Title
Chapter Four Ovarian Toxicity from Reactive Oxygen Species

Author
Luderer, Ulrike

Publication Date
2014

DOI
10.1016/b978-0-12-800095-3.00004-3

Peer reviewed

Ovarian Toxicity from Reactive Oxygen Species

Ulrike Luderer, Professor of Medicine, Developmental and Cell Biology, and Public Health,

University of California Irvine, Irvine, CA

Abstract

Oxidative stress occurs when cellular mechanisms to regulate levels of reactive oxygen species (ROS) are overwhelmed due to overproduction of ROS and/or deficiency of antioxidants. This chapter describes accumulating evidence that oxidative stress is involved in ovarian toxicity caused by diverse stimuli, including environmental toxicants. There is strong evidence that ROS are involved in initiation of apoptosis in antral follicles caused by several chemical and physical agents. Although less attention has been focused on the roles of ROS in primordial and primary follicle death, several studies have shown protective effects of antioxidants and/or evidence of oxidative damage, suggesting that ROS may play a role in these smaller follicles as well. Oxidative damage to lipids in the oocyte has been implicated as a cause of persistently poor oocyte quality after early life exposure to several toxicants. Developing germ cells in the fetal ovary have also been shown to be sensitive to toxicants and ionizing radiation, which induce oxidative stress. Recent studies have begun to elucidate the mechanisms by which ROS mediate ovarian toxicity.

Keywords: oxidative stress, ovary, ovarian follicle, oocyte, granulosa cell, phthalates, polycyclic aromatic hydrocarbons, methoxychlor, cyclophosphamide, ionizing radiation

Ovarian follicular development and its hormonal regulation

Ovarian follicles are the functional unit of the mammalian ovary. Follicles consist of the oocyte (germ cell) surrounded by and forming intercellular connections with specialized somatic cells called granulosa cells, which are in turn surrounded by and make connections with specialized somatic cells called theca cells (Figure 1). Follicles progress through various stages of development from primordial to preovulatory/Graafian (Figure 1). Primordial follicles are quiescent, with oocytes arrested in the first meiotic prophase since before birth. Throughout reproductive life, primordial follicles are continually recruited into the growing pool. When primordial follicles are activated, the oocyte grows and granulosa cells differentiate from a fusiform appearance to a more cuboidal form and proliferate. A follicle with a single layer of cuboidal granulosa cells is called a primary follicle. A follicle with more than one layer of granulosa cells is called a secondary follicle (sometimes referred to as preantral). Eventually fluid filled vesicles appear in the granulosa cell layer, forming early antral follicles. The vesicles coalesce to a single fluid-filled antrum. Granulosa cell proliferation then slows and the last stages of follicular growth are mainly due to expansion of the antrum, eventually forming a preovulatory or Graafian follicle.

Ovarian follicular development and ovulation are regulated by hypothalamic (Gonadotropin releasing hormone, GnRH) and pituitary (luteinizing hormone, LH, and follicle stimulating hormone, FSH) hormones (Figure 2). GnRH is secreted into the portal circulation to

act on the gonadotrope cells in the anterior pituitary gland to stimulate synthesis and secretion of the gonadotropins, LH and FSH. FSH and LH act on their specific receptors in the plasma membranes of granulosa cells and theca cells, respectively, of growing follicles. Follicles are responsive to gonadotropins from the secondary stage on, and preovulatory follicles have an absolute requirement for gonadotropins for survival. Large antral, preovulatory follicles are capable of ovulating in response to a mid-cycle gonadtropin surge. The ovarian steroids, estradiol (E_2) and progesterone (P), and the peptide hormone, inhibin, are synthesized in the granulosa cells and theca cells. These hormones feed back to regulate the synthesis and secretion of GnRH, LH, and FSH. The majority of ovarian follicles do not ovulate, but undergo an apoptotic process of degeneration called atresia at the small antral follicle stage (Hirshfield 1988) (Figure 1).

Stimulation of preovulatory follicle development in rodents via injection of equine chorionic gonadotropin (eCG, also called pregnant mare's serum gonadotropin, PMSG), which has FSH and LH receptor binding activity, followed 46-48 hours later by an ovulatory dose of human chorionic gonadotropin (hCG), which has only LH receptor binding activity, is commonly used in experiments assessing the effects of gonadotropin hormones on ovarian gene expression and other endpoints and for generating preovulatory follicles or ovulated oocytes for other studies.

Reactive oxygen species (ROS) and oxidative stress

ROS and reactive nitrogen species (RNS) include free radicals such as hydroxyl radical (OH•), superoxide anion radical (O_2-•), and nitric oxide (NO•); non-free radical ROS/RNS

include hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO-) (Spitz et al. 2004) (Figure 3). O_2-• and OH• are short-lived in biological systems because their unpaired electron makes them highly reactive with cellular macromolecules (Wiseman and Halliwell 1996; Roberts et al. 2009; Roede and Jones 2010). H_2O_2 is more long-lived and membrane-permeable and can diffuse from its site of origin within cells or among cells (Spitz et al. 2004; Giorgio et al. 2007). O_2-•, H_2O_2, and OH• are formed by the sequential addition of electrons to molecular oxygen. This occurs in mitochondria as a result of incomplete reduction of molecular oxygen to water during oxidative phosphorylation; it is estimated that about 0.5% of oxygen consumed by mitochondria is converted to O_2-• and H_2O_2 (Giorgio et al. 2007). Multiple enzyme systems that generate H_2O_2 exist within cells, including NADPH oxidase and phagocytic oxidases on cell membranes, peroxisomal oxidases in peroxisomes, sulfhydryl oxidase in endoplasmic reticulum, superoxide dismutase 1 (SOD1), and cyclooxygenase in cytoplasm, and superoxide dismutase 2 (SOD2) and p66shc in mitochondria (Giorgio et al. 2007). In steroidogenic tissues like the ovary, ROS can be generated as a result of uncoupling of electron transfer from substrate hydroxylation by steroidogenic cytochrome P450 enzymes (Hanukoglu 2006). ROS are also formed as a result of metabolism of xenobiotics, as will be discussed in detail below.

