

Chapter 7

Ovarian Toxicity of Environmental Contaminants: 50 Shades of Grey

M.A. Dominguez, J.C. Sadeu, M.T. Guerra, H.C. Furlong, Sharnjit Baines, and Warren G. Foster

Abstract Exposure to environmental contaminants is thought to be important in the development of adverse effects on reproductive health. While the adverse effects of environmental contaminants on semen quality and testicular function have been well studied, effects on ovarian function are less well defined. Epidemiological studies have linked exposure to environmental contaminants with adverse effects on menstrual cycle characteristics, infertility, and earlier age of menopause onset; yet direct evidence of effects on ovarian function is lacking. Environmental contaminant concentrations have been quantified in human ovarian follicular fluid establishing target tissue exposure; however, such data is sporadic and limited to women undergoing assisted reproductive therapies making generalization of results to the broader population of women difficult. We note that the relationship between serum and follicular fluid concentrations can be orders of magnitude different and thus target tissue distribution requires further study. Animal studies revealed effects of environmental contaminants on ovarian follicle dynamics, oocyte maturation, steroidogenesis, and epigenetic changes. Issues of dosing such as concentration of test chemicals used, route of administration, and use of multiple dose groups remain important limitations of the current literature. While animal studies establish a basis for biological plausibility of effects and support conclusions of reproductive hazard, we conclude that exposures in the general human population are too low to present a demonstrable risk to human ovarian function.

M.A. Dominguez

Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Tamaulipas, Cd. Victoria, Tamaulipas, Mexico

J.C. Sadeu • H.C. Furlong • S. Baines • W.G. Foster (✉)

Department of Obstetrics & Gynaecology, McMaster University,
1280 Main Street West, Hamilton, ON L8S 4K1, Canada
e-mail: fosterw@mcmaster.ca

M.T. Guerra

Department of Morphology, Biosciences Institute, UNESP,
Botucatu, São Paulo CEP 18618-970, Brazil

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7.1 Introduction

Worldwide, estimates of infertility range widely from 50 to 186 million couples of which 7.3 million American women alone are infertile (Boivin et al. 2007; Chandra et al. 2005; Mascarenhas et al. 2012; Rutstein and Shah 2004; Stephen and Chandra 2006). Established risk factors for infertility include: advanced age, diet, prescription medication use, pre-existing health status, genetic mutations, and infections; however, for many women the cause of their infertility is unknown. Thus, exposures to environmental contaminants are thought to adversely affect human fertility. Indeed, an association between farm or industrial work and infertility was demonstrated using a questionnaire on reproductive and occupational health histories, lifestyle and demographics (Fuortes et al. 1997), while residue levels of specific compounds were not measured. Infertile women had higher odds of working in industry (OR, 95 % CI=6.7, 2.3–19.6) or farms (1.8, 1.2–2.7), adjusted for age, date of outcome and smoking compared to a fertile control group. Among specific infertility types, ovulatory dysfunction was highest and shows an association with industry/occupation (10.9, 3.5–33.7). In a National survey conducted in French in vitro fertilization (IVF) clinics between 2011 and 2012 (Alvarez and Devouche 2012), one in eight women reported sexual problems (mostly dyspareunia, insensitivity and bleeding). Of the 348 responding women (mean age: 34.9 ± 5.3 years), 20 % smoked cigarettes, 1.4 % cannabis, and 23 % consumed alcohol, whereas 8.6 % reported occupational exposure to chemicals. Work stress was reported by 41 % and family stress by 14 %. Taken together, these data suggest that exposures to environmental contaminants are potentially associated with reduced fertility, however, the strength of this association and potential mechanisms are poorly defined.

While the effects of environmental contaminants on male reproductive health have received research attention, the effects on female fertility and ovarian function in particular remain to be elucidated. We propose that environmental contaminants affect reproductive health in part via impaired ovarian function (Augood et al. 1998; Foster 2003; Pocar et al. 2003; Sadeu et al. 2010). Moreover, we postulate that the ovary is potentially vulnerable to the adverse effects of environmental contaminant exposure owing to its rich blood supply, rapid cell division associated with follicle development, and ovulation. Moreover, environmental contaminant effects on the ovary have far reaching consequences on reproduction and general health owing to its central role in the production of the gonadal steroids estradiol (E_2) and progesterone (P_4), the finite and non-renewable number of follicles present in the ovary at birth, and development of female gametes (oocytes).

7.2 Epidemiology

Determining the effect of environmental contaminants on reproductive health in women and on ovarian function in particular is difficult to assess. The relationship between environmental contaminant exposure and reproductive function in women has primarily been limited to measurement of time-to-pregnancy (TTP), infertility, menstrual cycle characteristics, duration of menstrual bleeding, amenorrhea, dysmenorrhea, age at menopause, and premature ovarian failure (POF). Measurement of circulating follicle stimulating hormone (FSH), ovarian hormones (activin and inhibin), gonadal steroid hormones (E_2 and P_4), and more recently quantification of anti-Müllerian hormone (AMH) concentrations have been employed as markers of impaired reproductive health that provide indirect insight into ovarian function.

A questionnaire on reproductive health and lifestyle histories of women who presented with natural menopause revealed that only women who had been breast-fed as babies showed a significantly earlier natural menopause ($p=0.014$), while alcohol consumption ($p=0.080$) and smoking ($p=0.081$) had no significant effect on timing of natural menopause (Dvornyk et al. 2006). Other factors such as the use of oral contraceptives also had no significant effect. In contrast, a survey of 443 hairdressers, aged 21–55, revealed a greater likelihood of premature ovarian failure (POF; 3.2 vs. 1.4 %, $p=0.06$) compared to 508 women employed in other occupations (Gallicchio et al. 2009). While hairdressers also smoked significantly more than the controls, the overall relative risk (RR, 95 % CI) for POF was 1.90, (0.76–4.72) and thus was non-significant. Hairdressers are exposed to several chemicals such as solvents, bleaches, hair dyes, non-lye relaxers, alcohols, ethylene glycol, methacrylate and phthalates; all of which are potentially hazardous to ovarian function. Sub-analysis of the data revealed that Caucasians over 40 years had a RR of 5.58 (1.24–25.22) illustrating that age is an important variable in assessing the impact of environmental factors on ovarian function. In another study, ovarian failure was reported (Koh et al. 1998) in 16 laborers exposed to cleaning solvent containing 2-bromopropane (n-propyl bromide). Amenorrhea was reported in 26 women after occupational exposure in South Korea, of which 16 were diagnosed with primary ovarian failure. Between these two groups, there were significant differences ($P<0.05$) in levels of LH: 32.9 mIU/ml (range=10.1–93.0) vs. 8.5 (1.1–13.9); FSH: 87.7 mIU/ml (31.8–119.7) vs. 9.8 (3.3–28.3); and E_2 : 11.0 pg/ml (7.0–28.0) vs. 48.0 (12.0–205.0). While the effects of occupational exposure to environmental contaminants are well known, effects arising from exposure to environmental contaminants at exposure levels representative of the concentrations reported in contemporary biomonitoring studies involving the general population are less clear.

Persistent Organic Pollutants (POPs) are a group of chemicals including the chlorinated organic chemicals that have been used in the manufacture of a diverse group of chemical compounds such as pesticides, chlorine, bleaches, plastics, flame-retardants and metal production. Since they are stable and lipophilic, they easily accumulate in the food chain, and humans may acquire them via food or

