a T-1/2 of 35 It. The steady state concentration of BPA and its metabolites were investigated in rainbow trout (Oncorhynchus mykiss) exposed to 100 mug/BPA/1. The toxicokinetic parameters from zebrafish and rainbow trout were compared; including previously published data on the rainbow trout. The data indicate that the smaller estrogenic sensitivity observed for the zebrafish may be caused by a more rapid metabolism of BPA in the zebrafish liver.

XXI. ENVIRONMENTAL MONITORING AND ANALYTICAL METHODS

Ackermann, G.E., Brombacher, E. and Fent, K. (2002). Development of a fish reporter gene system for the assessment of estrogenic compounds and sewage treatment plant effluents. Environ Toxicol Chem 21:1864-1875.

This study reports on the development and application of a fish-specific estrogen-responsive reporter gene assay. The assay is based on the rainbow trout (Oncorhynchus mykiss) gonad cell line RTG-2 in which an acute estrogenic response is created by cotransfecting cultures with an expression vector containing rainbow trout estrogen receptor a complementary DNA (rtERalpha cDNA) in the presence of an estrogen-dependent reporter plasmid and an estrogen receptor (ER) agonist. In a further approach, RTG-2 cells were stably transfected with the rtERalpha cDNA expression vector, and clones responsive to 17beta-estradiol (E2) were selected. The estrogenic activity of E2, 17alpha-ethinylestradiol, 4-nonylphenol, nonylphenoxy acetic acid, 4-tert-octylphenol, bisphenol A, o,p'-DDT, p,p'-DDT, o,p'-2,2-bis(chlorophenyl)-1,1-dichloroethylene (o,p'-DDE), p,p'-DDE, o,p'-2,2-bis(chlorophenyl)-1,1-di-chloroethane (o,p'-DDD), p,p'-DDD, and p,p'-2,2-bis(chlorophenyl)acetic acid (p,p'-DDA) was assessed at increasing concentrations. All compounds except o,p'-DDT, p,p'-DDE, and p,p'-DDA showed logistic dose-response curves, which allowed the calculation of lowest-observed-effect concentrations and the concentrations at which half-maximal reporter gene activities were reached. To check whether estrogen-responsive RTG-2 cells may be used to detect the estrogenic activity of environmental samples, an extract from a sewage treatment plant (STP) effluent was assessed and found to have estrogenic activity corresponding to the transcriptional activity elicited by 0.05 nM of E2. Dose-response curves of nonylphenol, octylphenol, bisphenol A, and o,p'-DDD revealed that the RTG-2 reporter gene assay is more sensitive for these compounds when compared to transfection systems recombinant for mammalian ERs. These differences may have an effect on the calculation of E2 equivalents when estrogenic mixtures of known constitution, or environmental samples, such as STP effluents, are assessed.

Andreescu, S. and Sadik, O.A. (2004). Correlation of analyte structures with biosensor responses using the detection of phenolic estrogens as a model. Anal Chem 76:552-60.

The apparent increase in hormone-induced cancers and disorders of the reproductive tract in wildlife and humans has led to a search for an accurate and reliable method for monitoring endocrine-disrupting chemicals (EDCs). This study presents a generic approach that may allow researchers to establish screening procedures for potential EDCs by correlating the analyte structures with biosensor responses and explain possible reaction mechanisms. A simple amperometric tyrosinase-based biosensor (Tyr-CPE) has been developed for the detection of phenolic EDCs. The investigation of the enzymatic oxidation of selected phenolic estrogens was first carried out using UV-vis spectroscopy. The result was used to correlate sensor responses to enzymatic activity. Natural phytoestrogen polyphenols, including resveratrol (RES), genistein (GEN), and quercetin (QRC), were compared with synthetic estrogens, for example, bisphenol A (BPhA), nonylphenol (NPh), and diethylstilbestrol (DES). The Tyr-CPE biosensor resulted in rapid, simple, and accurate measurement of phenolic estrogens with varying degrees of sensitivity, selectivity, and response times. The sensor responses have been evaluated for the detection of binary and tertiary mixtures of EDCs and natural estrogens. The results showed that BPhA could be successfully discriminated in a composite mixture containing NPh and DES at various ratios. In the case of natural phenolic estrogens GEN, RES, and QRC, the sensor allows the determination of a total phenolic content. The sensor was also validated for the detection of BPhA in a real environmental water sample, and the results was compared with standard ASTM method 9065. Mechanistically, our results indicated that the number of OH groups, the nature and the position of aryl ring substituents, or both could affect the detection limit and the biosensor sensitivity.

Belfroid, A., van Velzen, M., van der Horst, B. and Vethaak, D. (2002). Occurrence of bisphenol A in surface water and uptake in fish: evaluation of field measurements. Chemosphere 49:97-103.

In this study the actual presence of the suspected endocrine disrupter Bisphenol A (BPA) in water systems was studied in the Netherlands. BPA was shown to be present in Dutch surface water at levels up to 330 ng/l, and one occasional observation of 21 microg/l. During the three sampling periods, 60-80% of the samples, most from marine and estuarine locations, contained BPA levels below the limit of quantification (14-40 ng/l). At a selected number of locations the presence of BPA in fish was studied, which showed that BPA varied from 2 to 75 ng/g in the liver and 1 to 11 ng/g in the muscle. Based on present measured concentrations in surface water and on literature derived toxicity data it was concluded that ecotoxicological effects nor estrogenic effects are likely to occur in the field situation.

Brock, J. W., Y. Yoshimura, J. R. Barr, V. L. Maggio, S. R. Graiser, H. Nakazawa and L. L. Needham (2001). Measurement of bisphenol A levels in human urine. Journal of Exposure Analysis Environmental Epidemiology 11(4): 323-328.

We report a new approach for assessing human exposure to bisphenol A (BPA) by measuring BPA in urine after enzymatic deglucuronidation. This method involves addition of C-13(12)-labeled BPA, enzymatic deconjugation, solid-phase extraction, and derivatization with pentafluorobenzyl bromide. The product of the derivatization is separated by gas chromatography followed by mass spectrometric detection using negative chemical ionization and selected ion monitoring. Using this analysis method, urine samples fortified with both a constant level of labeled BPA and a range of unlabeled BPA levels (0.27-10.6 ng/ml) demonstrated constant percentage recovery. In addition, a range of urine sample volumes (0.25-10.0 ml) with constant amounts of added internal standard produced a linear response (r(2)=0.99). The method limit of detection was 0.12 ng/ml. This method was validated by duplicate analyses using gas chromatography coupled to a high-resolution mass spectrometer.

Estevez-Alberola, M.C. and Marco, M.P. (2004). Immunochemical determination of xenobiotics with endocrine disrupting effects. Anal Bioanal Chem 378:563-575.

This paper is a review with more than 100 references discussing the immunochemical methods reported in the literature for the most important man-made chemicals with suspected endocrine disrupting activity. Details regarding immunizing hapten design, antibody production, and the features (limit of detection, dynamic range, specificity) of the most important immunochemical methods developed (ELISA, FIIA, immunosorbents, immunosensors, etc.) are presented for important environmental pollutants such as bisphenol A, phthalates, alkylphenol polyethoxylates, alkylphenols, polychlorinated biphenyl compounds, and dioxins. Availability of commercial reagents and methods is reported.

Kang, J.H. and Kondo, F. (2005). Bisphenol A degradation in seawater is different from that in river water. Chemosphere 60:1288-1292.

The purpose of this study was to identify a relationship between changes of bacterial counts and bisphenol A (BPA) degradation in seawater under aerobic or anaerobic conditions, and at temperatures of 4, 25, and 35 degrees C. Water samples (seawater and river water) spiked with 1000ng/ml of BPA was placed for 60d. The BPA from water samples was extracted by OASIS HLB cartridges and was detected by high-performance liquid chromatography with fluorescence detection. BPA in river water was degraded under aerobic conditions and was below a detection limit (0.5ng/ml) on the seventh day at both 25 and 35 degrees C. The more the level of bacterial counts increased, the more BPA degradation decreased. In the case of seawater samples, there was no relationship between BPA degradation and the change of bacterial counts. Bacterial counts at 25 and 35 degrees C increased rapidly at 5 and 3d, respectively, but decreased since then. The concentration of BPA was not changed for 30d at both 25 and 35 degrees C, but decreased from 40 to 60d in spite of low levels of bacteria. These results show that the different degradation way for BPA in seawater may exist. Moreover, our study suggest that BPA in seawater than in river water can continue for longer time with no degradation and the possibility of BPA contamination on a marine organism can be higher than that on freshwater organism.

Koda, T., Soya, Y., Negishi, H., Shiraishi, F. and Morita, M. (2002). Improvement of a sensitive enzyme-linked immunosorbent assay for screening estrogen receptor binding activity. Environ Toxicol Chem 21:2536-2541.