Many studies in recent years have shown that H_2O_2 and other ROS and RNS function as important signaling molecules within cells (Giorgio et al. 2007). One important mechanism by which this occurs is via oxidation of redox-sensitive cysteine residues in proteins, which reversibly alters protein function (Jones 2006; Jones 2008). Oxidation and reduction of these residues are regulated by two independent thiol-containing systems, thioredoxins (TXN) and glutathione (GSH) and its related enzymes, glutathione-*S*-transferases (GST), which catalyze

glutathionylation of proteins, and glutaredoxins (GRX), which catalyze de-glutathionylation (Jones 2006; Jones 2008; Anathy et al. 2012). Ovulation is an example of an ovarian process that is regulated by ROS. ROS levels rise and antioxidant levels fall in response to the preovulatory gonadotropin surges, and treatment with antioxidants inhibits ovulation (Laloraya et al. 1988; Miyazaki et al. 1991; Sato et al. 1992; Shkolnik et al. 2011). Oxidative stress occurs when the normal cellular redox balance is disturbed, resulting in dysregulation of redox-regulated processes and/or oxidative damage to cellular macromolecules (Jones 2006; Jones 2008).

Hormonal regulation of and effects of depleting or enhancing ovarian antioxidants

Cells possess numerous enzymatic (Figure 3) and nonenzymatic antioxidant systems that function to tightly regulate cellular levels of ROS/RNS. Deficiency of antioxidant vitamins has profound effects on female fertility. Expression of many ovarian antioxidant genes is regulated by gonadotropin hormones, suggesting that these antioxidants may play important roles in ovarian function. Numerous studies in recent years have examined the biological effects of the deletion of genes encoding antioxidant enzymes and their cofactors, including the effects on ovarian function. These studies demonstrate a role for some, but not all antioxidant genes examined in ovarian function.

Cytosolic, mitochondrial, and extracellular SODs (Cu-SOD or SOD1, Mn-SOD or SOD2, and SOD3, respectively) dismutate O_2-• to H_2O_2 and water (Figure 3). *Sod2* and *Sod3* mRNA expression, but not *Sod1* expression, are upregulated in rat ovary by eCG stimulation (Tilly and Tilly 1995). The eCG-induced increased *Sod2* expression is localized to theca interna cells of

growing follicles, and subsequent administration of an ovulatory dose of hCG increases theca cell expression further and upregulates expression in granulosa cells as well (Sasaki et al. 1994). Global deletion of *Sod1* has been reported by two groups using different gene-targeting strategies (Ho et al. 1998; Matzuk et al. 1998). Both groups reported that female *Sod1* null mice were subfertile, but reported somewhat different ovarian phenotypes. Matzuk et al observed fewer preovulatory follicles and corpora lutea in ovaries of adult *Sod1* null females, and concluded that *Sod1* deletion adversely affected antral follicle development and ovulation (Matzuk et al. 1998). Ho et al reported that *Sod1* null females had normal ovarian histology and ovulated similar numbers of oocytes during a natural estrous cycle, but had smaller litters due to postimplantation embryonic mortality (Ho et al. 1998). Another group subsequently reported that female *Sod1* null mice have decreased serum progesterone levels upon superovulation and during pregnancy and have small corpora lutea with increased numbers of apoptotic cells and fewer blood vessels and demonstrated that embryos null for *Sod1* developed normally when transplanted to wild type dams (Noda et al. 2012), suggesting that the postimplantation embryonic mortality observed by Ho et al may be due to luteal dysfunction. Mice null for Copper Chaperone of Superoxide Dismutase (CCS), which have decreased ability to incorporate copper into *Sod1*, display a similar ovarian phenotype to that reported by Matzuk et al (Matzuk et al. 1998) for *Sod1* null mice, with abnormal antral follicle development and absence of corpora lutea (Wong et al. 2000). Taken together, the results suggest that *Sod1* is required for normal antral follicle and corpus luteum development. Deletion of *Sod2* results in death before puberty, but transplantation of ovaries from *Sod2* null females to wild type recipients results in normal ovarian follicular development, ovulation, and fertility, suggesting that SOD2 is not required for ovarian function (Matzuk et al. 1998).

Glutathione peroxidases (GPX), catalase, peroxiredoxins (PRDX), and some glutathione-*S*-transferases (GST) can convert H_2O_2 to water (Figure 3). GPXs utilize the cysteine containing tripeptide glutathione (GSH, □-glutamyl cysteinyl glycine) as a cofactor. In the process of reducing peroxides, GSH becomes oxidized to the disulfide form (GSSG), which is reduced back to GSH by the action of glutathione reductase (GSR). GSTs also require GSH as a cofactor, but GSH is covalently linked with the substrate and consumed in these reactions (Hayes and Pulford 1995). GSH can also scavenge free radicals directly. Ovarian stimulation with eCG does not increase expression of *Gpx1* or catalase in rats (Tilly and Tilly 1995). Deletion of both of these genes (Ho et al. 1997; Ho et al. 2004) and inactivating mutations of *Gsr* (Pretsch 1999; Rogers et al. 2004; Rogers et al. 2006) reportedly did not affect fertility in mice, but detailed studies of ovarian effects of deleting these genes have not been conducted. Global deletion of *Gpx4* is embryonic lethal (Yant et al. 2003), but deletion of either the mitochondrial form or the nuclear form alone does not affect female fertility (Conrad et al. 2005; Schneider et al. 2009).