direct (occupational or accidental) exposure (Hombach-Klonisch et al. 2005). Polybrominated diphenyl ethers (PBDEs) are a family of structurally related chemicals used as flame retardants, and have been detected in human follicular fluid (Johnson et al. 2012). Several organochlorine compounds including the polychlorinated biphenyls (PCBs) and metabolites of the pesticide DDT, have also been quantified in follicular fluid (De Felip et al. 2004; Foster 1995; Mahalingaiah et al. 2012; Meeker et al. 2009; Petro et al. 2012; Schlebusch et al. 1989; Younglai et al. 2002) (Table 7.1). In a longitudinal study of 501 couples who discontinued contraception to achieve pregnancy (Bloom et al. 2007) the fecundability odds ratios (FOR) was decreased by 18–21 % in women with PCB congeners 118, 167 & 219. The strongest effect found was for PCB167 (FOR=0.79; 95 % CI=0.64–0.97). These correlations suggest that PCB exposure is associated with adverse effects on fertility although the direct ovarian effects of PCBs remain equivocal. Mean total PCB concentrations (3.1 ± 1.9 $\mu\text{g/L}$) were positively correlated with menstrual cycle length reported by 2314 women ($p=0.02$) (Cooper et al. 2005). The majority of 37 follicular fluid samples from an IVF clinic contained 0–1 and 1–2 $\mu\text{g/kg}$ of PCB138, 153, or 180, but the highest levels reached 15–16 $\mu\text{g/kg}$ (Schlebusch et al. 1989). In women attending a fertility clinic, follicular fluid concentrations of PCB180 were lower in women who became pregnant vs. those who did not (85 ± 14 vs. 147 ± 11 pg/ml , respectively, $p<0.05$). German women had higher serum PCB concentrations (0.35 ± 0.049 vs. 0.17 ± 0.02 $\mu\text{g/kg}$, $p<0.01$) compared to women in Tanzania (Weiss et al. 2006). In contrast, follicular fluid levels did not show regional differences (0.26 ± 0.02 vs. 0.22 ± 0.06 $\mu\text{g/kg}$). These studies suffer from relatively small sample sizes and recruitment exclusively from women attending fertility clinics and thus generalization of results to the entire population is difficult. Although 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD) is widely regarded as one of the most toxic environmental contaminants, adverse effects on ovarian function are poorly defined. A long-term study was carried out in Italy after a chemical plant explosion in 1976 that exposed a town to TCDD (Eskenazi et al. 2005). Serum residue concentrations of 43.7 ppt (range, 2.5–6320) were found in 616 women that were exposed between 1 month and 40 years of age. While there was a trend toward earlier menopause with increasing TCDD concentrations, suggesting potential effects on ovarian function, significance could not be documented.

The common pesticide 1,1-bis-(4-chlorophenyl)-2,2,2-trichloroethane (*p,p'*-DDT) has been banned in many industrialized countries, but continues to be used in developing nations. Although widely studied, the relationship between exposure to this pesticide and its metabolites on fertility and ovarian function remains equivocal. The DDT metabolite 1,1-bis-(4-chlorophenyl) 2,2-dichloroethene (*p,p'*-DDE) is frequently detected in serum and follicular fluid (De et al. 2004; Mahalingaiah et al. 2012; Petro et al. 2012; Younglai et al. 2002) although effects on fertility have been mixed (Table 7.1). A study that compared the fertility of 289 women born in the USA between 1960 and 1963 to that of their daughters' 30 years later showed a 32 % decrease in the probability of pregnancy of the daughters for every 10 $\mu\text{g/L}$ in maternal serum DDT, but also an unexpected 16 % increase in the probability of pregnancy for every 10 $\mu\text{g/L}$ in maternal serum DDE (Cohn et al. 2003). While in Brazil, sales

Table 7.1 Concentration of persistent organic pollutants (POPs) in the blood or follicular fluid of women participating in assisted reproductive (ART) programs

Study design	N	Age	Location	Exposure	Tissue	Concentration	Citation
Observational	28	28–38	Canada	Background	FF	hexachloroethane 23.2 ± 2.7	Younglai et al. (2002)
						1,2,4-trichlorobenzene 21.2 ± 2.0 pg/ml	
						mirex 3595 ± 590 pg/ml	
						cotinine 606 ± 147 ng/ml	
						PCB-49 62.4 ± 6.8 pg/ml	
						PCB-153 73.3 ± 7.0 pg/ml	
Observational	31 21	Not reported	Germany & Tanzania	Background	S	PCB-180 62.2 ± 5.2 pg/ml	Weiss et al. (2006)
					FF	PCBs 0.35 ± 0.05 vs. 0.17 ± 0.02 µg/kg	
						0.26 ± 0.02 vs. 0.22 ± 0.06 µg/kg	
Prospective	79	26–34	United States	Background	S	PCBs 5.27 ng/g serum	Bloom et al. (2007)
Case-control	765	35.9 ± 4.21 ^a	United States	Background	S ^b	HCB 18 ng/g lipid p,p'-DDE 226 ng/g lipid DDT 251 ng/g lipid	Mahalingaiah et al. (2012)

^amean ± SEM^bFollicular fluid concentrations were quantified but not reported

of pesticides in 1985 were correlated with reproductive alterations and cancer rates a decade later (Koifman et al. 2002), no association between serum DDE levels (mean value: 30.0 ± 19.6 $\mu\text{g/L}$) and menstrual cycle dysfunction (cycle length and missed periods) was found in two American studies (Cooper et al. 2005; Mahalingaiah et al. 2012). A study comparing German and Tanzanian women attending fertility clinics (Weiss et al. 2006) found that Tanzanian women had higher DDE levels (12.77 ± 9.7 vs. 0.78 ± 0.75 $\mu\text{g/kg}$, $P < 0.01$). In contrast, in a Canadian study (Younglai et al. 2002), pesticide concentrations in the follicular fluid were not related to pregnancy rates whereas in a Belgian study of infertility patients (Petro et al. 2012), an association between follicular fluid DDE levels (392 ± 348 pg/ml) and reduced fertilization rates and embryo quality was described. We postulate that equivocal findings may be related to study design, sample size, analytical methods, regional differences, and potential interactions amongst the myriad chemicals to which people are exposed.

Organophosphates are a widely used family of pesticides to increase crop productivity by eradicating pests. Despite substantial available information involving the impact of organophosphates on the environment and different animal species (Patel et al. 2007), evidence of toxicity in humans remains scarce. Exposures to high concentrations of organophosphates in a short period, especially during the manufacture, formulation, and application of chemicals can lead to toxic effects (Dyer et al. 2001). In Iowa and North Carolina, the association between pesticide use and menstrual function among 3103 women living on farms was examined (Farr et al. 2004). These women were premenopausal, between the ages of 21–40 years of age, not pregnant or breastfeeding, and not taking hormonal contraceptives. Results revealed that women who were exposed to pesticides experienced a non-significant increase in menstrual cycle length (odds ratio = 1.2, 95 % CI: 0.66, 2.2) and increased odds of missed periods (odds ratio = 1.5, 95 % CI: 1.2, 1.9) compared with women who have never used pesticides (Farr et al. 2004). Women who were exposed to organophosphates experienced longer menstrual cycles (odds ratio = 1.3, 95 % CI: 0.55, 2.9) and increased odds of missed periods (odds ratio = 1.4, 95 % CI: 0.93, 2.2) compared with women who had never used pesticides. Women with suspected exposure to hormonally active or ovotoxic pesticides (parathion and trichlorfon) had fewer cases of irregular menstrual cycles (odds ratio = 0.53, 95 % CI: 0.37, 0.78) but 60–100 % increased odds of long cycles (odds ratio = 1.6, 95 % CI: 1.0, 2.5), missed periods (odds ratio = 1.7, 95 % CI: 1.3, 2.2), and intermenstrual bleeding (odds ratio = 1.3, 95 % CI: 1.0, 1.6) than women who had never used pesticides (Farr et al. 2004). The findings suggest a possible but weak correlation between the use of certain hormonally active pesticides and decreased menstrual function among women living on farms. Further detailed data on menstrual cycle and organophosphate exposure is required to strengthen the association between organophosphate exposure and menstrual cycle characteristics.

Of the metals, exposure to cadmium (Cd), mercury (Hg), lead (Pb), selenium (Se) and zinc (Zn) are the most commonly encountered and linked with adverse reproductive effects (Dickerson et al. 2011; Jackson et al. 2011; Pollack et al. 2011; Yang et al. 2002). Cd, Hg and Pb have been measured in follicular fluid (Al-Saleh et al. 2008; Bloom et al. 2012b) providing direct evidence of target tissue exposure (Table 7.2). The effects of metals including Cd on ovarian function have been