A competitive enzyme-linked immunosorbent assay (ELISA) with estrogen receptor (alpha) and a fluorescence depolarization method with Full-Range Beacon were examined as estrogen receptor binding assays to prescreen endocrine-disrupting chemicals (EDCs). In this study, because it is difficult to measure the receptor binding ability of sparingly water-soluble chemicals using these methods, the competitive enzyme immunoassay was further modified for improved sensitivity by changing the operational parameters, such as receptor concentration, ligand concentration, and the reaction temperature. The method was applied to 10 test chemicals, including alkylphenols and bisphenol A (BPA). The diethylstilbestrol (DES) relative binding affinity (RBA) of ELISA kit was set equal to 1 (RBA = IC50/IC50 of DES). The RBAs of BPA, 4-nonylphenol (p-NP), and 4-t-octylphenol (p-t-OP) are 5386, 8619. and 8121 before using the improved competitive enzyme immunoassay and 883, 699, and 2832 using improved it respectively. Mixtures of BPA, p-NP, and p-t-OP gave results that the estrogen binding affinities of these chemicals are additive or slightly more than additive.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B. and Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national survey. Environ. Sci. Technol. 36:1202-1211.

This study was conducted by the U.S. Geological Survey to characterize pollutants in 139 streams in 30 states. They reported detecting bisphenol A in 40% of streams at concentrations up to 12 ppb (median 0.14 ppb; detection limit 0.09 ppb).

Kuch, H. M. and K. Ballschmiter (2001). "Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range." Environ. Sci. Technol. 36: 3201-3206.

Matsumoto, H., Adachi, S. and Suzuki, Y. (2005). Bisphenol A in ambient air particulates responsible for the proliferation of MCF-7 human breast cancer cells and its concentration changes over 6 months. Arch Environ Contam Toxicol 48:459-466.

To survey the estrogenic activity of the organic extracts from particulate matter of urban ambient outdoor air, samples were collected on glass fiber filters using a high-volume air sampler on the rooftop of our institute for 6 months (six filters/month). After extracting the organic materials and separating them into three fractions, i.e., acidic, neutral, and basic, we applied a cell-growth assay using MCF-7 human breast cancer cells to the original extract and the extracts of the fractions. Only the extract in the acidic fraction showed cell proliferation activity in a dose-response manner. To survey the chemical(s) responsible for the activity, a gas chromatography/mass spectrometry (GC/MS) analysis was conducted after silylating the extract. The presence of bisphenol A (BPA) was confirmed, because the retention times and the MS fragment patterns between the silylated derivative of a component in the sample and that of BPA itself were the same. By using a GC/MS-SIM (selective ion monitoring) technique, the average value was found to be 0.51 ng/m(3) of air (range: 0.02 approximately 1.92 ng/m(3) of air). The trend of the residual levels in air particulates showed seasonal variation, increasing from autumn to winter and decreasing from winter to spring. The only exception was that the value in January was lower than those in December and February. Considering the content of BPA in the extract of the acidic fraction and the strength of the activities with the extract and BPA itself, the estrogenic activity due to BPA in the fraction seemed to decrease. In spite of this decline, the possibility remains that the estrogenic activity mainly originated from BPA.

Onn Wong, K., Woon Leo, L. and Leng Seah, H. (2005). Dietary exposure assessment of infants to bisphenol A from the use of polycarbonate baby milk bottles. Food Addit Contam 22:280-288.

The residual bisphenol A (BPA) levels in 28 different brands of polycarbonate (PC) baby milk bottles available in the Singapore market were measured. With a detection limit of 3 mg/kg, BPA residues were detected in 19 out of the 28 PC baby milk bottles at levels between 4.01 and 141 mg/kg, with a mean of 28.1 +/- 31.4 mg/kg and a median of 17.2 mg/kg. The potential migration of BPA from each of the 28 PC milk bottles was also measured using food-simulating solvents and time conditions recommended by the US Food and Drug Administration (US FDA), but using temperatures more severe than actual use. The highest upper-bound mean BPA migration levels of 0.64 +/- 0.48 microg/in2 in 10% ethanol at 70 degrees C and 0.43 +/- 1.25 microg/in2 in corn oil at 100 degrees C were observed after incubating cut portions of the milk bottles for 240 h. With this migration data and using US FDA's procedure for estimation of dietary exposure, the worst-case dietary exposure assessment for the intake of BPA by infants between birth and three months of age was below the oral Reference Dose of 0.05 mg/kg bw/day established by the US Environmental Protection Agency. This study showed that the dietary exposure to BPA from actual uses of PC milk bottles is unlikely to pose a health risk in infants.

Peterman, P.H., Orazio, C.E. and Gale, R.W. (2000). Detection of tetrabromobisphenol A and formation of brominated 13C--bisphenol A's in commercial drinking water stored in reusable polycarbonate containers. Environmental Chemistry of Water: 2000 and Beyond, San Francisco. Available online.

Rudel, R. A., J. G. Brody, et al. (2001). Identification of selected hormonally active agents and animal mammary carcinogens in commercial and residential air and dust samples. Journal of the Air and Waste Management Association 51: 499-513.

In order to characterize typical indoor exposures to chemicals of interest for research on breast cancer and other hormonally mediated health outcomes, methods were developed to analyze air and dust for target compounds that have been identified as animal mammary carcinogens or hormonally active agents and that are used in commercial or consumer products or building materials. These methods were applied to a small number of residential and commercial environments to begin to characterize the extent of exposure to these classes of compounds. Phenolic compounds, including nonylphenol, octylphenol, bisphenol A, and the methoxychlor metabolite 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), were extracted, derivatized, and analyzed by gas chromatography/mass spectrometry (GC/MS)-selective ion monitoring (SIM). Selected phthalates, pesticides, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) were extracted and analyzed by GC/MS-SIM. Residential and workplace samples showed detectable levels of twelve pesticides in dust and seven in air samples. Phthalates were abundant in dust (0.3-524 micrograms/g) and air (0.005-2.8 micrograms/m3). Nonylphenol and its mono- and di-ethoxylates were prevalent in dust (0.82-14 micrograms/g) along with estrogenic phenols such as bisphenol A and o-phenyl phenol. In this 7-sample pilot study, 33 of 86 target compounds were detected in dust, and 24 of 57 target compounds were detected in air. In a single sample from one home, 27 of the target compounds were detected in dust and 15 in air, providing an indication of chemical mixtures to which humans are typically exposed.

Sajiki, J. and Yonekubo, J. (2004). Inhibition of seawater on bisphenol A (BPA) degradation by Fenton reagents. Environ Int 30:145-150.

To investigate bisphenol-A (BPA) degradation in seawater using Fenton reagents, changes in the BPA recovery and in the concentration of BPA metabolite, BPA-o-quinone in the three water samples; BPA free deionizad water (control water), 3% aq. NaCl and seawater as a function of time after BPA fortification in the presence of radical oxygen species (ROS) at 20 degrees C were investigated. The BPA recoveries were lower in both 3% aq. NaCl and seawater than in the control water. The BPA recovery in aq. NaOCl decreased as a function of NaOCl concentration, indicating that BPA could be degraded by the potent radical ion (OCl-) at the concentration of above 2 microM. A BPA metabolite, BPA-o-quinone was formed in all the water samples after addition of ROS which was produced by Fenton reaction (reaction of 0.11 M H2O2 and 0.44 mM FeCl3.6H2O). These results indicated that BPA degradation could occur by an addition of ROS and further accelerated by the formation of OCl- in salt containing water samples. BPA recovery was the highest in seawater immediately after addition of Fenton reagents and the amount of BPA-o-quinone was very low, which suggests that seawater possesses an inhibitory system on BPA degradation. There was a positive correlation ($p<0.01$) between the fortified iron concentration and turbidity in seawater. Turbidity might be originated from iron-binding substances. Degradation threshold of BPA was observed when Fenton reaction was employed in seawater fortified with high amount of BPA. The present study suggested that iron trapping caused an inhibition on BPA degradation by Fenton reagents.

Tabota, A., Kashiwada, S., Ohnishi, Y., Ishikawa, H., Miyamoto, N., Itoh, M. and Magara, Y. (2001). Estrogenic influences of estradiol-17ß, o-nonylphenol, and bisphenol A on Japanese medaka (Oryzias latipes) at detected environmental concentrations. Water Science and Technology 43:109-116.

Rivers in Japan were reported to have bisphenol A levels ranging from undetectable to 1.4 ppb. In two studies, bisphenol A was detected in 147/256 and 109/261 rivers.

Takahashi, A., Higashitani, T., Yakou, Y., Saitou, M., Tamamoto, H. and Tanaka, H. (2003). Evaluating bioaccumulation of suspected endocrine disruptors into periphytons and benthos in the Tama River. Water Sci. Technol. 47:71-76.