Ovarian stimulation with eCG increases protein expression of the modifier (GCLM) and catalytic (GCLC) subunits of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis, increasing GCL catalytic activity and GSH concentrations in the rat ovary (Aten et al. 1992; Luderer et al. 2001; Tsai-Turton and Luderer 2005). Studies in cultured rat antral follicles and granulosa cells show that FSH and estradiol interact to increase mRNA and protein expression of *Gclc* and *Gclm* (Tsai-Turton and Luderer 2006; Hoang et al. 2009). Deletion of *Gclc* in mice is embryonic lethal before gestational day 8.5 (Dalton et al. 2000; Shi et al. 2000). Mice with deletion of *Gclm* survive and reproduce, but have low tissue levels of GSH (Yang et

al. 2002; Giordano et al. 2006; McConnachie et al. 2007). GSH concentrations in the oocytes of *Gclm* null mice are less than 20% those of *Gclm*+/+ mice, and *Gclm*-/- females have decreased litter sizes due to preimplantation embryonic mortality (Nakamura et al. 2011). In vivo fertilization experiments showed that embryos derived from *Gclm*-/- females had decreased rates of formation of the second, male pronucleus at 0.5 days post coitum (dpc) and lower rates of survival to the blastocyst stage (Nakamura et al. 2011). Upon in vitro fertilization with wild type sperm, embryos derived from *Gclm*-/- oocytes progressed to the two-cell stage by 30 h after fertilization, but very few reached the blastocyst stage by 102 h (Nakamura et al. 2011). Together these results demonstrate that the preimplantation embryonic mortality in *Gclm*-/- females is driven by low GSH concentrations in the oocytes and not by low GSH concentrations in the female reproductive tract.

The enzyme γ-glutamyl transpeptidase-1 (*Ggt1*) is located on plasma membranes in many tissues. It catalyzes the first of two reactions that break down GSH into its three constituent amino acids, enabling them to be taken up by the cell (GSH itself cannot cross cell membranes). Deletion of *Ggt1* shortens lifespan and causes infertility in females, due to lack of ovarian large antral follicles, corpora lutea, and ovulatory response to exogenous gonadotropins (Lieberman et al. 1996; Kumar et al. 2000; Will et al. 2000). Ovarian GSH concentrations are normal in these mice, but ovarian cysteine concentrations are greatly decreased, and cysteine supplementation completely reverses the ovarian phenotype.

Non-enzymatic antioxidants in the ovary include the vitamins ascorbic acid (vitamin C), vitamin E, and vitamin A. Ascorbic acid directly scavenges free radicals. It is present in ovarian

cells at millimolar concentrations and at 50-200 micromolar in human follicular fluid (Luck et al. 1995; Zreik et al. 1999). Granulosa cells, theca cells, luteal cells, and oocytes all concentrate ascorbic acid, and its uptake and release from these cells is hormonally regulated (Aten et al. 1992; Luck et al. 1995; Musicki et al. 1996; Zreik et al. 1999). Rodents can synthesize ascorbic acid; humans and guinea pigs have an inactive form of the ascorbic acid synthesis gene and therefore have an absolute requirement for it in the diet. In the guinea pig, ascorbic acid depletion causes extensive follicular atresia and ovarian atrophy (Luck et al. 1995). Vitamin E refers to several compounds, chiefly □-, □-, □- and □-tocopherols, which are potent scavengers of peroxyl radicals (Niki and Traber 2012). Female infertility was one of the first reported effects of vitamin E deficiency, at a time when its molecular identity was unknown (Evans and Bishop 1922). Initially, infertility was thought to be entirely due to uterine effects, but more recently ovarian effects of vitamin E deficiency were also reported. Rats fed a vitamin E deficient diet beginning before puberty, have undetectable serum concentrations of LH, low estradiol levels, absence of the onset of normal estrous cycles, and ovaries with abnormal antral follicles with thickened granulosa cell layers (Das and Chowdhury 1999). Ovarian levels of vitamin A increase with eCG stimulation in rats and increase further during luteal regression (Aten et al. 1992). The vitamin A precursor □-carotene and □-tocopherol increase in bovine corpora lutea with increasing progesterone levels (Hanukoglu 2006). Vitamin A is required for the entry of germ cells into meiosis I, and vitamin A deficiency during prenatal development blocks the entry of ovarian germ cells into meiosis I during fetal life (Li and Clagett-Dame 2009). This action of vitamin A is mediated by retinoic acid, which is synthesized from retinol in target cells (Duester 2007). In addition, it was reported nearly a century ago that vitamin A deficiency during

adulthood in female rats disrupts normal completion of meiosis II in oocytes and prevents blastogenesis (Evans 1928).

Oxidative stress occurs in ovarian follicles when the normal hormonal support for follicular survival is removed

Culture of small and large (preovulatory) antral rat follicles without gonadotropin support leads to apoptotic death within 24 h, while FSH suppresses apoptosis (Chun et al. 1994; Chun et al. 1996). The antioxidants SOD, catalase, *N*-acetyl cysteine, and ascorbic acid are all able to inhibit apoptosis in cultured large antral follicles in the absence of FSH (Tilly and Tilly 1995). ROS levels, measured as increased fluorescence of the probes dichlorofluorescein and dihydrorhodamine, rise when preovulatory follicles are cultured without gonadotropin support before any increase in markers of apoptosis, and FSH significantly suppresses this rise in ROS, as well as inhibiting apoptosis of follicular granulosa cells (Tsai-Turton and Luderer 2006). Culture of preovulatory follicles without FSH is also associated with a decline in follicular GSH concentrations, whereas culture with FSH stimulates follicular GSH synthesis above initial levels (Tsai-Turton and Luderer 2006). Biochemical depletion of GSH with buthionine sulfoximine (BSO), a specific inhibitor of GCL, partially and significantly reversed the antiapoptotic effect of FSH, while supplementation with a cell-permeable form of GSH, glutathione ethyl ester, completely prevents the BSO-induced apoptosis (Tsai-Turton and Luderer 2006). Consistent with the in vitro effects of GSH depletion with BSO, in vivo treatment of adult female rats with BSO also significantly increases antral and secondary follicle atresia (Lopez and Luderer 2004). Taken

together these findings strongly support the conclusion that some of the anti-apoptotic effects of FSH in antral follicles are mediated by FSH upregulation of antioxidant defenses.