Table 7.2 Concentration of metals in the blood or follicular fluid of women

Study design	N	Age	Location	Exposure	Tissue	Concentration	Citation	
Observational	394	18–44	China	Occupational	Air at workplace	0.001 to 0.2 mg/m ³	Yang et al. (2002)	
Case-control	619	19–50	Saudi Arabia	Background	Follicular fluid	Cd=0.29±0.30 µg/L	Al-Saleh et al. (2008)	
						Pb=0.61 ± 1.17 µg/dL		
						Hg=2.12 ± 2.47 µg/L		
Case-control	252	18–44	United States	Background	Whole blood	Cd=0.30 µg/L	Jackson et al. (2011)	
						Pb=0.87 µg/dL		
						Hg=1.10 µg/L		
Case-control	30	26–42	United Kingdom	Background	Hair samples (Hg)	Hg=0.89±0.3 µg/g	Dickerson et al. (2011)	
						Serum (Zn & Se)		Zn=897.3±386.4 µg/g
								Se=100.2±19.7 µg/g
Case-control	46	28–43	United States	Background	Blood & Follicular fluid	Pb=0.82±0.31 µg/dL	Bloom et al. (2012a, b)	
						0.25±0.31 µg/dL		

evaluated by several research groups (Davis et al. 2001; Laughlin et al. 1987; Zhang and Jia 2007; Zhang et al. 2008). An inverse relationship between serum Cd concentrations and circulating concentrations of FSH were reported in the absence of any effect on circulating E_2 concentrations (Pollack et al. 2011). The relationship between menstrual cycle characteristics and circulating metal concentrations was investigated previously; however, only Cd was associated with higher serum E_2 concentrations (Jackson et al. 2011) which contrast with the results described by others who could not find an effect (Pollack et al. 2011). For each $\mu\text{g/l}$ increase in Cd there was a 24.3 % increase in circulating E_2 concentrations during the follicular phase (95 % CI=1.1–52.9). Although the source of exposure was not confirmed, cigarette smoking is thought to be the primary source. Cigarette smoking is associated with elevated levels of blood Cd that are 2.5–4 times higher than in non-smokers (Batariova et al. 2006; Wong and Lye 2008; Zhang and Jia 2007). However, these results contrast with studies that have reported decreased circulating concentrations of E_2 in female smokers (Windham et al. 2005).

While a positive association between blood Pb concentrations and circulating P4 levels have been reported (Pollack et al. 2011) an inverse association between follicular fluid Pb concentrations and fertilization rates (relative risk=0.68, $P=0.026$) was found in women undergoing fertility treatments in another study (Bloom et al. 2012b). The mean blood and follicular fluid Pb concentrations were 0.82 ± 0.31 and 0.25 ± 0.31 $\mu\text{g/L}$, respectively. In another study (Jackson et al. 2011), Pb levels of 0.87 $\mu\text{g/dl}$ were reported in human whole blood samples, but were not associated with toxic effects. An increased odds ratio for dysmenorrhea (1.66; 1.07–2.59) compared to non-exposed controls was found in a study of normal cycling women with occupational exposure to Hg vapor in a Chinese lamp factory (Yang et al. 2002). In another study (Dickerson et al. 2011), Hg, Zn and Se concentrations were measured in patients attending a fertility clinic. Only Hg showed a negative correlation with the number of oocytes collected and follicle number after ovarian stimulation, after adjusting for age and BMI. There were no effects on fertilization, cleavage rates or embryo quality. Despite evidence of reproductive hazard, the literature relating metal exposure with reproductive effects lacks consistency across studies and thus the evidence is judged as weak.

Unlike POPs and metals, newer generation commercial chemicals are engineered to be more labile and hydrophilic in order to obviate concerns with bioaccumulation and toxicity to mammalian systems. Of these bisphenol A (BPA) has received extensive research attention and has been linked with reproductive effects in women, yet considerable controversy continues to surround the health risk associated with this chemical. For example, urinary concentrations of BPA have recently been associated with a shorter luteal phase but not with adverse effects on the follicular phase length, TTP or early pregnancy loss (Jukic et al. 2015). In a subsequent study of women ($n=44$) attending a fertility clinic (Fujimoto et al. 2011), serum unconjugated (free) BPA concentrations were measured by HPLC. In this study, the median unconjugated BPA was 2.53 ng/ml and ranged from non-detectable to 67.4 ng/ml. Serum BPA concentrations were inversely associated with E_2 ($\beta=-0.16$; 95 % CI=-0.32, 0.01) and with E_2 concentration normalized to the number of mature-

sized follicles at the human chorionic gonadotropin (hCG) trigger ($\beta = -0.14$; 95 % CI = $-0.24, -0.03$). However, the concentration of unconjugated BPA in the serum was not associated with oocyte maturation rate, but intracytoplasmic sperm injection (ICSI) or conventional IVF patients had a 55 % decrease in the probability of fertilization for each doubling in BPA levels (adjusted relative risk = 0.45 , 95 % CI = $0.21-0.66$). Although preliminary, these data suggest that free BPA in the circulation is potentially associated with a reduced serum E_2 response to ovarian stimulation in IVF and reduced fertility. Limitations of the above studies are the focus on study participants who are attending fertility programs and thus are not necessarily representative of the population overall.

7.3 Exposure Data

Insight into whole body and target tissue exposure is essential for dose setting in experimental studies as well as for transparent and defensible evidence-based regulatory decisions and advice to guide industry action. However, exposure data is frequently lacking for many environmental contaminants and quantification of target tissue exposure is complicated by difficulty in accessing the ovary and ovarian follicular fluid. Therefore, understanding the relationship between circulating concentrations of contaminants and the concentrations in the follicular fluid is potentially valuable. Herein we summarize the available exposure data and explore the relationship between serum and follicular fluid concentrations.

Advances in analytical chemistry have enabled the measurement of increasingly smaller concentrations of environmental contaminants, many with reproductive and developmental toxicity, in the human circulation and reproductive fluids (Calafat et al. 2008; Silva et al. 2004). Environmental contaminants have been quantified in serum (Bloom et al. 2007, 2011; Fujimoto et al. 2011; Mahalingaiah et al. 2012; Younglai et al. 2002) and ovarian follicular fluid (Baukloh et al. 1987; Foster et al. 1995; Ikezuki et al. 2002; Jarrell et al. 1993; Jirsova et al. 2010; Neal et al. 2008; Trapp et al. 1984, 1990; Younglai et al. 2002) of women participating in assisted reproductive therapy (ART) programs. Although exposure to environmental contaminants has been linked to adverse effects on fertility (Bloom et al. 2011; Fujimoto et al. 2011; Ikezuki et al. 2002; Mahalingaiah et al. 2012; Takeuchi et al. 2004; Younglai et al. 2002), the available literature is limited to studies involving patients attending ART programs and thus is not representative of the general population. Thus improved methods of estimating target tissue exposure are needed.

Organofluorine compounds include the surfactants perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA); used as stain repellents in the manufacture of textiles, carpets and furniture (La Rocca et al. 2012), have been measured in follicular fluid (Petro et al. 2014). A study on the effects of long-term PFOS exposure in drinking water (Knox et al. 2011) found serum levels of 16.9 ± 9.9 , 19.1 ± 12.9 and 24.8 ± 16.3 ng/ml for 3 age groups (18–42, 43–51 and 52–65 years, respectively). PFOS exposure was negatively associated with estrogen levels (E_2 decreased from

~88 to 72 pg/ml, $p < 0.0001$ and ~32 to 22 pg/ml, $p < 0.007$, for the 1st compared to the 5th quintile of PFOS levels, respectively) in the two oldest age groups described above. Similarly there was an increased odds of experiencing early menopause for the 5th vs. the 1st quintile of PFOS concentrations (OR, 95 % CI=1.4, 1.1–1.8 and 2.1, 1.6–2.8 for both age groups, respectively). A positive effect of PFOA levels in follicular fluid on fertilization rate and proportion of viable embryos collected from IVF patients has been described (Petro et al. 2014); however, the mechanism for this effect is unclear. A study of 53 couples attending a fertility clinic in Italy (La Rocca et al. 2012) revealed that patients with unexplained infertility had detectable PFOS (>0.5 ng/g whole blood). Of those exhibiting infertility factors (PCOS, endometriosis or male-factor infertility), 30 women had >20 -fold lower limit (3–144 ng/g). Whole blood PFOA concentrations were below the detection limit in 90 % of samples. It is worth noting that serum and follicular fluid concentrations of the perfluorinated compounds are close to identical (Petro et al. 2014) suggesting that serum concentrations are useful surrogate markers of target tissue exposure.