There are two major routes through which fish are exposed to endocrine disruptors (EDs); one route is through water that is a habitat; the other is through aquatic food such as algae and benthos. Few studies on the bioaccumulation of EDs in food have been conducted. Therefore, we evaluated the concentration in food of nonylphenol (NP), bisphenol A (BPA) and 17beta-estradiol (E2), which were frequently detected in river water and in final discharge of Wastewater Treatment Plants (WWTPs) in Japan. We also evaluated the estrogenicity of samples using recombinant yeast. NP concentrations ranged 0.1-0.4 microg/L in the river water, while they ranged 8-130 microg/kg-wet in the periphytons and 8-140 microg/kg-wet in the benthos. BPA concentrations ranged 0.02-0.15 microg/L in the river water, while they ranged 2-8.8 microg/kg-wet in the periphytons and 0.3-12 microg/kg-wet in the benthos. E2 concentrations ranged 0.0001-0.0076 microg/L in the water, while they ranged 0.09-2.26 microg/kg-wet in the periphytons and <0.01-0.22 microg/kg-wet in the benthos. The estrogenicity ranged 0.0001-0.0464 microg-E2equivalent/L in the water, while it ranged 3.4-66.8 microg-E2equivalent/kg-wet in the periphytons and 7.4-5458 microg-E2equivalent/kg-wet in the benthos. Bioaccumulation factors of NP are estimated as 160-650 for the periphytons, and 63-990 for the benthos, respectively. Bioaccumulation factors of BPA are estimated as 18-650 for the periphytons, and 8-170 for the benthos, respectively. Bioaccumulation factors of E2 are estimated as 64-1,200 for the periphytons, and 100-160 for the benthos, respectively. The ratios of the periphytons and the benthos to the water in terms of the estrogenicity were larger than those in terms of the chemicals. In particularly, the ratio of the benthos to the water is about 10(6) in the maximum. The results suggest that food may be a more important route for fish exposed to EDs in water.

Watabe, Y., Takuya Kondo, Hiroe Imai, Masatoshi Morita, Nobuo Tanaka, and Ken Hosoya. (2004). Reducing Bisphenol A Contamination from Analytical Procedures To Determine Ultralow Levels in Environmental Samples Using Automated HPLC Microanalysis. Anal. Chem. 76:105-109.

A new high-performance liquid chromatography (HPLC) method has been developed to detect ultralow concentrations, below 1 ng/L (ppt), of bisphenol A (BPA) using column switching, and electrochemical detection. This HPLC method provided a detection limit of 0.36 ppt BPA and repeatability of 9.3% with a relative standard deviation at 1 ppt (r) 0.999 with a linear calibration curve over a concentration range of 1-100 ppt.). BPA is inherently ubiquitous in the environment, including the very tools and solvents used for its analysis, so to obtain meaningful results, the overall BPA contamination concentration must be below the detection limit for BPA using the analytical system. Therefore, purified water for preparing the standard BPA solution must be filtered with a hydrophobic membrane to suppress BPA background levels of contamination. In addition, all of the glassware used to prepare the standards and samples for analysis must be treated carefully to eliminate residual BPA contamination. Although BPA-free water and heattreated glassware were used as precautionary measures for analysis, manual preparation and injection resulted in considerable BPA levels that will confound the results. Furthermore, the use of manual injection syringes with a fixed cemented needle also contributed to substantial BPA contamination, even if

fluorescence detection was employed. However, manual injection syringes with a removable needle gave rather good results compared to that of the cemented needle type. By employing these precautionary measures and procedures to reduce BPA contamination from the analytical procedure, 1-10 ppt of BPA in environmental water samples was accurately determined using a columnswitching HPLC system. This paper describes a systematic procedure and solution for reducing BPA contamination introduced by methods and procedures to determine a full range of BPA concentrations in environmental samples, such as lake water, even at very low concentrations.

Yamamoto, T., Yasuhara, A., Shiraishi, H. and Natasugi, O. (2001). Bisphenol A in hazardous waste landfill leachates. Chemosphere 42:415-418.

Bisphenol A was found in 7/10 hazardous landfill sites in Japan at levels ranging from 1.3 ppb to 17,200 ppb (median = 269 ppb, detection limit 0.4 ppb). The highest levels of bisphenol A were associated with landfills containing household waste, mainly waste plastics), while low levels of bisphenol A were found at sites with little household waste or plastics.

Yasuda, S., Wu, P.S., Hattori, E., Tachibana, H. and Yamada, K. (2004). Simultaneous determination of isoflavones and bisphenol A in rat serum by high-performance liquid chromatography coupled with coulometric array detection. Biosci Biotechnol Biochem 68:51-58.

A method for simultaneous detection and quantification is presented to determine the presence of isoflavones and bisphenol A in a biological sample. A coulometric array detector was used with reversed-phase high-performance liquid chromatography (HPLC). Daidzein (1), glycitein (2), genistein (3) and their glucoside conjugates, daidzin (4), glycitin (5) and genistin (6), were measured as phytochemicals. Also assayed here was equol (7), a metabolite from compound 1, and bisphenol A (8), an industrial chemical that acts as an endocrine disrupter. All chemicals were simultaneously detected by using a 600-mV single detection voltage with high efficacy. A mixture of 1, 3 and 8 was orally administered to rats, and the levels of these three chemicals in the serum were clearly increased after a 4 kU beta-glucuronidase treatment. The levels of compounds 1 and 3 in the serum were detected at 1665 and 2040 ng/ml, while 8 was at a low level of 417 ng/ml. Compound 7 in the serum was not detected until after enzymatic hydrolysis (72 ng/ml). These results suggest that this analytical method would be useful for metabolic and pharmacokinetic studies on isoflavones and bisphenol A.

XXII. LEACHING OF BISPHENOL A FROM PRODUCTS

Bae, B., Jeong, J.H. and Lee, S.J. (2002). The quantification and characterization of endocrine disruptor bisphenol-A leaching from epoxy resin. Water Sci Technol 46:381-7.

Bisphenol-A (BPA), a known endocrine disruptor, is a main building block of epoxy resin which has been widely used as a surface coating agent on residential water storage tanks. Therefore, BPA leaching from the epoxy resin can adversely affect human health. In this study, BPA leaching from three epoxy resins were quantified at 20, 50, 75 and 100 degrees C both in deionized water and the specified test water, respectively. BPA leached to the test water was identified using GC-MS and quantified with GC-FID after a sequential extraction and concentration. The results showed that BPA leaching has occurred in all three samples tested. The quantity of BPA from unit area of epoxy resin coating was in the range of 01.68-273. 12 microg/m2 for sample A, 29.74-1734.05 microg/m2 for sample B and 52.86-548.78 microg/m2 for sample C depending on the test temperature, respectively. In general, the amount of BPA leashing increased as the water temperature increases. This result implies a higher risk of BPA leaching to drinking water during a summer season. In addition, microbial growth, measured by colony forming units, in epoxy coated water tanks was higher than

that in a stainless steel tank. The results suggest that compounds leaching from epoxy resin may support the growth of microorganisms in a residential water holding tank.

Brede, C., P. Fjeldal, I. Skjevrak and H. Herikstad (2003). Increased migration levels of bisphenol A from polycarbonate baby bottles after dishwashing, boiling and brushing. Food Addit. Contam. 20(7): 684-9.

Bisphenol A leaching (detection limit, 0.1 ppb) was measured in twelve polycarbonate baby bottles after dishwashing, brushing and boiling. Water at 100 C was placed in the bottles for 1 hr. Bisphenol A leaching from new bottles was 0.23 ppb, diswashed 51 times = 8.4 ppb, diswashed 169 times = 6.7 ppb. TDI in Europe of 10 µg/kg/day is not exceeded, but many adverse effects in animals occur at lower exposures, assuming about one liter of formula consumed per day and a 5 kg baby, resulting in about 1.5 µg/kg/day.

Brotons, J. A., M. F. Olea-Serrano, M. Villalobos, V. Pedraza and N. Olea (1995). Xenoestrogens released from lacquer coating in food cans. Environ. Health Perspect. **103**:608-612.

We present data showing that some foods preserved in lacquer-coated cans and the liquid in them may acquire estrogenic activity. Hormonal activity was measured using the E-screen bioassay. The biological activity of vegetables packed in cans was a result of plastic monomers used in manufacturing the containers. The plastic monomer bisphenol-A, identified by mass spectrometry, was found as a contaminant not only in the liquid of the preserved vegetables but also in water autoclaved in the cans. The amount of bisphenol-A in the extracts accounted for all the hormonal activity measured. Although the presence of other xenoestrogens cannot be ruled out, it is apparent that all estrogenic activity in these cans was due to bisphenol-A leached from the lacquer coating. The use of plastic in food-packaging materials may require closer scrutiny to determine whether epoxy resins and polycarbonates contribute to human exposure to xenoestrogens.

Cowie, J. M. G. (1991). Polymers: Chemistry and Physics of Modern Materials. New York,, Chapman and Hall.

Cowie states that the polymerization reaction for polycarbonate may approach 100% but rarely achieves it. Usually some amount of reactants remain in the finished product, which is consistent with some leaching of bisphenol A from new polycarbonate products.

Factor, A. (1996). Mechanisms of thermal and photodegradations of bisphenol A polycarbonate. Polymer Durability: Degradation, Stabilization, and Lifetime Prediction. R. L. Clough, N. C. Billingham and K. T. Gillen. Washington, D.C., American Chemistry Society**:** 59-76.