Effects of exogenous H_2O_2 on ovarian follicles and granulosa cells

Although endogenous H_2O_2 is an important signaling molecule, high levels of H_2O_2 are toxic to cells. Exogenous H_2O_2 at concentrations ≥0.5 mM rapidly induces cytotoxicity in cultured COV434 cells, a human granulosa cell tumor line, which displays many characteristics of normal granulosa cells, such as synthesis of estradiol in the presence of androstenedione and FSH (Cortés-Wanstreet et al. 2009). Stable overexpression of mouse *Gclm* or *Gclc* in COV434 cells increases cellular GSH concentrations and protects against H_2O_2-induced cytotoxicity (Cortés-Wanstreet et al. 2009). In primary cultures of rat granulosa cells, H_2O_2 dose-dependently inhibits progesterone synthesis via inhibition of steroidogenic acute regulatory (StAR) protein expression (Yu et al. 2012). StAR regulates the rate-limiting step in steroid synthesis, the transfer of cholesterol to the inner mitochondrial membrane.

Neonatal rodent ovaries are enriched for primordial follicles, making cultured neonatal ovaries an ideal model system in which to study the effects of toxicants on primordial follicles. In contrast to the adverse effects of H_2O_2 on steroidogenically active granulosa cells, primordial and small primary follicles in cultured neonatal (postnatal day 4) rat ovaries are relatively resistant to H_2O_2 concentrations below 6 mM applied for 8 days (Devine et al. 2012). At 6 mM concentrations, primordial and primary follicles display morphological signs of atresia (Devine et al. 2012).

Involvement of ROS in ovarian toxicity caused by chemical toxicants

Phthalates

Phthalates are diesters of *o*-phthalic acid with side chains of various lengths, which are commonly used as plasticizers and in other consumer products such as cosmetics. They impart flexibility to plastics in toys, medical devices, vinyl flooring, and food wraps. Phthalate diesters are metabolized to monoesters, which are the toxicologically active metabolites. In adult female rats, di(2-ethylhexyl) phthalate (DEHP) or its monoester metabolite, mono(2-ethylhexyl) phthalate (MEHP), decrease estradiol levels via both intraovarian and extraovarian mechanisms. DEHP and several other phthalates increase the metabolism of estradiol to estrone by inducing hepatic 17□-hydroxysteroid dehydrogenase Type IV activity (Lovekamp-Swan and Davis 2003). In addition, DEHP/MEHP, but not other phthalates, suppress aromatase mRNA and protein expression and thereby decreasing estradiol secretion in cultured rat granulosa cells (Lovekamp-Swan and Davis 2003). This effect of MEHP is mediated by activation of the nuclear receptors and transcription factors peroxisome proliferator activated receptors alpha and gamma (PPARα and PPARγ) (Lovekamp-Swan et al. 2003).

Both DEHP and MEHP inhibit the growth of cultured mouse antral follicles via an oxidative stress-dependent mechanism (Wang et al. 2012; Wang et al. 2012). Inhibition of growth is preceded by upregulation of expression of the proapoptotic *Bax* gene and downregulation of the antiapoptotic *Bcl2* gene and of several cell cycle regulatory genes (Wang et al. 2012). Both DEHP and MEHP at concentrations (10 μg/mL and ≥ 1 μg/mL, respectively) that inhibit growth

increase follicular ROS production, measured by an assay that utilizes the oxidation sensitive fluorophore dichlorofluorescein (Wang et al. 2012; Wang et al. 2012). Treatment with the GSH precursor and antioxidant, *N*-acetylcysteine, prevents the DEHP/MEHP-induced growth inhibition and the changes in apoptotic and cell cycle regulatory gene expression (Wang et al. 2012; Wang et al. 2012). These findings strongly support a role for ROS in initiating apoptosis in antral follicles exposed to DEHP/MEHP. It is not known whether cross-talk exists between the effects of DEHP/MEHP on estradiol synthesis in granulosa cells and follicle apoptosis; however, this may prove a fruitful avenue for further investigation.

Methoxychlor

Methoxychlor is an organochlorine insecticide, which was widely used as a less persistent alternative to DDT for many years, but which has been banned in the United States since 2002 (http://www.epa.gov/oppsrrd1/REDs/methoxychlor_red.htm). Administration of 32 mg/kg methoxychlor, but not lower doses, for 20 days to adult female mice causes atresia of antral follicles, but not of smaller follicles (Borgeest et al. 2002). Treatment regimens that induce antral follicle atresia also increase ovarian H_2O_2 concentrations and ovarian oxidative DNA and protein damage, detected by immunostaining for 8-hydroxy-2'-deoxyguanosine (8-OHdG) and nitrotyrosine, respectively, while decreasing ovarian enzymatic activity and mRNA expression of the antioxidants catalase, GPX, and SOD1 (Gupta et al. 2006). Culture of mouse antral follicles with methoxychlor (1-100 µg/mL) inhibits growth and increases atresia in a concentration- and time-dependent manner (Gupta et al. 2006). There is an initial upregulation of antioxidant gene expression (*Gpx* and catalase) after 48 h of 100 µg/mL methoxychlor treatment, before the onset of atresia, but expression of *Gpx*, catalase, and *Sod1* decrease in all concentration groups by 96

h, when marked atresia is evident (Gupta et al. 2006). Co-treatment with *N*-acetylcysteine prevents methoxychlor-induced growth inhibition, atresia, and alterations in antioxidant gene expression (Gupta et al. 2006). Together these findings support a role for oxidative stress in the induction of atresia in antral follicles by methoxychlor.