The concentrations of total BPA (free + conjugated) in the serum from healthy non-pregnant and pregnant women, and follicular fluid from women attending an IVF clinic have been reported (Ikezuki et al. 2002). Concentrations ranged from 1.4 ± 0.9 to 2.4 ± 0.8 ng/ml in most cases suggesting exposure to BPA in women. While these data suggest exposure of estrogen sensitive target tissues at critical stages of steroid regulated development, these reports are limited to measures of total BPA and thus the concentration of the bioactive form of BPA, free BPA, in ovarian follicular fluid remains unknown. Polycystic ovarian syndrome (PCOS) and serum concentrations of BPA were linked through two independent studies, one conducted in Japan and the other in Greece (Kandaraki et al. 2011; Tsutsumi 2005). Serum BPA concentrations were 0.64 ± 0.1 for normal cycling women compared to 1.04 ± 0.1 ng/ml for women with PCOS (Tsutsumi 2005). In the second study (Kandaraki et al. 2011), women referred to a PCOS clinic were stratified by BMI and serum BPA concentrations were again higher in women with PCOS and a BMI ≥ 25 (0.96 ± 0.46 vs. 0.72 ± 0.39 ng/ml, $p < 0.05$) or < 25 (1.13 ± 0.63 vs. 0.70 ± 0.36 ng/ml, $p < 0.001$) compared to healthy cycling women, respectively. However, the antibody based ELISA method employed to quantify circulating BPA concentrations in these studies employed an antibody with significant cross reactivity with BPA metabolites and thus the assay cannot discriminate between free (bioactive) BPA and its metabolites. Therefore, it is unclear, on the basis of this evidence, what role if any BPA may play in the development or progression of this disease. Free BPA could not be detected in the follicular fluid of women (Krotz et al. 2012) raising further concern that the reported link between BPA and PCOS may be spurious; however, we note that the data in this study is limited to only five study subjects and thus cannot be taken as conclusive. Regardless of the findings with free BPA, the authors were able to quantify phthalate metabolites in ovarian follicular fluid of these same five women suggesting that technical limitations are unlikely to account for the failure to detect free BPA in these samples. The phthalate metabolites quantified in the follicular fluid included: mono-2-ethylhexyl phthalate (m-EHP, 9.34 ± 3.33 ng/mL), monoethyl phthalate (m-EP, 3.19 ± 2.97 ng/mL), mono-n-butyl

phthalate (m-BuP, 1.62 ± 0.59 ng/mL) and monomethyl phthalate (m-MP, 1.19 ± 0.25 ng/mL) (Krotz et al. 2012) but these concentrations are considered to be 200–1000 times lower than those harmful to reproductive function in laboratory animals (Kay et al. 2013). Therefore, although human exposure is recognized as wide-spread, the concentrations present in the ovary are thought too low to have an adverse effect. While regulatory action seems inappropriate, health conscious individuals who remain concerned can minimize their personal exposures to these chemicals through consumption of fresh foods and reducing personal exposure to plastics and canned goods.

Finally, parabens are another group of contemporary chemicals used as antimicrobial agents in the production of cosmetics, food and pharmaceuticals (Ahn et al. 2012; Taxvig et al. 2008). Urinary parabens concentrations were measured in patients attending a fertility clinic (Smith et al. 2013) yielding median concentrations of 158, 35.5 and 1.53 $\mu\text{g/L}$ for methyl, propyl and butyl parabens (mPB, pPB and bPB), respectively. Although serum or follicle fluid levels were not measured, the authors suggested a correlation between urinary paraben concentrations and ovarian reserve. This provocative suggestion requires additional study in both clinical samples and animal studies to confirm and explore potential mechanisms of action. Although widely appreciated, we feel compelled to remind the reader that evidence of exposure cannot be equated with evidence of an adverse health effect or increased risk of an adverse health outcome at a later date. Risk is the product of exposure, evidence of a health hazard usually derived from experimental animal studies and an uncertainty factor that accounts for differences between experimental animals and humans.

7.4 Experimental Animal Evidence

Well-controlled animal studies allow for testing with known concentrations of pure test compounds, using relevant routes of exposure, to genetically homogenous animals at well-defined developmental stages, and under carefully regulated environmental conditions that cannot be replicated in human populations. Consequently, animal studies are important to understanding the potential health hazards associated with exposure to environmental contaminants and contribute essential data to evidence based regulatory decisions.

Different reproductive and developmental toxicology test protocols have been developed by government bodies such as the Organization for Economic Cooperation and Development (OECD), the United States-Environmental Protection Agency (US-EPA), and the National Toxicology Program (NTP). While these protocols exploit good laboratory practices rendering their results highly reproducible and thus attractive for regulatory purposes, they have been criticized for the superficial nature of the outcome measures employed and lack of mechanistic insight. Animal testing for reproductive and developmental effects using these protocols provides indirect evidence for toxicant effects on ovarian function as shown by changes in

estrous cycle length, sexual maturation, fertility index, weight of reproductive organs and histology, and circulating reproductive hormone concentrations. By comparison, academic scientists operate in less restrictive environments and thus are able to develop innovative protocols designed to assess reproductive function and provide mechanistic insight that is lacking in traditional regulatory toxicity testing paradigms. For example, a series of elegant studies have shown that BPA exposure can affect oogenesis and aneuploidy in mouse and non-human primate oocytes (Hunt et al. 2003, 2012), insight that could not be achieved through traditional testing paradigms. Moreover, access to primary (Haney et al. 1984) and immortalized ovarian granulosa cell lines (Kwintkiewicz et al. 2010) has also allowed for direct assessment of test chemical effects. Recent developments have seen the introduction of isolated ovarian follicle cultures employed to assess test chemical effects on stage dependent effects of follicle growth, steroidogenesis, follicle survival, and ovulation (Cortvrindt et al. 1998; Cortvrindt and Smitz 2002; Lenie et al. 2004; Lenie and Smitz 2009; Neal et al. 2007, 2010; Sadeu and Foster 2011a, b; Van Wemmel et al. 2005), a model that has been extended to three dimensional cultures of follicles (Songsasen et al. 2011). While these studies provide much needed mechanistic insight, they suffer from the need for sophisticated testing, and high level of expertise in the field. Regardless of their limitations, results arising from both regulatory and academic scientific studies contribute essential data to the risk assessment process and thus are central to evidence based regulatory decisions. Chief among the effects detected are depletion of the ovarian follicle reserve, interference with the cumulus oocyte complex (COC) communication, and steroidogenesis.

7.4.1 Effects on Ovarian Reserve

With each menstrual cycle, a cohort of primordial follicles enters one of the waves of growing follicles (Baerwald et al. 2003a, b). Typically one follicle from the growing pool of follicles is selected to ovulate while the remainder become atretic. Of the estimated 500,000 follicles present in the human ovary at the start of reproductive life, only about 400 reach the pre-ovulatory stage and ovulate whereas follicle atresia is the fate for the vast majority of female germ cells (Byskov 1978). The number of follicles formed *in utero*, the rate of recruitment into the growing pool, and rate of follicle destruction are all potentially modifiable by environmental contaminant exposure with important implications to fertility, sterility, premature ovarian failure, and menopause. Indeed, contaminant exposures that disrupt the number of follicles formed during development, affect the rate of follicle recruitment, follicle development, or follicle loss can have serious implications for circulating steroid concentrations, fertility, and ultimately the reproductive life span for a woman. Since estrogens are important mediators of cell proliferation, neurogenesis, growth and maintenance of bone, and cardiovascular health, the consequences to general health arising from ovarian toxicity are potentially profound and reach across the life span with important implications to health care needs and costs.

Contaminant exposure in adult animals has been shown to decrease ovarian follicle counts but the most sensitive follicle stage to the toxic effects of environmental contaminants is variable. For example, 4 vinylcyclohexene diepoxide (VCD) has been shown to selective target primordial and primary follicles (Appt et al. 2006; Sahambi et al. 2008; Sobinoff et al. 2010), the follicles at the earliest stages of development. Similar effects have also been shown with polycyclic aromatic hydrocarbons such as 9,10- dimethyl benzanthracene, 3-methylcholanthrene, and benzo[a] pyrene and (Borman et al. 2000), all of which involve activation of the apoptosis pathway. Of note, repeated exposure to low concentrations may be more toxic than exposure to a single high concentration of an environmental contaminant (Borman et al. 2000), an effect that could have important implications for humans who are typically exposed to low concentrations of contaminants over long periods of time. Similar effects on follicle counts have been demonstrated with several different POPs. For example, PCB treatment-related increase in ovarian follicle loss (Baldrige et al. 2003) has been documented with fewer preantral follicles (15 ± 0.6 vs. 56 ± 0.7 /section, $p < 0.05$), and fewer small and large antral follicles (18 ± 0.9 vs. 41 ± 2 and 8 ± 0.9 vs. 30 ± 0.6 /section, respectively, $p < 0.05$), as well as more atretic follicles (59 ± 0.9 vs. 26 ± 1.2 , $p < 0.05$). Similarly, in a study on the long-term reproductive effects of dioxin exposure (Franczak et al. 2006), prepubertal rats were treated orally with vehicle or 10 μg TCDD/kg at 29 days of age (acute treatment) while pregnant rats were treated (0, 50 or 200 ng/kg) on GD 14, 21 and postnatal D7 and 14. Subsequently, the female pups were given the same weekly doses until 8 months of age (chronic treatment). The acute treatment delayed ($p < 0.05$) vaginal opening (37.8 ± 1.5 vs. 33.2 ± 1.0 d), reduced reproductive lifespan (287.2 ± 9.7 vs. 319.2 ± 4.1 d), and prolonged diestrus (0.65 ± 0.01 vs. 0.58 ± 0.02 of cycle). Treatment also reduced E_2 output ($p < 0.05$). Chronic treatment had no effect on follicle and corpus luteum numbers or structure but at 8 months only 25–30 % of treated females showed normal cycles (vs. 100 % in controls, $p < 0.05$). Although the two largest doses of TCDD used accelerated reproductive ageing (fewer had normal cycles at 9 and 11 months, $p < 0.05$), there was no effect of TCDD treatment on follicle counts (Shi et al. 2007). In another study TCDD (0 or 5 $\mu\text{g}/\text{kg}$, i.p.) treatment decreased ER gene expression in the ovary and other tissues in 6-to-8-week-old mice (Tian et al. 1998). Available studies have primarily evaluated the effect of test chemicals under basal conditions. In a provocative study (Jung et al. 2010), TCDD treatment decreased ovulation rate (2.7 ± 1.9 vs. 20.3 ± 3.6 , $p < 0.05$) and increased Aryl hydrocarbon Receptor (AhR) expression in granulosa cells in 25-day-old rats treated with 1 dose of TCDD (0 or 32 $\mu\text{g}/\text{kg}$, by gavage) plus hCG and LH (1 and 3 d later, respectively) to induce follicle development and ovulation. Treatment also decreased the number of follicles available for ovulation. Taken together these data suggest that TCDD may have direct effects upon the ovary although the mechanisms remain to be determined.