Factor notes that “Perhaps the most important but most easily overlooked aspect of bisphenol A-polycarbonate stability is its vulnerability to reaction with water … both during melt processing and in end-use applications involving exposure to water at elevated temperatures, such as sterilization by autoclaving”. Industry tries to minimize the susceptibility by removing catalytic residues, carefully drying before melt processing, and adding certain stabilizers. However, bisphenol A is released as a hydrolysis breakdown product of the repeated washing in the sanitizing cage washer and as unreacted molecules due to an increase in the surface area of the cage interior as a result of wear. The polysulfone co-polymer is predicted to be less susceptible to hydrolysis due to the ether bond between polysulfone co-polymers, relative to the ester bond in polycarbonate. This has been shown in studies reported by Union Carbide Corporation.

Howdeshell, K. L., P. H. Peterman, B. M. Judy, J. A. Taylor, C. E. Orazio, R. L. Ruhlen, F. S. vom Saal and W. V. Welshons (2003). Bisphenol A is released from used polycarbonate animal cages into water at room temperature. Environ. Health Perspect. 111:1180-1187.

Polycarbonate and polysulfone are both strong thermoplastics that are, in most cases, made using bisphenol A. Technically called co-polymers, they are produced by reacting two different molecules together resulting in combined units (n = 50-100 units) and molecular mass to approximately 25,000 Da. Bisphenol A is a symmetrical aromatic molecule that reacts on both phenolic ends in polymerization reactions. Bisphenol A is produced by an acid-catalyzed reaction of acetone and phenol. For polycarbonate, bisphenol A typically reacts with phosgene forming an ester linkage, while for polysulfone, BPA typically reacts with dichlorodiphenyl sulfone to form an ether linkage. The co-polymers strength comes from the rigid aromatic rings while inherent flexibility comes from the ether and ester C-O single bonds which are freer to rotate. Both co-polymers are amorphous (i.e. they do not form a crystalline structure), but can be melted and formed or reformed into strong structures thus suitable for use as thermoplastics.

Howdeshell reported that new polycarbonate cages released low but detectable (by GC-MS) amounts of free bisphenol A into water placed into the cages at room temperature, and that old polycarbonate cages that were discolored due to repeated washing (using normal animal cage cleaning procedures in an AALAC-accredited facility) released up to 310 µg/liter free bisphenol A into water standing in the cage for 7 days at room temperature. This amount is greater than the bisphenol A that leached from the cages in the Hunt study described as “severely damaged” due to being washed in an alkaline detergent by Hunt et al. (2003). Taken together, these findings demonstrate that just based on normal use in animal research facilities, polycarbonate cages and water bottles will release enough bisphenol A as a result of repeated washings to adversely impact the exposed animals. If this can occur in female mice due to drinking from visibly worn polycarbonate bottles, there should be concern about similar adverse effects on the meiosis-I arrested oocytes in newborn children drinking from old, visibly worn polycarbonate baby bottles.

Hunt, P. A., K. E. Koehler, M. Susiarjo, C. A. Hodges, A. Hagan, R. C. Voigt, S. Thomas, B. F. Thomas and T. J. Hassold (2003). Bisphenol A causes meiotic aneuploidy in the female mouse. Current Biol. **13**:546-553.

An adverse effect of exposure to very low doses of bisphenol A is profound disruption of chromosomes during meiosis in oocytes in female mice exposed to bisphenol A that leached out of polycarbonate animal cages and water bottles after being washed in an alkaline detergent. Specifically, Hunt and colleagues report that after washing polycarbonate cages and water bottles in an alkaline detergent (which greatly accelerates the breakdown of the polycarbonate ester bonds), there was a dramatic increase in the incidence of abnormal alignment of chromosomes during the first meiotic division in oocytes. This results in aneuploidy, or abnormal numbers of chromosomes in oocytes, such as occurs in Down’s syndrome; these authors thus refer to bisphenol A as a “potent meiotic aneugen”. Aneuploidy is thought to be a major cause of embryonic mortality in humans; with the exception of chromosome 21 (Down's syndrome), abnormal numbers of chromosomes are typically lethal. Hunt reported that severe oocyte chromosome abnormalities increased in peripubertal female mice from a baseline frequency of 1.8% in control animals (not housed in damaged cages) to 20% due to housing females in damaged polycarbonate cages, 30% due to the use of damaged polycarbonate water bottles, and 41% due to combined use of both damaged cages and water bottles. In a subsequent experiment the researchers intentionally damaged polycarbonate water bottles by washing them different numbers of times in the detergent. The polycarbonate water bottles were found by gas chromatography-mass spectrometry (GC-MS) analysis to release between 100 (mild damage) and 260 µg/liter (severe damage) free bisphenol A into water placed into the bottles, resulting in daily exposure of the female mice ranging between 15-72

µg/kg/day. When peripubertal female mice housed in undamaged new cages were fed bisphenol A one time per day in oil at the very low doses of 20, 40 and 100 µg/kg/day to simulate exposure within the range released by the polycarbonate, there was a significant dose-related increase in the incidence of aneuploidy.

Kang, J. H. and F. Kondo (2002). Determination of bisphenol A in canned pet foods. Res. Vet. Sci. 73:177-82.

A total of 26 samples (15 samples of cat food and 11 samples of dog food) were prepared for analysis by high-performance liquid chromatography. The concentration of BPA ranged from 13 to 136 ng/g in canned cat food and from 11 to 206 ng/g in dog food. Also, to confirm that the BPA had originated from the can coating, distilled water was added to each washed empty can and the cans were autoclaved at 121 degrees C for 30 min. The concentration of BPA leached from empty cans was between 7 and 31 ng/ml. The migration of bisphenol A from pet food cans was thus less than the legal limit of 3 µg/g set by the European Union in 1990.

Kang, J. H. and F. Kondo (2002) Bisphenol A migration from cans containing coffee and caffeine. 19, 886-890.

This study was conducted to reconfirm the possibility and level of bisphenol A (BPA) migration from cans containing coffee and test the relationship between caffeine concentration and BPA migration from the can coating. BPA migration from cans containing decaffeinated and non-decaffeinated instant coffee averaged 66.2 and 84.0 ng ml(-1), respectively. In our study, the possibility of BPA migration from cans containing coffee after processing was found. In addition, the more caffeine content in the water solution of caffeine increased, the more BPA migration grew. This means that caffeine can have an effect on BPA migration from the can coating.

Kang, J. H., K. Kito and F. Kondo (2003) Factors influencing the migration of bisphenol A from cans. 66, 1444-1447.

The objective of this study was to determine whether there is a relationship between bisphenol A (BPA) migration from metal cans and container contents (glucose, sodium chloride, and vegetable oil), heating time, and/or temperature. Cans containing 5 to 20% glucose solution, 1 to 10% sodium chloride solution, and vegetable oils (corn, olive, and soybean oil) were heated at 121 degrees C for 30 min. Water samples were heated at 105 degrees C for 30 min and at 121 degrees C for 15, 30, and 60 min, respectively. In the test involving water samples, it was found that temperature's effect on BPA migration from cans can be more extensive than that of heating time. When cans were heated at 121 degrees C, the presence of 1 to 10% sodium chloride or vegetable oils greatly increased the migration of BPA from the cans. Moreover, the presence of 5 to 20% glucose in cans heated to 121 degrees C resulted in increased BPA migration relative to that for water controls.

Kang, J. H. and F. Kondo (2003) Determination of bisphenol A in milk and dairy products by high performance liquid chromatography with fluorescence detection. 66, 1439-43.

This study was conducted to develop a selective and sensitive method for the determination of bisphenol A (BPA) levels in milk and dairy products. A method based on solvent extraction with acetonitrile and solid-phase extraction (SPE) was developed for the analysis of BPA in milk, yogurt, cream, butter, pudding, condensed milk, and flavored milk, and a method using two SPE cartridges (OASIS HLB and Florisil cartridge) for skim milk was also developed. The developed methods showed good recovery levels (77 to 102%) together with low detection limits (1 microg/liter for milk, yogurt, pudding, condensed milk, flavored milk, and skim milk and 3 microg/liter for cream and butter). These methods are simple, sensitive, and suitable for the analysis of BPA in milk and dairy products. When 40 milk and dairy products were analyzed by the proposed methods, BPA was not identified in noncanned products, but its levels

ranged from 21 to 43 microg/kg in canned products, levels that were 60- to 140-fold lower than the migration limits in the European Union and Japan.

Krishnan, A. V., P. Stathis, S. F. Permuth, L. Tokes and D. Feldman (1993). Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinol. 132:2279-86.