Although, as noted above, methoxychlor does not cause atresia of small follicles in the adult mouse ovary at doses up to 32 mg/kg/day, treatment of neonatal mice with 100 mg/kg/day, but not 50 mg/kg/day, methoxychlor for 7 days decreases the proportion of primordial follicles, while increasing proportions of transitional and primary follicles (Sobinoff et al. 2010). These findings suggest that methoxychlor increases recruitment of primordial follicles into the growing pool, and transcriptomic analysis showed that methoxychlor increases expression of genes in the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, as well as in other signaling pathways that are known to be involved in follicle recruitment and growth (Sobinoff et al. 2010). In addition, the same neonatal treatment regimen with methoxychlor, causes dose-dependently decreased in vitro sperm binding and sperm fusion to zona pellucida-free oocytes collected after superovulation from 6 wk old mice (Sobinoff et al. 2010). Incubation of superovulated oocytes with the ovotoxic methoxychlor metabolite, 2,2-bis-(p-hydroxyphenyl)-1,1, 1-trichloroethane, for 2 h increases lipid peroxidation measured by decreased ratio of red:green BODIPY fluorescence (Sobinoff et al. 2010). The latter results suggest that oxidative damage to oocyte plasma membrane lipids due to neonatal exposure to methoxychlor causes permanent decrements in oocyte quality. There is as of yet no direct evidence linking ROS signaling or oxidative stress to increased recruitment of primordial follicles, but culture of neonatal mouse ovaries with methoxychlor for 96 h induces oxidative DNA damage (Sobinoff et al. 2010), and

PI3K/Akt signaling pathways are known to respond to and to be modulated by oxidative stress (Sedding 2008).

Polycyclic aromatic hydrocarbons (PAHs)

PAHs are formed during the incomplete combustion of organic materials, such as wood, tobacco, fossil fuels, and foods. They are ubiquitous environmental pollutants to which all Americans are exposed (NHANES 2009). The parent PAH compounds are relatively nontoxic, but metabolism leads to the formation of DNA-reactive compounds and ROS generation (Winn and Wells 1997; Xue and Warshawsky 2005). It has been known for decades that the PAHs benzo[a]pyrene (BaP), 9,10-dimethyl-1,2-benzanthracene (DMBA), and 3-methylcholanthrene (3-MC) are potent ovarian toxicants, which dose-dependently destroy primordial and primary follicles in peripubertal mice and rats after single high doses (Mattison 1979; Mattison and Thorgeirsson 1979; Mattison 1980) or repeated lower doses (Borman et al. 2000). Human primordial follicles in ovarian explants placed into mice are also sensitive to depletion by DMBA (Matikainen et al. 2001). Exposure of mice to tobacco smoke, which is rich in PAHs, also depletes primordial follicles (Tuttle et al. 2009), and PAHs in tobacco smoke may be responsible for the earlier onset of menopause in women who smoke (Mattison et al. 1989; Harlow and Signorello 2000). Very little information is available on the possible roles of ROS in mediating PAH-induced primordial and primary follicle toxicity. One study showed varying effects of in vivo tobacco smoke exposure on several markers of oxidative stress; ovarian protein levels of heat shock protein 25 increased, while SOD2 protein levels decreased, but no changes were observed in oxidative protein and DNA damage or GSH concentrations (Gannon et al. 2012). Another recent study showed that DMBA treatment of neonatal mice (1 mg/kg/day for 7 days) or

cultured neonatal mouse ovaries (50 nM) increased activation of primordial follicles (measured by PCNA, phosphorylated Akt immunostaining), while increasing apoptosis in primary and secondary follicles (immunostaining for activated caspases 2 and 3 and TUNEL) (Sobinoff et al. 2011). Microarray analysis showed upregulation of methionine metabolism pathway, which was interpreted by the authors as suggesting a role for DMBA-induced oxidative stress in the neonatal ovarian toxicity of DMBA. The same group subsequently reported similar results for the PAHs BaP and 3-MC (Sobinoff et al. 2012; Sobinoff et al. 2012). In addition, they reported that neonatal treatment with BaP (1.5 or 3.0 mg/kg/day for 7 days) results in increased mitochondrial O_2-• generation measured using the fluorophore MitoSOX, increased lipid peroxidation measured using the fluorophore BODIBY, and decreased in vitro sperm binding and sperm fusion in oocytes ovulated at 6 weeks of age (Sobinoff et al. 2012).

In addition to destroying primordial and primary follicles, 3-MC also targets secondary follicles and DMBA also targets antral follicles (Mattison 1980; Borman et al. 2000). Culture of rat large antral follicles with DMBA concentration-dependently (≥1μM) induces granulosa cell apoptosis, increasing immunostaining for the proapoptotic protein BAX by 24 h, and increasing caspase 3 activation and DNA fragmentation by TUNEL staining by 48 h (Tsai-Turton et al. 2007). Follicular ROS levels, measured by dichlorofluorescein fluorescence, increase by 12 h and remain elevated, and supplementation of GSH protects against DMBA-induced apoptosis, while GSH depletion potentiates DMBA-induced apoptosis (Tsai-Turton et al. 2007). While these results suggest that the rise in ROS mediates induction of apoptosis by DMBA, the lack of protection against DMBA-induced apoptosis by two other antioxidants, dithiothreitol and butylated hydroxytoluene, suggests that other actions of GSH besides its antioxidant actions may

be involved (Tsai-Turton et al. 2007). Further studies are needed to clarify the role of ROS in DMBA-induced antral follicle toxicity.

The fetal ovary appears to be even more sensitive to PAH ovarian toxicity than the peripubertal ovary. Treatment of pregnant mice by oral gavage with 0, 10, 40, or 160 mg/kg/day BaP from gestational days 7 to 16 dose-dependently decreases fertility of the F1 female offspring, with very few follicles observed in the ovaries of mice exposed prenatally to the highest dose (MacKenzie and Angevine 1981). Support for a role of ROS in mediating BaP fetal ovary toxicity comes from a recent study, which showed that F1 female embryos deficient in GSH synthesis due to deletion of *Gclm* are more sensitive to the transplacental ovarian toxicity of BaP than their wild type littermates (Lim et al. 2013). F1 female *Gclm-/-* offspring exposed to 2 or 10 mg/kg/day BaP from gestational day 7 to 16 have greater decrements in fertility and in ovarian follicle numbers compared to vehicle treated controls of the same genotype than did their *Gclm*+/+ littermates (Lim et al. 2013).

Cyclophosphamide (CP)

Treatment of cancer and autoimmune diseases in women with alkylating antineoplastic drugs often causes temporary amenorrhea or premature ovarian failure, consistent with destruction of large, growing follicles and primordial follicles, respectively (Nicosia et al. 1985; Howell and Shalet 1998; Byrne 1999; Meirow and Nugent 2001; Chemaitilly et al. 2006). Even if menstrual cycles resume after a period of amenorrhea, women may suffer from premature ovarian failure due to partial depletion of the primordial follicle pool.