In addition to direct effects of TCDD in adults, evidence has been brought forward in the literature that suggests effects may be transmitted across generations. In a transgenerational study (Nilsson et al. 2012), F3 females also exhibited fewer primordial follicles than controls (~ 14 to 9, $p < 0.005$), with no effect on antral follicle

counts, but developed cystic structures similar to PCOS following treatment of pregnant rats with TCDD (0 or 100 ng/kg BW/d i.p.) on gestation day (GD) 8–14. However, the underlying mechanisms remain unclear.

Clinical markers of adverse effects of environmental contaminants on ovarian function are sparse; however, AMH has received increasing interest as a marker of ovarian reserve and has been used in toxicology to assess Methoxychlor (MXC) effects on the ovary. In a developmental study (Masutomi et al. 2003), rats were treated with MXC (0, 24, 240, or 1200 ppm in the diet) from GD 3 to PND 21. Only the highest dose (1200 ppm) decreased body and ovarian weight, accelerated vaginal opening, and caused irregular estrous cycles. Furthermore, longer estrous cycles (6.28 ± 1.91 vs. 3.76 ± 0.25 days, $p < 0.001$), fewer corpora albicantia from previous cycles, fewer corpora hemorrhagica, and more degenerate oocytes were found in MXC treated rats (Quignot et al. 2012a, b). MXC (0, 1, 10, 50, 100, or 500 mg/kg/d) exposure from 3 to 10 days of age resulted in antral follicle loss but increased the growing follicle pool, and increased AMH expression at 20 days of age (Uzumcu et al. 2006). Doses of 50, 100, and 500 led to 1.6 ± 0.2 , 1.85 ± 0.6 and 2.2 ± 0.5 times the AMH expression of the control group ($p < 0.05$), as measured using immunohistochemistry. Antral follicle counts were reduced (~ 6 /section after 100 or 500 mg, vs. ~ 17 in controls, $p < 0.05$), whereas preantral follicles increased (~ 13 and 15 after 100 and 500 mg, vs. ~ 8 in controls, $p < 0.05$). The effects of VCD on circulating AMH concentrations (Sahambi et al. 2008) further support the notion that AMH is a potentially useful minimally invasive marker of toxicant effect.

The adverse effects of legacy chemicals such as the POPs on ovarian follicle counts are not unique and can be extended to contemporary commercial chemicals such as BPA. In a study on prenatal exposure (Markey et al. 2003), pregnant mice were exposed to BPA on GD 9 to term (via s.c. pump delivering 0, 25 or 250 $\mu\text{g}/\text{kg}/\text{day}$). Treatment did not affect age at vaginal opening, but more mice showed persistent estrus/metestrus (≥ 4 days) after exposure to 25 μg (55.7 %) and 250 μg (55.6 %) than controls (37.1 %, $p < 0.05$). A proportion of exposed females showed blood-filled ovarian bursae at 3 months (11 and 16 vs. 0 %), and those exposed to 250 μg had a greater ovarian area occupied by antral follicles (15.5 ± 2.7 vs. 6.4 ± 2.8 %, $p < 0.05$). In a transgenerational study (Nilsson et al. 2012), pregnant rats were treated with BPA (0 or 50 mg/kg BW/d i.p.) on GD 8–14, F3 females exhibited fewer primordial follicles than controls (~ 14 to 9, $p < 0.005$), with no effect on antral follicle counts, but developed cystic structures similar to PCOS.

In another study on pre- and postnatal exposure (Signorile et al. 2012), pregnant mice were treated with BPA (0, 100 or 1000 $\mu\text{g}/\text{kg}$) on GD 1-PND 7. Exposed females showed fewer primordial follicles (median numbers: 7.75, 3.8 and 2.65, respectively, $p < 0.001$), reduced numbers of developing follicles (11.9, 5.5 and 4.5, $p < 0.001$), and increased the number of atretic follicles (1.55, 3.3 and 3.4, $p < 0.001$). In another study (Xi et al. 2011), female mice and their pups were treated with BPA (0, 12, 25 or 50 mg/kg/d, by gavage) from GD 1 to PND 20, and from PND 20 to 49, respectively. Exposure led to a dose-dependent increase in E_2 (~ 150 , 300, 550 and 600 pMol/L), which altered feedback signals within the hypothalamic-pituitary-gonadal axis.

Neonatal exposure to BPA (PND 1, 3, 5 and 7) was examined (Rodriguez et al. 2010) in rats treated with oil (negative control), DES (positive control, 0.2 or 20 $\mu\text{g}/\text{kg}$) or BPA (0.05 or 20 mg/kg). BPA treatments (20 mg/kg) led to the highest proportion of recruited follicles on PND 8: ~30 % of follicles for control, ~45 % for DES0.2 ($p < 0.001$), ~40 % for DES20 ($p < 0.01$), ~55 % for BPA20 ($p < 0.001$), and ~30 % for BPA0.05 (NS). The BPA (20 mg/kg) dose also led to more EsR1-positive follicles than negative controls (~65 vs. 15 %, $p < 0.05$), which may explain the higher recruitment rate. In contrast, EsR2 positive staining was not affected by treatment. Taken together the data consistently show BPA exposure induced primordial follicle loss and suggest accelerated follicle recruitment into the growing pool.

In contrast to the examples of BPA, more primordial follicles and more primary follicles and altered gene expression for steroidogenic enzymes such as *StAR* and *Cyp11a1* were found (Ahn et al. 2012) in rats treated with methyl, propyl or butyl parabens (mPB, pPB and bPB, respectively, at 0, 62.5, 250 or 1000 $\text{mg}/\text{kg}/\text{d}$ each, s.c.) or E_2 (40 $\mu\text{g}/\text{kg}/\text{d}$, positive control) for 7 days (PND 1-7). In a gestational exposure study (Taxvig et al. 2008), rats were exposed to s.c. oil, ethyl paraben (ePB, 400 $\text{mg}/\text{kg}/\text{d}$) or bBP (200 or 400 $\text{mg}/\text{kg}/\text{d}$) on GD 7-21. The females and the fetuses showed no histologic alterations in the ovaries, but treatment increased EsR2 gene expression. Additionally, female rats maternally exposed to bPB from GD 6 to PND 20 (100 $\text{mg}/\text{kg}/\text{day}$) presented early vaginal opening with no alterations in ovarian weight and histology (Kang et al. 2002), possibly due to the estrogenic action of parabens in the female HPG axis during development. In contrast, prepubertal mPB and isopropyl paraben exposure (250 and 1000 mg/kg from PND 21-40) leads to a delay in vaginal opening, a decrease in the number of corpora lutea and an increase in the number of cystic follicles in the ovary (Vo et al. 2010).