In studies to determine whether Saccharomyces cerevisiae produced estrogens, the organism was grown in culture media prepared using distilled water autoclaved in polycarbonate flasks. The yeast- conditioned media showed the presence of a substance that competed with [3H]estradiol for binding to estrogen receptors (ER) from rat uterus. However, it soon became clear that the estrogenic substance in the conditioned media was not a product of the yeast grown in culture, but was leached out of the polycarbonate flasks during the autoclaving procedure. [3H]Estradiol displacement activity was monitored by ER RRA, and the active substance was purified from autoclaved medium using a series of HPLC steps. The final purified product was identified as bisphenol-A (BPA) by nuclear magnetic resonance spectroscopy and mass spectrometry. BPA could also be identified in distilled water autoclaved in polycarbonate flasks without the requirement of either the organism or the constituents of the culture medium. Authentic BPA was active in competitive RRAs, demonstrating an affinity approximately 1:2000 that of estradiol for ER. In functional assays, BPA (10-25 nM) induced progesterone receptors in cultured human mammary cancer cells (MCF-7) at a potency of approximately 1:5000 compared to that of estradiol. The BPA effect on PR induction was blocked by tamoxifen. In addition, BPA (25 nM) increased the rate of proliferation of MCF-7 cells assessed by [3H]thymidine incorporation. Thus, BPA exhibited estrogenic activity by both RRA and two functional bioresponse assays. Finally, MCF-7 cells grown in media prepared with water autoclaved in polycarbonate exhibited higher progesterone receptor levels than cells.grown in media prepared with water autoclaved in glass, suggesting an estrogenic effect of the water autoclaved in polycarbonate. Our findings raise the possibility that unsuspected estrogenic activity in the form of BPA may have an impact on experiments employing media autoclaved in polycarbonate flasks. It remains to be determined whether BPA derived from consumer products manufactured from polycarbonate could significantly contribute to the pool of estrogenic substances in the environment.

Koehler, K. E., R. C. Voigt, S. Thomas, B. Lamb, C. Urban, T. J. Hassold and P. A. Hunt (2003). When disaster strikes: Rethinking caging materials. Lab Anim. **32**:32-35.

These authors report that there was a significant increase in mortality in mice at the time the cages that caused aneuploidy in the Hunt et al. (2003) study were being used in their animal colony. They reported that some polycarbonate cage manufacturers are now only recommending use of the cages for 20 autoclave cycles.

Kuo, H.W. and Ding, W.H. (2004). Trace determination of bisphenol A and phytoestrogens in infant formula powders by gas chromatography-mass spectrometry. J Chromatogr A 1027:67-74.

This investigation describes a reliable and sensitive method for simultaneously determining bisphenol A (BPA) and two major phytoestrogens, daidzein and genistein, in powdered milks and infant formulas by gas chromatography-mass spectrometric analysis after trimethylsilylation. To reduce the matrix interference associated with the constituents of the formulas, the dissolved formula solutions were firstly ultra-centrifuged and the analytes in the supernatant were then extracted using a C18 solid-phase extraction column. The accuracy and precision of the method were determined and the technique was successfully employed to measure trace concentrations of BPA, daidzein and genistein in powdered formulas. The results show that BPA, daidzein and genistein were detected in all the testing samples (n = 6) at concentrations from 45 to 113 ng/g (except one infant formula), 20

to 2050 ng/g and 21 to 6510 ng/g, respectively. The highest concentrations of daidzein and genistein (i.e., 2050 and 6510 ng/g) were detected in a soy-based powdered infant formula. The quantitation limits were 1.0 ng/g for BPA, and 10 ng/g for daidzein and genistein using 0.5 g powdered milk samples.

Lopez-Cervantes, J. and Paseiro-Losada, P. (2003). Determination of bisphenol A in, and its migration from, PVC stretch film used for food packaging. Food Addit Contam 20:596-606.

Bisphenol A (BPA) is used as an additive in polyvinyl chloride (PVC) products, including stretch films used for food packaging. The BPA contents were investigated of several brands of stretch film bought locally but marketed internationally or throughout Spain and which were presumably produced at different manufacturing plants. Their major components were identified by FTIR (Fourier Transform Infrared Spectrometry) and horizontal attenuated total reflectance, and the migration of BPA from these materials into the standard European Union food simulants was determined by high-performance liquid chromatography (HPLC) using both fluorescence (FL) and ultraviolet (UV) detection, the identity of the analyte being confirmed by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). The two HPLC detection methods had different detection limits (30 microg x l(-1) for UV, 3 microg x l(-1) for FL), but afforded virtually identical BPA determinations for the samples tested. BPA contents ranging from 40 to 100 mg x kg(-1) were found in three of the five PVC-based films analysed, and a content of 500 mg x kg(-1) was found in a fourth; for these determinations, extraction into acetonitrile was used. In standard tests of migration into water, 3% acetic acid and olive oil over 10 days at 40 degrees C, migration from a given film was in all cases greatest into olive oil. Migration from the films with non-zero BPA contents ranged from 3 to 31 microg x dm(-2), values higher than those reported for many other food-contact materials, but lower than the European Union specific migration limit for BPA. PVC stretch film nevertheless may make a significant contribution to contamination of foodstuffs by BPA, and should be taken into account in estimating BPA intake or exposure to this substance.

Munguia-Lopez, E.M., Peralta, E., Gonzalez-Leon, A., Vargas-Requena, C. and Soto-Valdez, H. (2002). Migration of bisphenol A (BPA) from epoxy can coatings to jalapeno peppers and an acid food simulant. J Agric Food Chem 50:7299-302.

Effects of heat processing, storage time, and temperature on migration of bisphenol A (BPA) from an epoxy type can coating to an acid food simulant and jalapeno peppers were determined. Commercial jalapeno pepper cans (8 oz, dimensions 211 x 300) were stored at 25 degrees C for 40, 70, and 160 days. A solution of 3% acetic acid was canned in 211 x 300 cans from the same batch used for jalapeno peppers. Heat processing was applied to two-thirds of the cans, and the remaining cans were not heat processed. Cans were stored at 25 and 35 degrees C for 0, 40, 70, and 160 days. Results showed that there is a minimal effect of heat treatment. An effect of storage time on migration of BPA during the first 40 days at 25 degrees C was observed. An increase on migration of BPA was observed with storage time at 35 degrees C. The highest level of migration was 15.33 microg/kg of BPA at 160 days at 35 degrees C. A correction factor of approximately 0.4 was calculated for migration under simulating conditions of storage compared to the real ones. The highest level of BPA found in jalapeno peppers cans, surveyed from three supermarkets, was 5.59 +/- 2.43 microg/kg. Migration of BPA, performed according to the European and Mercosur conditions, was 65.45 +/- 5.29 microg/kg. All the migration values found in this study were below those legislation limits (3 mg/kg).

Nerin, C., Fernandez, C., Domeno, C. and Salafranca, J. (2003). Determination of potential migrants in polycarbonate containers used for microwave ovens by high-performance liquid chromatography with ultraviolet and fluorescence detection. J Agric Food Chem 51:5647-53.

The determination of several compounds present in a commercial polycarbonate container intended to be used in microwave ovens which could be considered as potential migrants has been carried out by reversed-phase high-performance liquid chromatography (HPLC) with both ultraviolet (UV) and fluorescence detectors. Total dissolution with dichloromethane and polymer reprecipitation with methanol have been used to evaluate 100% potential migration as the worst case. The extract consisted of a complex mixture containing monomers, oligomers, UV stabilizers, antioxidants, degradation products, and other additives. Phenol, Bisphenol A, 2,4-di-tert-butylphenol, Cyasorb UV5411, bis(2-ethylhexylphthalate), Irganox 1076, and Irgafos 168 were identified by both retention times and fluorescence-to-UV ratios. Additional confirmation was achieved by HPLC with diode array detection and gas chromatography-mass spectrometry. Recovery percentages were in the range of 73.8-94.4%, the lowest one being for the antioxidant Irgafos 168 due to its transformation into the phosphate form and 2,4-di-tert-butylphenol. The concentrations of the studied analytes present in the polycarbonate container ranged between 0.9 and 240 microg.g(-)(1). The total dissolution conditions that may affect the final concentration of analytes, mainly Bisphenol A, are discussed.

Olea, N., R. Pulgar, P. Perez, F. Olea-Serrano, A. Rivas, A. Novillo-Fertrell, V. Pedraza, A. M. Soto and C. Sonnenschein (1996). Estrogenicity of resin-based composites and sealants used in dentistry. Environ. Health Perspect. **104**:298-305.

We tested some resin-based composites used in dentistry for their estrogenic activity. A sealant based on bisphenol-A diglycidylether methacrylate (bis-GMA) increased cell yields, progesterone receptor expression, and pS2 secretion in human estrogen-target, serum-sensitive MCF7 breast cancer cells. Estrogenicity was due to bisphenol-A and bisphenol-A dimethacrylate, monomers found in the base paste of the dental sealant and identified by mass spectrometry. Samples of saliva from 18 subjects treated with 50 mg of a bis-GMA-based sealant applied on their molars were collected 1 hr before and after treatment. Bisphenol-A (range 90-931 micrograms) was identified only in saliva collected during a 1-hr period after treatment. The use of bis-GMA- based resins in dentistry, and particularly the use of sealants in children, appears to contribute to human exposure to xenoestrogens.

Ram, A., O. Zilber and S. Kenig (1985). Life expectation of polycarbonate. Polymer 7. Engineering Sci. **25**: 535-540.

Although the solubility of water in polycarbonate is very low, enough water dissolves in it when immersed in boiling water so that, when cooled, it appears hazy because small water droplets are released. Ram et al. found no mechanical defects from immersing polycarbonate in water at room temperature for one year, but 16% shrinkage after 30 days at 40°C, 55% after 30 days at 60°C, and total “tensile breakdown” after just 14 days at 80°C. Even the most stable grade of polycarbonate failed in hot water, although not as quickly.