The anticancer activity of alkylating agents like CP results from their ability to alkylate DNA of cancer cells; however, DNA damage to nontarget cells is responsible for many of their side effects. CP is metabolically activated by cytochrome P450 enzymes to 4-hydroxycyclophosphamide, which undergoes ring-opening to aldophosphamide, which spontaneously decomposes to phosphoramide mustard (PM) (Chang et al. 1993; Dirven et al. 1994; Gamcsik et al. 1999). PM is thought to be the active metabolite both in terms of its anticancer activity and its ovarian toxicity (Plowchalk and Mattison 1991; Gamcsik et al. 1999). CP destroys follicles at all stages of development in women and in rodent models (Kumar et al. 1972; Warne et al. 1973; Shiromizu et al. 1984; Jarrell et al. 1987; Plowchalk and Mattison 1991; Davis and Maronpot 1996). Mice are more sensitive to the destruction of primordial follicles by in vivo treatment with CP (Shiromizu et al. 1984; Meirow et al. 1999), while rats are more sensitive to the destruction of secondary and antral follicles (Davis and Heindel 1998). Consistent with these in vivo findings, treatment of cultured neonatal mouse and rat ovaries, which are enriched in primordial follicles, with PM concentrations 3 and 30 μM, respectively, depletes primordial follicles (Desmeules and Devine 2006; Petrillo et al. 2011). Caspase activation, a classical marker of apoptosis, is not observed in primordial follicles treated with PM, and inhibition of caspase activation does not protect against primordial follicle depletion (Desmeules and Devine 2006; Petrillo et al. 2011). Double stranded DNA breaks are detected by immunostaining for phosphorylated histone H2AFX in primordial follicle oocytes prior to the onset of follicle degeneration at doses that induce follicle degeneration at later time points, consistent with a role for DNA damage in initiating follicular destruction (Petrillo et al. 2011). Although double-stranded DNA breaks can be caused by oxidative DNA damage (Lloyd et al.

1998), no studies have measured ROS or oxidative damage in primordial follicles exposed to PM.

In contrast to primordial follicles, there is evidence for ROS and caspase activation playing a role in the mechanism of follicular destruction by CP in secondary and antral follicles. A single i.p. dose of ≥50 mg/kg CP in normally cycling adult rats significantly increases the percentages of secondary and antral follicles that have apoptotic granulosa cells, as evidenced by TUNEL staining and immunostaining for activated caspases 3 and 9 (Lopez and Luderer 2004; Devine et al. 2012). COV434 human granulosa cells have been used as a model system in which to study the mechanisms by which CP induces ovarian apoptosis (Tsai-Turton et al. 2007). Treatment of COV434 cells with 0, 1, 10, or 50 μM 4-hydroperoxycyclophosphamide (4HC), a preactivated form of CP that decomposes in solution to 4-hydroxycyclophosphamide (Gamcsik et al. 1999; Flowers et al. 2000) causes rapid (within 2 h), concentration-dependent declines in GSH concentrations and increases in ROS assessed by dichlorofluorescein fluorescence (Tsai-Turton et al. 2007). These are followed at 12 and 24 h by oxidative DNA damage measured by 8-OHdG immunofluorescence, activation of caspase 3, and DNA fragmentation by TUNEL staining (Tsai-Turton et al. 2007). Supplementation with a cell-permeable form of GSH or with the antioxidants ascorbic acid or dithiothreitol, is protective against 4HC-induced apoptosis, whereas depletion of GSH with BSO potentiates 4HC-induced apoptosis (Tsai-Turton et al. 2007). Together, the available evidence is consistent with an early increase in ROS during CP metabolism initiating apoptosis in granulosa cells of secondary and antral follicles.

Chromium

The heavy metal chromium (Cr) is used in numerous industrial processes, including leather tanning, electroplating, wood preservative manufacture, and steel alloy production (Keegan et al. 2008). Cr contamination of drinking water from industrial and natural sources is an important exposure route for the general population in parts of the world. Cr exists in several oxidation states. CrVI is a known carcinogen, which is rapidly reduced to CrIII, the most stable oxidation state, in biological systems by ascorbic acid, GSH, and cysteine. However, during reduction, reactive Cr species and ROS are generated (O'Brien et al. 2003). CrIII is excreted in breast milk, and lactation is thus a source of exposure to the offspring.

Administration of Cr in drinking water causes follicular atresia in adult mice (Murthy et al. 1996). Administration of 200 mg/L potassium dichromate (a CrVI compound) in the drinking water to lactating female rats from the day of parturition until weaning at postnatal day (PND) 21 decreases primordial and primary follicle numbers in the F1 female offspring at PND 21, 45, and 65, and decreases secondary and antral follicle numbers at PND 21 and 45, with recovery by PND 65 (Banu et al. 2008). Co-administration of 500 mg/L ascorbic acid with Cr prevents the decreases in follicle numbers (Banu et al. 2008). A subsequent dose response study by the same group showed that 50, 100, and 200 mg/L Cr administration dose-dependently increases ovarian follicular atresia and apoptosis over the same age range and that ascorbic acid administered by gavage at 500 mg/kg/day is protective (Stanley et al. 2013). Cr administration also dose-dependently decreases serum estradiol, progesterone, and testosterone concentrations and increases FSH concentrations at all time points in F1 female offspring; ascorbic acid co-administration prevents or mitigates these effects (Banu et al. 2008; Stanley et al. 2013). Cr treatment is associated with increased systemic and ovarian H_2O_2 and lipid hydroperoxide

concentrations in the F1 female offspring through PND 65, and again ascorbic acid prevents or mitigates these increases (Stanley et al. 2013). Cr administration dose-dependently decreases plasma and ovarian enzymatic activities of the antioxidant enzymes GPX, GSR, SOD, and catalase, as well as ascorbic acid concentrations, and ascorbic acid supplementation mitigates these effects (Stanley et al. 2013). Similarly, another recent study found that F1 female offspring which were exposed to 200 mg/L Cr in the drinking water from GD9 through PND65, first via dosing of their mothers and then via direct dosing, had elevated malondialdehyde, an indicator of lipid peroxidation, and H_2O_2 concentrations in the ovaries at various ages from birth until PND65 compared to controls that received drinking water without Cr (Samuel et al. 2012). Together these in vivo studies are consistent with increased ROS and the resulting oxidative stress mediating the destruction of ovarian follicles and ovarian endocrine dysfunction caused by developmental Cr exposure.