In summary, multiple contaminants from different chemical classes have been shown to affect ovarian follicle counts and thus establish the ovary as a potentially important target organ for adverse effects of environmental contaminant exposure. Moreover, we postulate that the accelerated loss of primordial follicles has the potential to shorten reproductive life span as well as advance the age of onset for health problems such as osteoporosis and cardiovascular disease arising from loss of estrogen. The mechanisms of ovarian follicle loss are unclear and may include decreased number of oocytes and follicles formed during development (Yin et al. 2015), increased recruitment of follicles into the growing pool (Rodriguez et al. 2010), and increased rate of follicle loss through either apoptosis (Jurisicova et al. 2007; Matikainen et al. 2001; Takai et al. 2003) or autophagy (Gannon et al. 2012, 2013). Regardless of the mechanism, the underlying initiating events remain to be elucidated and have important implications for fertility preservation as well as life-long health benefits. Tissue culture studies have shown that the adverse effects of polycyclic aromatic hydrocarbons such as benzo[a]pyrene can be attenuated by co-treatment with AhR antagonists such as resveratrol and 3,4 dimethoxyflavone (Casper et al. 1999; Neal et al. 2010). These data raise the encouraging possibility that the ovary can be protected from the adverse effects of some contaminants on ovarian follicle loss. Fertility preservation is an exciting area of reproductive biology that is receiving renewed interest which we anticipate will uncover novel therapeutic interventions to protect ovarian function.

7.4.2 Steroidogenesis

Steroid production in the ovary is regulated by gonadotrophins and involves androgen production by theca cells and subsequent conversion of androgens to estrogens by granulosa cells. Results of animal studies demonstrate that CdCl₂ exposure decreased serum E₂ and P₄ concentrations by altering expression and/or activity of granulosa cell steroidogenic enzymes P450_{scc} and StAR in adult female rats injected s.c. with CdCl₂ (0, 2.5, 5 and 7.5 mg/kg) (Zhang and Jia 2007; Zhang et al. 2008). CdCl₂ has a MW of 183, but only 112 of it is Cd. 1M would be 112 g/L; 1 μM would be 112 μg/L; 20 μM (lowest significant effect) would be 2.24 mg/L, a level much higher than the 0.29 ± 0.30 μg/l reported in follicular fluid in Saudi Arabian women for both controls and women under fertility treatment (Al-Saleh et al. 2008). Lead acetate was given to adult rhesus monkeys over several years at different doses (3.6, 5.9 and 8.1 mg/kg/d) in drinking water (Laughlin et al. 1987). Lead was detected in blood at concentrations that ranged between 51 and 88 μg/dl. Although the monkeys were still fertile, treated females showed menstrual cycles that were longer (50.9 vs. 32.9 days) and more variable, with shorter menses (2.3 vs. 3.4 d, *p* < 0.05). Chronic lead exposure suppressed circulating concentrations of LH, FSH and E₂ in cynomolgus monkeys (Foster 1992) and suppressed luteal function at moderate blood levels (Foster et al. 1996).

Toxic effects of phthalates and phenols in experimental animal studies have been examined by several authors (Guerra et al. 2010; Kimura et al. 2006; Laskey and Berman 1993; Masutomi et al. 2003; Nagao et al. 2000; Willoughby et al. 2005) and have recently been reviewed elsewhere (Kay et al. 2013). Pregnant rats were exposed to di-*n*-butyl-phthalate (DBP) from GD 12 to PND 21 (0 or 100 mg/kg/d by gavage); the dose used did not affect ovarian development, puberty, hormone levels or fertility (Guerra et al. 2010). In another study (Masutomi et al. 2003), rats were treated with diisononyl phthalate (DINP, 0, 400, 4000, or 20,000 ppm in the diet) from GD 3 to PND 21; the highest dose decreased body and ovarian weight, whereas the lower doses had no adverse effects. Using a different approach, adult rats were treated with bis(2-diethylhexyl) phthalate (DEHP) (0 or 1500 mg/kg/day by gavage) for 10 days and their ovaries collected at different cycle stages for culture and measurement of steroid output (Laskey and Berman 1993). In diestrus, treated rats produced more E₂ and testosterone than controls, whereas in estrus, they produced more E₂. Another study (Mlynarcikova et al. 2009) also evaluated the effects of phenols and phthalates, by treating cumulus-oocyte-complexes (COCs) with 4-Chloro-3-methyl phenol (CMP), di(2-ethylhexyl) phthalate (DEHP) and benzyl butyl phthalate (BBP), in a range of 10²–10⁻⁴ μM. Only the highest levels of CMP and BBP decreased COC expansion rate. The highest level of CMP decreased oocyte maturation rate (50.0 % reached metaphase II (MII) vs. 81.5 % in controls; *p* < 0.001) and only the second highest level of DEHP increased P₄ output (~50 % more than control *p* < 0.05). In another study from this group (Mlynarcikova et al. 2007), porcine granulosa cells were treated with 10⁻⁸M to 10⁻⁴M 4-octylphenol (OP), 4-nonylphenol (NP), and 4-*tert*-octylphenol (tOP), diisononyl phthalate

(DiNP), diisodecyl phthalate (DiDP) or dioctyl phthalate (DOP), with or without FSH. OP (10^{-5} M) reduced P_4 output ($p < 0.05$), and NP (10^{-8} M) decreased E_2 output ($p < 0.05$). FSH stimulated E_2 concentration in the media was reduced by OP, NP ($p < 0.05$), as well as DiDP and DiNP ($p < 0.01$) treatment. Taken together these data suggest that ovarian steroidogenesis can be affected by some of the phenols and phthalates used as plasticizers. To evaluate the effect of phthalate treatment on oocyte maturation equine COCs were incubated with di-(2-ethylhexyl) phthalate (DEHP) at 0, 0.12, 12 or 1200 μ M (Ambruosi et al. 2011). The lowest concentration of DEHP (0.12 μ M) inhibited oocyte maturation ($P < 0.05$) with increased apoptosis and reduced ROS levels ($p < 0.001$). Higher concentrations did not affect oocyte maturation, but apoptosis and ROS were higher ($p < 0.0001$). Treated oocytes also exhibited higher ATP content ($p < 0.05$). Taken together these data suggest that the COC is sensitive to the adverse effects of environmental contaminants; however, the underlying mechanisms are uncertain. We postulate that contaminant effects on gap junction communication are an area that may yield interesting results (Ganesan and Keating 2014; Gittens et al. 2003, 2005; Kidder and Mhawi 2002; Li et al. 2007).

While contaminant effects on ovarian steroidogenesis have been established, the mechanisms remain ill-defined and thus different culture methods have been employed in an effort to clarify this issue. Cocultures of granulosa and theca cells were isolated from pig antral follicles (Ptak et al. 2006) and treated with 4-Chlorobiphenyl (PCB3) or two of its metabolites (4-OH or 3,4-diOH PCB3, at 6 ng/ml). PCB3 and metabolite treatment decreased P_4 output by 55–65 % and 70–80 % of control ($p < 0.05$), respectively. All 3 compounds increased E_2 output (130–330 % of controls, $p < 0.05$), via increasing P_4 conversion to androgen and subsequently androgen aromatization. Chronic TCDD exposure upregulated the expression of 19 genes of known function (e.g. Cyp11a1, indicating activation of AHR) and downregulated the expression of 31 genes (e.g. 17 α -OHase); FSH receptor, aromatase and inhibin were not affected (Valdez et al. 2009). Thus, TCDD alters ovarian steroidogenesis by affecting genes related to androgen synthesis. Similarly, rat granulosa cells from punctured follicles treated with 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE, the main metabolite of methoxychlor) produced a dose-dependent inhibition of P_4 output, blocked FSH-induced P450_{scc}, 3 β -HSD and P450_{arom} mRNAs, but not StAR (Zachow and Uzumcu 2006). Thus, HPTE alters steroidogenesis by inhibiting several of the corresponding enzymes. The cells were incubated for 48 h with androgens (as aromatase substrates) and FSH (30 ng/ml) in the presence or absence of HPTE (0.1, 1, or 10 μ M). The effects of methoxychlor (MXC) on ovarian steroidogenesis have been demonstrated by treating granulosa cells from pig antral follicles for 48 h with DDE (10^0 – 10^3 μ M) or MXC (10^{-2} – 10^1 μ M), E_2 (0.1 μ M, positive control) or negative control (10 % new born calf serum), with a final FSH exposure (Chedrese and Feyles 2001). In addition to stimulating cell proliferation, DDE affected FSH-induced cAMP production. MXC inhibited P_4 but not cAMP. The authors concluded that the mechanism appeared to be non-estrogenic. In rats, exposed prenatally (GD 19–22) and postnatally (PND 0–7) to 0, 20 or 100 μ g/kg/d MXC exhibited 83–100, 78–100 and 0–11 % regular cycles and E_2 levels of ~27, 20, 18 pg/ml, respectively ($p < 0.05$), at 13–15 months of age (Gore et al. 2011).