Reports, C. (1999). Baby alert: New findings about plastics. Consumer Reports **May**:28-29.

Robeson, L.M. et al. (1985) *Hydrolytic Stability of High Tg Engineering Polymers: Relevance to Steam Sterilization*, Union Carbide Corporation Technical Bulletin F-49806. This reference was also published as an article in *Polymer News*, No. 12, June 1986, pp. 359-365.

This 1985 technical bulletin from Union Carbide Corporation provided evidence that hydrolytic attack or molecular breakdowns are less of an issue for polysulfone and polyethersulfone

co-polymers relative to polycarbonate. The effect of hot water and hot water plus base (NaOH) were compared for their effects on five different polymers: polysulfone, polyethersulfone, polycarbonate, polyestercarbonate and polyetherimide. At water temperature of 98 °C polysulfones maintained their molecular weight while polycarbonates, including polyestercarbonate (which a co-polymer of polycarbonate and a phthalate ester), showed severe molecular weight degradation due to hydrolysis. The hydrolysis causes the generation of bisphenol A in polycarbonate, particularly at and near the exposed surface. Combining a basic environment combined with high temperature (10% NaOH solution, 96 °C) resulted in weight loss of the immersed co-polymers polycarbonate, polyestercarbonate and polyetherimide, but not polysulfone or polyethersulfone due to molecular breakdown by base catalyzed hydrolysis. The molecular fragments generated from the hydrolysis process were sufficiently small that they were either dissolved or ablated from the surface into the basic solution. The weight losses observed were accompanied by proportionate reductions in thickness of the test parts.

Sajiki, J. and J. Yonekubo (2004). Leaching of bisphenol A (BPA) from polycarbonate plastic to water containing amino acids and its degradation by radical oxygen species. Chemosphere 55:861-7.

In this study, (1) the change in the concentration of bisphenol A (BPA) leached from polycarbonate plastic (PCP) tube to water samples containing phosphate, sodium barbital, glycine, methionine or albumin at 37 degrees C as a function of time, and (2) the degradation rate of BPA leached from PCP tube to amino acid solutions in the presence of radical oxygen species (ROS) were investigated. The BPA leaching velocity (BPA-LV) from PCP tube to 50 mM glycine at pH 6 or 7 was twice that to control water, and the leaching was enhanced above pH 8. At pH 11, BPA-LV was significantly higher in 50 mM glycine and methionine solutions than in 50 mM NaOH. These results indicate that basic pH and amino acids contained in water could accelerate BPA leaching. The BPA-LV in phosphate buffer was different from the BPA-LVs in other buffers (barbital and glycine) at the same pH. BPA leached to the glycine or methionine solutions at pH 11 was degraded time dependently in a similar manner as the control water in the presence of ROS. The degradation of leached BPA was inhibited in the glycine solution, but was accelerated in the methionine solution. However, degradation of BPA added to freshly prepared methionine was inhibited in a similar manner to BPA in glycine. BPA degradation could be influenced by some kinds of amino acids, but glycine and methionine might be involved in BPA degradation in different ways.

Sasaki, N., Okuda, K., Kato, T., Kakishima, H., Okuma, H., Abe, K., Tachino, H., Tuchida, K. and Kubono, K. (2005). Salivary bisphenol-A levels detected by ELISA after restoration with composite resin. J Mater Sci Mater Med 16:297-300.

Bisphenol-A diglycidylether methacrylate (Bis-GMA), which is synthesized from bisphenol-A (BPA), a compound with exogenous endocrine disrupter action, is widely used as a dental material. During clinical filling with sealants and composite resins, the compounds are solidified by polymerization and then used. However, it has been noted that unpolymerized monomers may become dissolved in saliva. In this study using a competitive ELISA system, we investigated the changes in the BPA concentration in saliva after restoration with composite resins. Commercial composite resins from nine companies were tested. Mixed saliva was collected from 21 subjects. Based on the dynamics of salivary BPA detected by this ELISA system, we concluded that several tens to 100 ng/ml of BPA were contained in saliva after filling teeth with composite resin but that sufficient gargling can remove it from the oral cavity. Our data suggest that sufficient gargling after treatment is important for risk management.

ScienceNewsOnline (1999). Food for thought: What's coming out of baby's bottle? Science News Online, www.sciencenews.org/sn_arc99/8_7_99/food.htm. Volume 156(6): 1-4.

Amounts of bisphenol A leaching from new and used polycarbonate baby bottles and tableware used in Japanese primary schools, as well as canned products, is reported. The findings are that use, scratched surface, or heat increased substantially the amount of leaching of bisphenol A.

Takahashi A, Higashino F, Aoyagi M, Kyo S, Nakata T, Noda M, Shindoh M, Kohgo T, Sano H. 2004 Oct 15. Bisphenol A from dental polycarbonate crown upregulates the expression of hTERT. J Biomed Mater Res 71B:214-21.

Bisphenol A (BPA) is one of the endocrine-disrupting chemicals (EDCs) that possess estrogen-like biologic activity. Many dental materials have been reported to release BPA. However, there are few reports available on the release of BPA from dental polycarbonates. The purpose of this study was to investigate the release of BPA from dental polycarbonate crowns and to evaluate the estrogenic activity of BPA. Polycarbonate crowns were immersed in five solvents (water, ethanol, n-hepthane, acetic acid, and acetonitrile) at 37 or 65 degrees C for 24 h. The elution from the material was analyzed by high-performance liquid-chromatography (HPLC) and mass-spectrometry (MS) analysis. BPA release was detected corresponding to the degradation of dental polycarbonates under the some storage conditions (ethanol, acetic acid, and acetonitrile). A previous report proved that estrogen increased human telomerase catalytic subunit (hTERT) mRNA, whereas the effect of EDCs on the hTERT promoter has never been reported. The estrogenic activity of BPA was analyzed by luciferase assay with the use of the hTERT promoter. This assay revealed that BPA was a positive regulator of hTERT transcription. In addition, quantitative real-time PCR analysis showed that BPA increased the expression level of hTERT mRNA in MCF7 cells. Herein, it is demonstrated that hTERT is a new target of BPA.

Takao, Y., H. Chul Lee, Y. Ishibashi, S. Kohra, N. Tominaga and K. Arizono (1999). Fast screening method for bisphenol A in environmental water and in food by solid-phase microextraction (SPME). J. Health Sci. 45:39.

Takao, Y., H. C. Lee, S. Kohra and K. Arizono (2002). Release of bisphenol A from food can lining upon heating. J. Health Sci. 48:331-334.

Tarumi, H., Imazato, S., Narimatsu, M., Matsuo, M. and Ebisu, S. (2000). Estrogenicity of fissure sealants and adhesive resins determined by reporter gene assay. J Dent Res 79:1838-1843.

It is controversial whether the dental resinous materials containing 2,2-bis[4-(2-hydroxy-3-methacryloyloxypropoxy)phenyl]propane (Bis-GMA), which is synthesized from the estrogenic compound bisphenol A (BPA), include unreacted BPA and/or can mimic the effects of natural steroid hormones. In the present study, the estrogenic activities of 3 fissure sealants and 5 adhesive resins, which were all unpolymerized, were determined by means of a reporter gene assay, and the relevance of the components to the estrogenicity was investigated. Two commercially available sealants were confirmed to have estrogenic activity, although none of the tested materials contained BPA. In contrast, hydrophobic monomer bisphenol A dimethacrylate (BPA-DMA), which is also estrogenic, was found to be included in these estrogenic sealants in an amount greater than the minimum concentration to show estrogenicity. This suggests that the estrogenicity of the two proprietary sealants was associated with BPA-DMA rather than with BPA.

Terasaki, M., Shiraishi, F., Nishikawa, T., Edmonds, J.S., Morita, M. and Makino, M. (2005). Estrogenic activity of impurities in industrial grade bisphenol A. Environ Sci Technol 39:3703-3707.

The estrogenicities of 10 compounds found as impurities in industrial grade bisphenol A (BPA) were measured by yeasttwo-hybrid assays incorporating the human estrogen receptor alpha(hERalpha) orthe medaka fish (Oryziaslatipes) estrogen receptor alpha (medERalpha). Five impurities showed greater activity than BPA itself in an agonist assay for hERalpha. p-Cumylphenol, the most active of the impurities in the hERalpha assay, was 12 times as active as BPA. The REC10 (10% relative effective concentration: 10% of the activity of 10(-8)M 17,B-estradiol) was 710 nM. Five impurities showed greater activity than BPA in an agonist assay for medERa: 4,4'-(1,3-dimethylbutylidene) bisphenol and 2-(4'-hydroxy-phenyl)-2,4,4-trimethylchroman were nearly equipotent and 9 times as active as BPA, and the REC10 values of these compounds in the medERalpha assay were 280 and 320 nM, respectively. Comparison of the experimentally determined estrogenicities of mixtures of BPA and 4,4'-(1,3-dimeth-ylbutylidene) bisphenol and those calculated by the concentrations addition (CA) method confirmed the suitability of the method for the prediction of the estrogenicities of the mixtures of BPA and its phenolic analogues. The measured estrogenicities of four samples of industrial grade BPA and laboratory grade (pure) BPA were not significantly different in either the hERalpha assay or the medERalpha assay ($p > 0.05$ in each case). We conclude that the impurities in industrial grade BPA, although some are of much higher estrogenic activity than BPA itself, do not significantly increase the estrogenicity of the industrial compound and therefore do not increase possible adverse health effects from such activity.