Studies by the same group using cultured primary rat granulosa cells and theca cells or spontaneously immortalized granulosa cells (SIGC) provide additional insights into the mechanisms of Cr-induced ovarian toxicity. Treatment of cultured rat granulosa cells with 10 μM potassium dichromate for 12 and 24 h induces apoptosis, measured by TUNEL, caspase 3 activation, and cleavage of PARP; treatment with a caspase inhibitor prevents Cr-induced apoptosis (Banu et al. 2011). Induction of apoptosis in granulosa cells appears to be via the mitochondrial pathway, as Cr treatment decreases cellular levels of the antiapoptotic proteins BCL-2 and BCL-XL and increases translocation of the proapoptotic proteins BAX and BAD and of activated p53 to the mitochondria and increases translocation of cytochrome c from the mitochondria to the cytosol (Banu et al. 2011). The same Cr treatment regimen also induces cell

cycle arrest and inhibits proliferation of cultured rat granulosa cells (Stanley et al. 2011). All of these effects of Cr in granulosa cell cultures are mitigated by co-treatment with ascorbic acid (Banu et al. 2011; Stanley et al. 2011). More recently, this group showed that treatment of cultured granulosa cells, theca cells, and SIGC with 10 μM potassium dichromate increases H_2O_2 and lipid hydroperoxide concentrations by 12 h, and ascorbic acid prevents this Cr-induced oxidative stress (Stanley et al. 2013). Further, the same Cr treatment decreases ascorbic acid concentrations, mRNA expression of the antioxidant genes *Sod1, Sod2, catalase, Glrx1, Gsr, Gstm1, Gstm2, Gsta4, Txn1, Txn2, Txnrd2,* and *Prdx3*, and enzymatic activity of GPX, GSR, and GST in all three cell types by 12 h, and these effects are largely mitigated by ascorbic acid in granulosa cells and SIGC and to a lesser extent in theca cells (Stanley et al. 2013). Together these results suggest that increased ROS and resultant oxidative stress initiate apoptosis via the mitochondrial pathway in granulosa cells and theca cells upon exposure to Cr.

Menadione

Menadione or 2-methyl-1,4-naphthoquinone is a vitamin K analog, which can undergo one electron reduction by enzymes including microsomal NADPH-cytochrome P450 reductase and mitochondrial NADH-ubiquinone oxidoreductase (complex I), resulting in redox cycling, or it can undergo detoxification via two electron reduction by NAD(P)H quinone oxidoreductase (Criddle et al. 2006).

In vivo treatment of neonatal mice from PND 4 to 10 with 0, 7.5 or 15 mg/kg/day menadione dose-dependently decreased the proportion of primordial follicles and increased the proportions of activating/transitional and primary follicles, while decreasing the total number of

follicles per section (Sobinoff et al. 2010). Ovaries from the mice treated in vivo with menadione and neonatal ovaries cultured with 5 μM menadione showed increased PCNA staining in granulosa cells and oocytes of primordial follicles, consistent with increased activation of primordial follicles by menadione (Sobinoff et al. 2010). Ovaries cultured with menadione also showed widespread immunostaining for the oxidative DNA lesion, 8-OHdG, widespread TUNEL staining in follicles of all stages, and immunostaining for activated caspase 3 in granulosa cells and caspase 2 in oocytes of primary and secondary follicles. Oocyte quality, measured by in vitro sperm binding and sperm fusion to zona pellucida-free oocytes collected at 6 weeks of age from mice treated neonatally with the same regimen of menadione described above, was significantly decreased at both menadione doses (Sobinoff et al. 2010). Treatment of cultured superovulated oocytes from 6-8 week old mice with menadione at concentrations from 0-25 μM concentration-dependently induced lipid peroxidation in oocytes as measured by BODIPY immunofluorescence (Sobinoff et al. 2010). These results are consistent with the possibilities that menadione-induced oxidative stress initiates apoptosis in ovarian follicles at all stages of development and also increases activation of primordial follicles. In addition, follicles that survive neonatal menadione treatment show persistent abnormalities of oocyte quality, which may be caused by lipid peroxidation of the oocyte plasma membrane.

Involvement of ROS in ovarian toxicity caused by ionizing radiation

Women who are treated for cancer with ionizing radiation to the pelvis frequently develop temporary amenorrhea and/or premature ovarian failure, similar to the effects of antineoplastic drugs (Meirow and Nugent 2001; Lo Presti et al. 2004). Higher radiation doses

and older age increase the risk for developing premature ovarian failure. It has been estimated that the dose of radiation required to destroy half of the primordial follicle pool in young women is less than 2 Gray (Wallace et al. 1989; Wallace et al. 2003). Ionizing radiation destroys follicles of all developmental stages in women and in rodents (Jarrell et al. 1987; Kim and Lee 2000; Meirow and Nugent 2001; Hanoux et al. 2007).