In tissue culture experiments (Grasselli et al. 2010), granulosa cells aspirated from pig antral follicles were treated with BPA (0, 0.1, 1, or 10 μM) and cell proliferation, E_2 , P_4 , and VEGF output were measured. Cell proliferation was not affected by BPA whereas the lowest dose of BPA increased estradiol, while the two higher ones inhibited it ($p < 0.001$), and all BPA levels inhibited progesterone ($p < 0.01$). VEGF output was increased after treatment with 1 and 10 μM BPA ($p < 0.05$). The authors concluded that BPA alters follicle steroidogenesis and VEGF production. In one study (Mlynarcikova et al. 2005), granulosa cells from pig antral follicles were treated with BPA or BPA-dimethacrylate (DMA) at 10^{-4} – 10^{-8} M and E_2 and P_4 output was determined. P_4 concentrations were increased by 10^{-6} BPA treatment ($p < 0.05$) whilst 10^{-4} BPA inhibited P_4 output ($p < 0.001$). At all concentrations tested, BPA inhibited FSH-induced E_2 production ($p < 0.05$). In another study (Mlynarcikova et al. 2009), porcine COCs were treated with BPA (10^2 – 10^{-4} μM) during FSH-induced oocyte maturation. Subsequently, cumulus expansion, oocyte maturation and P_4 output were determined. Only the highest BPA concentration tested reduced the COC expansion rate compared to controls (16.9 % vs. 39.3 %, $p < 0.05$), oocyte maturation rate (50.0 % reached MII vs. 81.5 %, $p < 0.001$), and P_4 output (47.4 % less than controls, $p < 0.01$). Although equivocal, the data lend some support to the observation that BPA is associated with an impaired response to ovulation induction (Fujimoto et al. 2011).

Human cells have also been used to study potential ovariotoxic effects. KGN cells, an ovarian granulosa-like tumour cell line that expresses a functional FSH receptor, as well as human granulosa cells (GLC) from IVF patients were treated with BPA (0, 40, 60, 80 or 100 μM), with or without FSH (Kwintkiewicz et al. 2010). In the KGN cells, BPA exhibited a dose-dependent reduction on FSH-induced IGF1, aromatase and E_2 . In the GLC, only aromatase was examined, with similar effects to those seen in KGN cells (but the cells were more sensitive to lower doses). BPA also induced PPAR γ , an aromatase inhibitor, which was seen as a possible mechanism for lower aromatase expression. Human fetal (21–22 weeks) ovarian explants were incubated with BPA (0 or 30 μM) for 1, 2 and 3 weeks (Brieno-Enriquez et al. 2012). Oocytes and fibroblasts were isolated for RNA extraction. BPA led to higher expression ($p < 0.05$) of genes related to double-strand break generation, signaling and repair (*Rpa*, *Spo11*, *H2ax*, *Blm*, *Stra8*, *Nalp5*), onset of meiosis (*Stra8*), estrogen receptors and primordial follicle formation (*Nalp5*). However, the importance of these findings to risk assessment is unclear in the absence of accurate measures of free BPA concentrations in the follicular fluid. Assuming that the total BPA concentrations previously reported in follicular fluid were composed entirely of free BPA then the concentrations as added to the culture media used in this study would be approximately 4500 times greater than the concentrations measured in follicular fluid.

Isolated ovarian follicle culture techniques have been developed to assess the effect of test chemicals on stage-dependent follicle development and ovulation (Sun et al. 2004). In one study (Lenie and Smitz 2009), mouse follicles were isolated and cultured for 12 days to determine follicular development. Ovulation was stimulated (with recombinant hCG and recombinant epidermal growth factor), and evaluated

on day 13. During development, follicles were exposed to the active metabolite of di (2-ethylhexyl) phthalate (DEHP), mono (2-ethylhexyl) phthalate (MEHP, 10–200 μM). MEHP led to a fivefold increase in P_4 output by day 12, but no change after ovulation stimulus. Testosterone/ E_2 ratios showed that, despite increased testosterone output, less E_2 was produced with increasing MEHP. In another study (Treinen et al. 1990), MEHP was added to cultures of rat granulosa cells collected from freshly ruptured follicles (the rats had been treated with DES to induce granulosa cell proliferation). MEHP decreased up to 40 % cAMP production. Treatments containing 10, 25, 50 and 100 μM MEHP produced 30, 35, 40 and 70 % less P_4 than controls ($p < 0.05$), and cAMP production was similarly inhibited. Human granulosa cells from IVF patients were treated with MEHP (0 or 0.6–500 μM) and aromatase mRNA, cell viability and steroid output were measured. MEHP decreased aromatase and E_2 output (Reinsberg et al. 2009) whereas P_4 output and cell viability decreased only at the highest dose. The large variety of phthalate compounds appears to have varying effects, and again, most studies involve laboratory animals, where significant effects are seen only at higher concentrations. The lowest dose with an effect was 0.12 μM of DEHP (Ambruosi et al. 2011), a dose equivalent to 47 $\mu\text{g/L}$ (or ng/mL); a concentration that is substantially higher than the highest concentration of a phthalate metabolite (9.3 ng/ml) previously measured in follicular fluid (Krotz et al. 2012). In preliminary data from our lab, using an isolated mouse ovarian follicle culture system, BPA treatment significantly decreased the number of follicles as a percentage of the controls progressing to the preantral (18.04 ± 5.5 %), antral (28 ± 13.7 %) and preovulatory (54.5 ± 20.4 %) stage, and follicle survival (50.6 ± 18.8 %), but only at the highest concentration (5.0 μM) tested (Fig. 7.1). Treatments had no effect on gonadal steroid output in these cultures (Fig. 7.2). Thus, effective concentrations as added in cultures ranged between 1.14 and 2.28 mg of BPA/ml; concentrations far in excess of human exposure.

7.4.3 Genetic and Epigenetic Effects

The mutagenic effects of environmental toxicants have been well documented and effects on DNA repair mechanisms have also been described (Ganesan et al. 2013, 2014; Ganesan and Keating 2015). In addition to transcriptional regulation of genes through toxicant changes in transcription factor expression and microRNA (miRNA), contemporary studies have revealed that contaminant exposures are also associated with changes in the epigenome involving histone modifications including DNA methylation. Marks on the DNA that regulate gene expression and may be carried across generations.

Epigenetic effects of dietary factors such as phytoestrogens and environmental contaminants have been documented on the rodent uterus and lung whereas effects on the ovary remain largely unexplored (Zama and Uzumcu 2010). In one study identified in our literature search (Zama and Uzumcu 2009), MXC induced treatment-related effects on estrous cyclicity, ovarian steroidogenesis, and produced

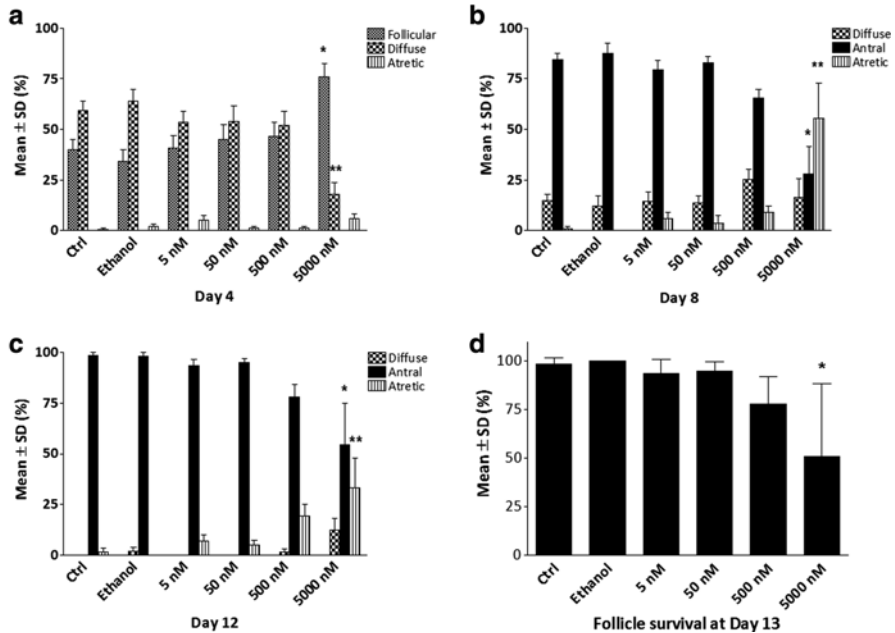


Fig. 7.1 Effect of BPA-exposure on *in vitro* follicle growth and survival. Follicular development at (a) 4, (b) 8, and (c) 12 days of culture and (d) follicular survival at 12 days of culture. All the follicles were follicular at day 0 (start) of culture. Each experiment was repeated at least 3 times in duplicate plate/condition, $n=826$ follicles (10–16 follicles/plate). Bars with different letters (within the same follicular stages) differ significantly ($p < 0.05$)