Thompson, T. and P. P. Klemchuk (1996). Light stabilization of bisphenol A polycarbonate. Polymer Durability: Degradation, Stabilization, and Lifetime Prediction. R. L. Clough, N. C. Billingham and K. T. Gillen. Washington, D.C., American Chemical Society. **303:** 303-317.

Thompson and Klemchuk report that polycarbonate as an ester is susceptible to hydrolysis and base-catalyzed hydrolysis, mainly at elevate d temperatures, while it is more resistant to hydrolysis at ambient temperatures. However, while the findings of Howdeshell et al. (2003) confirm that this applies to new polycarbonate cages, these findings show that as polycarbonate cages age, associated with discoloration and cracking, there is a marked increase in leaching of free BPA into water at room temperature.

Thomson, B.M. and Grounds, P.R. (2005). Bisphenol A in canned foods in New Zealand: an exposure assessment. Food Addit Contam 22:65-72.

Exposure to bisphenol A (BPA) from the consumption of canned and bottled food has been determined for New Zealand adults. Eighty different canned foods purchased from retail outlets in Christchurch, New Zealand, between November 2003 and February 2004 were analysed for BPA concentration by gas chromatography/mass spectrometry. BPA was detected in all foods analysed except for soft drinks. Concentrations ranged from < 10 to 29 microg kg(-1), except for individual samples of tuna, corned beef and coconut cream, which were 109, 98 and 191 microg kg(-1) , respectively. The limit of quantitation was <10 microg kg(-1) for foods of low fat content (< 1%) and <20 microg kg(-1) for foods containing >1% fat. Mean concentration data were combined with 24-h dietary recall information for 4399 individual consumers. Mean and maximum exposures were 0.008 and 0.29 microg kg(-1) bw day(-1), respectively, well below the temporary tolerable daily intake of 10 microg kg(-1) bw day(-1) given by the European Commission in 2002. The results of the present survey suggest that the levels of BPA identified in canned foods are unlikely to be of concern to adult health, and there is no reason for consumers to change their consumption patterns as a result of these findings. When the concentration data found in the current survey are applied to an oestrogenicity model for an adult male, the contribution of BPA to the total oestrogenicity from 16 food components is 7%. The impact of this level of oestrogenicity remains unclear.

Wada, H., Tarumi, H., Imazato, S., Narimatsu, M. and Ebisu, S. (2004). In vitro estrogenicity of resin composites. J Dent Res 83:222-226.

Previously, we have reported that sealants incorporating bisphenol A dimethacrylate showed estrogenicity by a reporter gene assay. This study tested the hypothesis that commercial composites, which contain various monomers and additives, exhibit estrogenic activity in vitro. The estrogenic activities of eluates obtained from 24 composites and 18 chemicals identified from the composites tested were examined with the use of the reporter gene assay. Among the 24 composites, 6 products were estrogenic, and among the 18 constituents, 1 photostabilizer, 2-hydroxy-4-methoxy-benzophenone (HMBP), 1 photoinitiator, 2,2-dimethoxy-2-phenyl-acetophenone (DMPA), and 1 inhibitor, 2,6-di-tert-butyl-p-cresol (BHT) had significant estrogenic activity. The concentration of HMBP in 4 estrogenic eluates was greater than the minimum concentration required for estrogenicity, and DMPA was found at a higher level than the minimum estrogenic concentration in the remaining 2 estrogenic specimens. These results suggest that the observed estrogenic activity of 6 composites is associated with the elution of either HMBP or DMPA.

Yoshida, T., M. Horie, Y. Hoshino and H. Nakazawa (2001). Determination of bisphenol A in canned vegetables and fruit by high performance liquid chromatography. Food Additives Contamin. **18**:69-75.

A high performance liquid chromatography (HPLC) method was developed for the determination of bisphenol A (BPA) that had migrated into canned fruit and vegetables. BPA was extracted with acetonitrile from the solid portion of canned food, and with an OASIS HLB cartridge from the aqueous portion, respectively. Both extracts were cleaned up on a Florisil cartridge. The HPLC separation was carried out on a Wakosil II 3C18 RS column (4.6 x 150 mm) with acetonitrile-water (40:60, v/v) as a mobile phase with a flow rate of 0.8 ml/min. BPA was detectable by UV detector at 228 nm and determined with the similarity of chromatographic peak spectrum by multiwavelength detector (similarity index was 0.99 or above). The quantification limits were 10 ng/g for the solid portion and 5 ng/ml for the aqueous portion, respectively (note that this is substantially higher than the detection limit with GC-MS). BPA was mainly detected in the solid portion of canned food and found at the maximum level of 11 micrograms per can. To verify migration into the solid portion of canned food, a partitioning experiment was carried out.

XXIII. ESTROGENIC ACTIVITY OF BISPHENOL A DIMETHACRYLATE IN DENTAL SEALANTS

Darmani H, Al-Hiyasat AS. 2004. Reproductive toxic effect of bisphenol A dimethacrylate in mice. J Biomed Mater Res 69A:637-43.

The current study evaluated the effect of bisphenol A dimethacrylate (Bis-DMA) on mouse fertility. Adult male and female mice were exposed to intragastric Bis-DMA (0, 5, 25, and 100 microg/kg) daily for 28 days and then mated with sexually mature untreated mice and after mating fertility was assessed. Females mated by males that had been exposed to Bis-DMA had significant reductions in pregnancy rates and significant increases in the total number of resorptions out of the total number of implantations. Bis-DMA exposed males had significant reductions in body weights and relative testes weights and significant increases in seminal vesicle and preputial gland weights. Testicular and epididymal sperm counts as well as the efficiency of sperm production were also significantly reduced in these groups. Female mice exposed to Bis-DMA showed significant reductions in pregnancy rates, number of implantation sites, number of viable fetuses, and total number of resorptions out of the total number of implantations. Significant reductions in the body

weights were observed at all doses, and significant increases were found in the relative weights of the ovaries and the uterus. The results suggest that Bis-DMA has adverse effects on the fertility and reproductive systems of male and female mice.

Wada H, Tarumi H, Imazato S, Narimatsu M, Ebisu S. 2004. In vitro estrogenicity of resin composites. Journal of Dental Research 83(3):222-6.

Previously, we have reported that sealants incorporating bisphenol A dimethacrylate showed estrogenicity by a reporter gene assay. This study tested the hypothesis that commercial composites, which contain various monomers and additives, exhibit estrogenic activity in vitro. The estrogenic activities of eluates obtained from 24 composites and 18 chemicals identified from the composites tested were examined with the use of the reporter gene assay. Among the 24 composites, 6 products were estrogenic, and among the 18 constituents, 1 photostabilizer, 2-hydroxy-4-methoxy-benzophenone (HMBP), 1 photoinitiator, 2,2-dimethoxy-2-phenyl-acetophenone (DMPA), and 1 inhibitor, 2,6-di-tert-butyl-p-cresol (BHT) had significant estrogenic activity. The concentration of HMBP in 4 estrogenic eluates was greater than the minimum concentration required for estrogenicity, and DMPA was found at a higher level than the minimum estrogenic concentration in the remaining 2 estrogenic specimens. These results suggest that the observed estrogenic activity of 6 composites is associated with the elution of either HMBP or DMPA. HMBP is a principal component of many UV screening agents and cosmetics (Lewerenz et al., 1972. [Toxicology of UV-absorber MOB. Nahrung 16:133-134]).

XXIV. BISPHENOL A AND CANCER

Huff, J. (2001). Carcinogenicity of bisphenol-A in Fischer rats and B6C3F1 mice. Odontology 89:12-20.

Bisphenol-A (BP-A; 4,4'-isopropylidenediphenol) is a monomer of plastics commonly used in various consumer products, and is used as an intermediate in the manufacture of epoxy, polycarbonate, and polyester-styrene resins. A National Toxicology Program carcinogenesis bioassay of BP-A (>98% pure) was conducted by feeding diets containing 0, 1000, or 2000 ppm BP-A to groups of 50 male and 50 female Fischer (F)344 rats; 0, 1000, or 5000 ppm to groups of 50 male B6C3F1 mice; and 0, 5000, or 10 000 ppm to groups of 50 female B6C3F1 mice for 103 weeks. The mean body weights of the low- and high-dose rats and of female mice and high-dose male mice were lower than those of the controls throughout much of the study. Lower body weight gains in rats were likely caused by reduced food consumption. Survivals were comparable among groups. Regarding neoplasia, leukemias occurred at increased incidences in BP-A-dosed rats of both sexes: male, 13/50 controls vs 12/50 low-dose and 23/50 high-dose ($P < 0.03$); in females, the respective findings were 7/50, 13/50, and 12/50. Interstitial-cell tumors of the testes were increased in BP-A-dosed male rats: 35/49 controls vs 48/50 ($P < 0.01$) and 46/49 ($P < 0.01$); and an increasing trend was observed for mammary gland fibroadenomas in male rats ($P < 0.05$, 0/50 controls vs 0/50 and 4/50). In male mice, lymphomas/leukemias were increased: 2/49 controls vs 9/50 ($P < 0.05$) and 5/50. Multinucleated giant hepatocytes were observed in male mice (1/49 controls vs 41/49 and 41/50), whereas there was no increase of liver tumors. In their BP-A bioassay report, the National Toxicology Program concluded that there was no convincing evidence that BP-A was carcinogenic for rats or mice. However, the marginal increases in leukemias in male and female rats, along with increases in the combined incidence of lymphomas and leukemias in male mice, suggest that BP-A may be associated with increased cancers of the hematopoietic system. Increases in interstitial-cell tumors of the testes

in rats were also evidence of carcinogenesis, as was the unusual occurrence of mammary gland fibroadenomas in male rats.