In addition to the direct ionization of DNA, ionizing radiation also ionizes water molecules, resulting in ROS formation, and leads to persistent increases in mitochondrial ROS production (Wiseman and Halliwell 1996; Spitz et al. 2004; Kim et al. 2006). Therefore, the toxic effects of ionizing radiation on ovarian follicles may be mediated by ROS. Degeneration of primordial and primary follicles is observed as early as 2 h after gamma irradiation of prepubertal mice with a dose of 8.3 Gray (Kim and Lee 2000; Lee et al. 2000). Pretreatment with 100 μg of the antioxidant melatonin significantly protects against radiation-induced primordial follicle destruction at all time points, while 10 μg is less protective; beneficial effects of melatonin are less consistent for primary and larger growing follicles (Kim and Lee 2000). Rapid, sustained increases in ROS occur in human COV434 granulosa cells within 30 min after 1 or 5 Gray gamma irradiation, followed by apoptotic death at 6 h (Cortés-Wanstreet et al. 2009). Stable overexpression of one or both subunits of the rate-limiting enzyme in GSH synthesis, *Gclc* and *Gclm*, increases GSH synthesis, prevents the radiation-induced rise in ROS, and prevents apoptotic death of the cells (Cortés-Wanstreet et al. 2009). Taken together the available data support a role for ROS in initiating apoptosis in granulosa cells and ovarian follicles following exposure to ionizing radiation, but detailed understanding of the mechanisms awaits further study.

The fetal ovary is also sensitive to the effects of ionizing radiation. Exposure to 1.5 Gray ionizing radiation causes double stranded DNA breaks, measured using phosphorylated histone H2AFX immunostaining, in oogonia of cultured human fetal ovary pieces (weeks 6-10 of gestation) and GD 12.5 mouse ovary treated in vivo or in vitro (Guerquin et al. 2009). At one hour after irradiation, the number of phosphorylated histone H2AFX foci is similar in fetal testes and ovaries, but the number of foci declines more rapidly thereafter in mouse ovaries than in testes, consistent with faster DNA repair in ovaries (Guerquin et al. 2009). Doses ≥1.5 Gray induce germ cell apoptosis and significantly deplete ovarian germ cells by more than 50% in cultured human and mouse fetal ovaries by 48 h after irradiation by a mechanism that does not involve p53 or p63 (Guerquin et al. 2009). These results contrast with those for fetal testes, where germ cell apoptosis is induced at lower doses and induction of germ cell apoptosis involves p53 activation (Lambrot et al. 2007; Guerquin et al. 2009). As of yet, there is no direct evidence for ROS involvement in the destruction of fetal ovarian germ cells by ionizing radiation exposure. Future studies should examine the effects of ionizing radiation on fetal ovary ROS production and test the protective effects of antioxidants.

Summary and conclusions

In recent years, the importance of ROS and oxidative stress in ovarian toxicity by diverse stimuli have been increasingly recognized. There is strong evidence that ROS are involved in initiation of apoptosis in antral follicles upon gonadotropin hormone withdrawal, exposure to chemicals including phthalates, PAHs, methoxychlor, and cyclophosphamide, as well as ionizing

radiation. Although less attention has been focused on the roles of ROS in primordial and primary follicle atresia, several studies have shown protective effects of antioxidants and/or evidence of oxidative damage, suggesting that ROS may play a role in these smaller follicles as well. Oxidative damage to lipids in the oocyte has been implicated as a cause of persistently poor oocyte quality after early life exposure to several toxicants. Developing germ cells in the fetal ovary have also been shown to be sensitive to toxicants and ionizing radiation, which induce oxidative stress. Recent studies have begun to elucidate the mechanisms by which ROS mediate ovarian toxicity. Future studies should test the ability of in vivo supplementation with antioxidants to prevent the adverse effects of ovarian toxicants. It will also be important to translate the findings to humans by investigating whether women with polymorphisms in antioxidant genes are at greater risk of ovarian failure or infertility.

Figure Legends

Figure 1: Ovarian follicular development. Ovarian follicles progress from the least mature primordial stage (green arrow in A) through primary (yellow arrowhead in A), secondary (yellow arrow in A), antral (B and C) and preovulatory stages. B) Portion of a healthy small antral follicle with multiple fluid filled vesicles that have not yet coalesced. Mitotic figures can be seen in the granulosa cell layer. O: oocyte. G: granulosa cells. T: theca cells. C) Portion of an atretic antral follicle, characterized by pyknotic granulosa cells (arrows) and loss of intercellular connections between oocyte and granulosa cells and among granulosa cells. Images are hemotoxylin and periodic acid Schiff-stained sections from mouse ovary. Original magnification 400x.

Figure 2: Feedback Loops in the Hypothalamic-Pituitary-Ovarian Axis. Arrows indicate positive feedback/stimulatory effects; blunt ends indicate negative feedback/inhibitory effects. GnRH: Gonadotropin-Releasing Hormone. FSH: Follicle-Stimulating Hormone. LH: Luteinizing Hormone. A: Androgens (androstenedione and testosterone). E_2: Estradiol. P: Progesterone. GnRH from the hypothalamus stimulates synthesis and secretion of FSH and LH by the anterior pituitary. FSH and LH act on specific membrane receptors in granulosa cells and theca cells of the ovary, stimulating follicular growth and hormone synthesis. The peptide hormone inhibin is synthesized and secreted by granulosa cells and specifically inhibits FSH synthesis and secretion by the pituitary. P is synthesized in granulosa and theca cells, converted to A in theca cells, and A is aromatized to E_2 in granulosa cells. E_2 and P negatively feed back to the hypothalamus and pituitary to suppress GnRH, LH, and FSH. When E_2 levels surpass a threshold, E_2 exerts a positive feed back effect on hypothalamic GnRH, which is the trigger for ovulation.

Figure 3: Reactive oxygen species (ROS) generation and detoxification. ROS are formed by the sequential addition of electrons to molecular oxygen, forming superoxide anion radical (O_2-•), H_2O_2, and hydroxyl radical (OH•). Peroxynitrite ($ONOO^-$) is formed when superoxide anion radical reacts with nitric oxide (NO•). Key antioxidant enzymes (in bold, italicized) and the reactions they catalyze are shown. CAT: catalase. GPX: glutathione peroxidase. GSR: Glutathione reductase. GST: Glutathione-S-transferase. PRDX: peroxiredoxin. SOD: superoxide dismutase. GPXs and GSTs require glutathione (GSH) as a cofactor, and GSH can also scavenge free radicals through direct chemical reactions. GSR reduces the oxidized form of GSH (GSSG, Glutathione disulfide). Modified from Devine et al (Devine et al. 2012).

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