changes in DNA methylation with potential effects across generations. Effects of MXC on DNA methylation have been documented in pregnant rats exposed to MXC (0, 20 μg or 100 $\text{mg}/\text{kg}/\text{d}$ i.p.) from GD 19 to PND 7. Female pups were also treated from day 0 to PND 7 and euthanized after a cycle on PND 50–60. DNA methylation showed that 10 ovarian genes were hypermethylated after the highest MXC dose, including genes related to follicle maturation and ovulation, such as *PAPP-A*. Ovarian *EsR1* was not affected, but *EsR2* expression was reduced with 100 mg MXC (19.9 ± 1.06 vs. 36.7 ± 5.47 , $p < 0.05$). Environmental contaminant-induced changes in ovarian epigenetic marks is an important emerging area in reproductive biology that holds great promise for increased mechanistic insight.

7.5 Future Directions

Understanding the hazard to reproductive health and ovarian function arising from exposure to environmental contaminants is difficult to appreciate in the absence of direct measures of exposure and more sensitive markers of an adverse effect. While

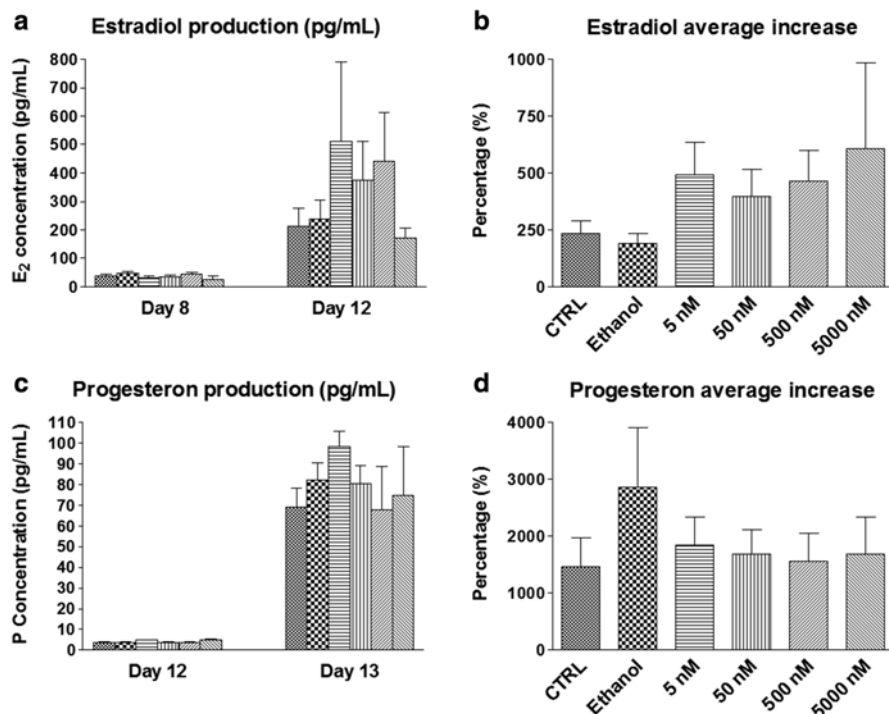


Fig. 7.2 Effect of BPA-exposure on steroid outputs during *in vitro* follicle growth. (a) Estradiol (E_2) and (c) progesterone (P_4) outputs after antral (Day 8) and preovulatory (Day 12) follicle development. Relative average increase of (b) E_2 between Days 8 and 12 [(Day 12/Day 8) \times 100] and of (d) P_4 between Days 12 and 13 [(Day 13/Day 12) \times 100]. Values are means \pm S.E.M. E_2 : n=4–8 replicates; P_4 : n=4–5 replicates

circulating concentrations of AMH provides insight into the primordial follicle count/ovarian reserve, there are a number of limitations with this marker that have yet to be adequately resolved. Specifically, changes in circulating concentrations of AMH as women age as well as relationship with BMI and health status such as PCOS and diabetes are yet to be defined and normal ranges are only generally established.

While the concentrations of some chemicals in the serum are representative of concentrations that can be measured in ovarian follicular fluid (e.g. perfluorinated compounds), this is not always the case as shown for *p,p'*-DDE which can be three times higher in the serum than the follicular fluid (Younglai et al. 2002). It is encouraging that good correlation has been shown between serum and ovarian follicular fluid concentrations of DDT and its metabolites (Mahalingaiah et al. 2012). Therefore, we suggest that the pharmacokinetics and pharmacodynamics of environmental contaminants are essential data for establishing relevant target doses for animal and tissue culture studies as well as for risk assessment purposes.

We note that animal studies are clearly indispensable in the assessment of potential hazards to human health and reproductive function. We propose that their contribution to the risk assessment process could be enhanced with greater attention to issues of comparative endocrinology. For example, emerging evidence suggests that women may have more than one wave of follicles developing for ovulation in a given cycle (Baerwald et al. 2003a, b; Baerwald and Pierson 2004). However, many questions remain and the relevance of these observations to human health, fertility and toxicology has yet to be defined. We further postulate that while the academic investigators enjoy a much less restrictive environment that favors development of innovative testing strategies to enhance understanding of the mechanisms underlying toxic phenomenon, greater insight into the dose used, route of exposure, and use of multiple dose groups would enhance uptake of study results by regulatory bodies. Finally, there have been many calls for studies of mixtures and we add to the cacophony of voices calling for such testing.

Finally, although the literature suggests that effects of environmental contaminants at concentrations typically measured in contemporary biomonitoring studies are unlikely, precaution is never a poor option. Overall, regulatory decisions appear to be effective in protecting the health of general population against adverse effects on ovarian function; however, we note that chemicals continue to enter the environment. Manufacturing practices have improved substantially over the last decades with reductions in chemical emissions or releases into the environment. However, leaching of chemicals from finished products continues to be a problem and thus is an issue for engineering to insure safety. While calls to regulatory authorities for chemical bans continue, we caution against replacement of suspected chemicals with alternatives that are potentially more hazardous to human health such as Bisphenol S and Bisphenol F, replacements for BPA (Eladak et al. 2015; Rosenmai et al. 2014). Toxicity testing has progressed substantially over the last several decades as techniques have improved and understanding of reproductive physiology and endocrinology have advanced. We note that it will never be possible to prove absolute safety; however, enhancements to existing regulatory testing paradigms and continued interest of academic investigators promises to insure that potential health effects of environmental contaminants are identified and brought forward in the literature for use in the risk assessment process. In addition to partnering with chemical engineers to identify safer alternatives, academic investigators are encouraged to explore mechanisms of action as well as potential therapeutic interventions that can protect the ovary and aid in fertility preservation. Renewed interest in this area could have spill-over benefits for women undergoing cancer therapies that are well known to damage ovarian health.

7.6 Conclusions

Human exposure to potential ovarian toxicants has not been well defined and of greater concern is the lack of data on internal exposure dose and measurement of parent and metabolite concentrations in the ovary. While there is a robust animal

literature illustrating the potential hazard to ovarian function and reproductive health from exposure to environmental contaminants, the concentrations at which effects were documented were frequently orders of magnitude beyond human exposure. While a robust literature was uncovered, the environmental contaminants addressed represent only a very small proportion of the many thousands of chemicals in commercial use that enter the environment, the food chain, and ultimately result in human exposure. Thus the available literature provides only an incomplete picture of the potential impact of environmental contaminants on reproductive health and ovarian function. We note that some environmental contaminant exposures have been linked with decreased circulating concentrations of E_2 , shorter or longer menstrual cycle length, attenuated response to ovulation induction strategies, impaired fertility, and earlier menopause. However, the available literature is equivocal and highly variable with respect to study design, populations studied, sample size, exposure assessment, analytical methods employed, and outcomes assessed. Therefore, we propose that the state-of-the science is presently inadequate to conclude that exposure to environmental contaminants are or are not associated with adverse effects on ovarian function or human fertility.

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