Huff, J. (2002). Carcinogenicity of bisphenol A revisited. Toxicol Sci 70:281-3; author reply 283-4.

Ichihara, T., Yoshino, H., Imai, N., Tsutsumi, T., Kawabe, M., Tamano, S., Inaguma, S., Suzuki, S. and Shirai, T. (2003). Lack of carcinogenic risk in the prostate with transplacental and lactational exposure to bisphenol A in rats. 28:165-171.

The current study was designed to examine the modulating effects of bisphenol A (BPA) on prostate cancer risk in male offspring exposed transplacentally and lactationally. BPA was administered to F344 female rats by gavage at 0, 0.05, 7.5, 30, 120 mg/kg/day during pregnancy and lactation periods. When F1 males reached 5 weeks old, they were given 10 subcutaneous injections of 3,2'-dimethyl-4-aminobiphenyl (DMAB) or corn oil vehicle and rats were then sacrificed under ether anesthesia at week 60. There were no observable effects on the accessory sex organ weights of male offspring. Transplacental and lactational exposure to BPA did not affect the incidences of preneoplastic and neoplastic lesions in the accessory sex organs (prostate and seminal vesicle) of F1 rats and did not induce any proliferating lesions without DMAB. Our data suggest that maternal exposure to BPA during the period of pregnancy and lactation does not affect the risk of prostate carcinogenesis in male offspring.

Parry, E.M., Parry, J.M., Corso, C., Doherty, A., Haddad, F., Hermine, T.F., Johnson, G., Kayani, M., Quick, E., Warr, T. and Williamson, J. (2002). Detection and characterization of mechanisms of action of aneugenic chemicals. Mutagenesis 17:509-521.

A comprehensive evaluation of the genotoxic potential of chemicals requires the assessment of the ability to induce gene mutations and structural chromosome (clastogenic activity) and numerical chromosome (aneugenic activity) aberrations. Aneuploidy is a major cause of human reproductive failure and an important contributor to cancer and it is therefore important that any increase in its frequency due to chemical exposures should be recognized and controlled. The in vitro binucleate cell micronucleus assay provides a powerful tool to determine the ability of a chemical to induce chromosome damage. The application of an anti-kinetochore antibody to micronuclei allows their classification into kinetochore-positive and kinetochore-negative, indicating their origin by aneugenic or clastogenic mechanisms, respectively. The availability of chromosome-specific centromere probes allows the analysis of the segregation of chromosomes into the daughter nuclei of binucleate cells to evaluate chromosome non-disjunction. Quantitative relationships between the two major causes of aneuploidy, chromosome loss and non-disjunction, can be determined. The mechanisms leading to chromosome loss and non-disjunction can be investigated by the analysis of morphological and structural changes in the cell division apparatus by the application of specific stains and antibodies for various cell division components. We illustrate such analyses by the demonstration of the interaction of the monomer bisphenol-A with the centrosome of the mitotic spindle and the folic acid antagonist pyrimethamine with the centromeres of chromosomes. Both types of modifications lead to the induction of aneuploidy in exposed cells. Our studies also implicate the products of the p53 and XPD genes in the regulation of the fidelity of chromosome segregation at mitosis.

Schrader, T.J., Langlois, I., Soper, K. and Cherry, W. (2002). Mutagenicity of bisphenol A (4,4'-isopropylidenediphenol) in vitro: effects of nitrosylation. Teratog. Carcinog. Mutagen. 22:425-441.

Bisphenol A (4,4'-isopropylidenediphenol) is a common component of polycarbonate plastics and epoxy resins. Since bisphenol A-containing plastics and resins have found uses in food-contact

items, its potential migration into foodstuffs and possible health consequences have been the focus of many recent studies. However, the potential mutagenic activation of bisphenol A by nitrosylation has received little attention. Incubation of bisphenol A with sodium nitrite under acidic conditions produced a yellow-brown product. When nitrosylated bisphenol A was tested in the Ames Salmonella/microsome assay at 100 ng to 1 mg/plate, dose-dependent increases in mutagenicity were found in both TA98 and TA100 Salmonella strains. These results indicated the presence of a direct-acting mutagenic activity causing both frameshift and base pair mutations, respectively. When compared to colony formation in untreated controls, the addition of rat liver S9 for metabolic activation had little influence on revertant colony formation. Unreacted bisphenol A dissolved in DMSO, acidic buffer, or inactivated nitrosylation solution showed negligible mutagenicity. When the nature of the mutagenic changes was examined using the Ames II trade mark Assay, a variety of base pair changes was found including T:A to A:T - S9, G:C to A:T +/- S9,C:G to A:T +/- S9 and C:G to G:C +/- S9. Bisphenol A also induced frameshift mutations at G:C sites. In addition, the presence of electrophiles was shown by the production of an intensely coloured orange-red product upon incubation of nitrosylated bisphenol A with the nucleophile 4-(4'-nitrobenzyl)pyridine. These findings suggest that migration of bisphenol A into nitrite containing foodstuffs, or its ingestion in the presence of nitrite, could lead to the formation of mutagenic compounds.

Tsutsui, T., Tamura, Y., Yagi, E., Hasegawa, K., Takahashi, M., Maizumi, N., Yamaguchi, F. and Barrett, J.C. (1998). Bisphenol-A induces cellular transformation, aneuploidy and DNA adduct formation in cultured Syrian hamster embryo cells. Int, J, Cancer 75:290-294.

Bisphenol-A (BP-A) is a major component of epoxy, polycarbonate and other resins. For an assessment of in vitro carcinogenicity and related activity of BP-A, the abilities of this compound to induce cellular transformation and genetic effects were examined simultaneously using the Syrian hamster embryo (SHE) cell model. Cellular growth was reduced by continuous treatment with BP-A at doses > or = 100 microM. However, colony-forming efficiencies were not decreased significantly following treatment with up to 200 microM BP-A for 48 hr. Morphological transformation of SHE cells was induced by treatment of cells with BP-A at 50 to 200 microM for 48 hr. BP-A exhibited transforming activity at doses > or = 50 microM but was less active than the benzo[alpha]pyrene used as a positive control. Over the dose range that resulted in cellular transformation, treatment of SHE cells with BP-A failed to induce gene mutations at the Na+/K+ ATPase locus or the hprt locus. No statistically significant numbers of chromosomal aberrations were detected in SHE cells treated with BP-A. However, treatment of cells with BP-A induced numerical chromosomal changes in the near diploid range at doses that induced cellular transformation. 32P-Postlabeling analysis revealed that exposure of cells to BP-A also elicited DNA adduct formation in a dose-dependent fashion. Our results indicate that BP-A has cell-transforming and genotoxic activities in cultured mammalian cells and potential carcinogenic activity.

Tsutsui, T., Tamura, Y., Suzuki, A., Hirose, Y., Kobayashi, M., Nishimura, H., Metzler, M. and Barrett, J.C. (2000). Mammalian cell transformation and aneuploidy induced by five bisphenols. Int. J. Cancer 86:151-154.

Bisphenol-A (BP-A), a monomer of plastics used in numerous consumer products and a xenoestrogen, induces cellular transformation and aneuploidy in Syrian hamster embryo (SHE) cells. In this study, the abilities of 4 other bisphenols to induce cellular transformation and genetic effects in SHE cells were examined and compared to BP-A. Cellular growth was inhibited by all bisphenols in a concentration-related manner. The growth inhibitory effect of the bisphenols ranked: BP-5 > BP-4 > BP-3 > BP-2 or BP-A. Morphological transformation of SHE cells was induced by BP-A, BP-3, BP-4 and BP-5, and the induced-transformation frequencies were highest with BP-4. None of the

bisphenols induced gene mutations at the Na(+)/K(+) ATPase locus or the hprt locus, or chromosomal aberrations in SHE cells. By contrast, aneuploidy induction in the near-diploid range was exhibited by BP-A, BP-3, BP-4 or BP-5, corresponding to the transforming activity of each compound. The results indicate that BP-A, BP-3, BP-4 and BP-5 exhibit transforming activity in SHE cells, while BP-2 does not, and that aneuploidy induction may be a causal mechanism of the transforming activity.