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Minireview

Roles of Reactive Oxygen Species and Antioxidants in Ovarian Toxicity[1]

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ABSTRACT

Proper functioning of the ovary is critical to maintain fertility and overall health, and ovarian function depends on the maintenance and normal development of ovarian follicles. This review presents evidence about the potential impact of oxidative stress on the well-being of primordial, growing and preovulatory follicles, as well as oocytes and early embryos, examining cell types and molecular targets. Limited data from genetically modified mouse models suggest that several antioxidant enzymes that protect cells from reactive oxygen species (ROS) may play important roles in follicular development and/or survival. Exposures to agents known to cause oxidative stress, such as gamma irradiation, chemotherapeutic drugs, or polycyclic aromatic hydrocarbons, induce rapid primordial follicle loss; however, the mechanistic role of ROS has received limited attention. In contrast, ROS may play an important role in the initiation of apoptosis in antral follicles. Depletion of glutathione leads to atresia of antral follicles in vivo and apoptosis of granulosa cells in cultured antral follicles. Chemicals, such as cyclophosphamide, dimethylbenzanthracene, and methoxychlor, increase proapoptotic signals, preceded by increased ROS and signs of oxidative stress, and cotreatment with antioxidants is protective. In oocytes, glutathione levels change rapidly during progression of meiosis and early embryonic development, and high oocyte glutathione at the time of fertilization is required for male pronucleus formation and for embryonic development to the blastocyst stage. Because current evidence suggests that oxidative stress can have significant negative impacts on female fertility and gamete health, dietary or pharmacological intervention may prove to be effective strategies to protect female fertility.

follicle, oocyte, ovary, oxidative stress, toxicology

REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

Reactive oxygen and nitrogen species (ROS and RNS, respectively) include superoxide anion radicals, hydroxyl radicals, hydrogen peroxide (H_2O_2), peroxynitrite and other peroxides, nitric oxide, and others (Fig. 1). ROS are formed through leakage of electrons from the inner mitochondrial membrane during oxidative phosphorylation and ATP generation. In steroidogenic tissues such as the ovary, steroidogenic cytochrome P450 enzymes are also sources of ROS [1, 2]. Oxidative and nitrosative damage occur when ROS and RNS react with cellular lipids, proteins, and nucleic acids [3–5]. The redox (reduction-oxidation) state of a cell describes the relative concentrations of reduced versus oxidized states of proteins, enzymes, sulfhydryl-containing molecules, and other factors. The relationship between intracellular levels of ROS relative to endogenous antioxidants has a significant impact on this measure. Oxidative stress occurs when increased ROS levels disrupt cellular redox circuits, resulting in disturbances of redox-regulated cellular processes and/or oxidatively damage cellular macromolecules [6, 7].

ANTIOXIDANT DEFENSE MECHANISMS

Multiple enzyme systems and soluble factors maintain the redox state of cells. Some of these are shown in Figure 1. The antioxidant defense mechanisms in cells are complex and appear to be compartmentalized, such that nuclear, cytoplasmic, and mitochondrial levels of antioxidants behave independently [8, 9]. Ascorbate (vitamin C), present at millimolar levels in ovarian cells and at 50–200 micromolar in human follicular fluid [10, 11], reacts directly with ROS to detoxify them, as do tocopherols (vitamin E). Ascorbate is concentrated in granulosa cells, theca cells, luteal cells, and the oocyte, and its uptake is hormonally regulated [10–14]. Glutathione (GSH), a cysteine-containing tripeptide, is another key antioxidant present in millimolar concentrations in cells. It can scavenge free radicals through either direct chemical reactions or reduction of peroxides as a cofactor for GSH peroxidases, cycling between its reduced form (GSH) and an oxidized, dimerized form (GSSG) [15, 16]. The activity of GSH

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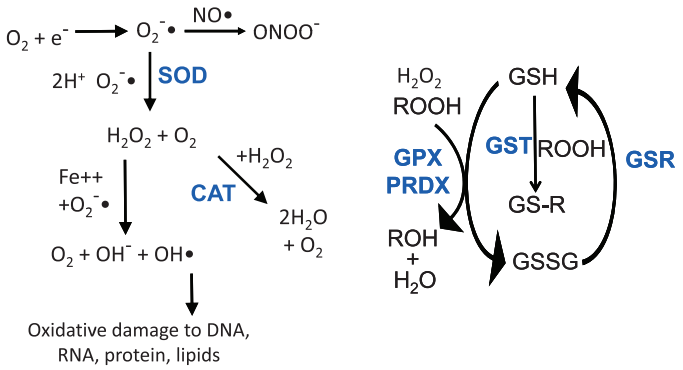

FIG. 1. Reactive oxygen species (ROS) generation and detoxification. ROS are formed by the sequential addition of electrons to molecular oxygen, forming superoxide anion radical, H_2O_2, and hydroxyl radical. Peroxynitrite ($ONOO^-$) is formed when superoxide anion radical reacts with nitric oxide (NO). Key antioxidant enzymes (in bold blue) 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, PRDXs, 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).

reductase, which requires NADPH as an energy source, ensures that most GSH is present in cells in the reduced form. Furthermore, glutathione transferases (GSTs), a large family of enzymes that covalently link reactive chemicals with GSH, aid in detoxification and excretion of toxic substances. The thioredoxin/thioredoxin reductase system serves a similar function, providing reducing potential for multiple biochemical reactions. Although homeostatic mechanisms ensure that the reduced forms of cysteine, GSH, and thioredoxins predominate, these various systems are not in thermodynamic equilibrium, as shown by the different Nernst potentials of oxidized and reduced forms of cysteine, GSH, and thioredoxins [8]. Together, these systems maintain proteins in the optimal reduced state and conformation, permit proper redox signaling, and even control some signal transduction pathways and gene expression [8, 9].

Other enzymes directly detoxify ROS (Fig. 1). Superoxide dismutases (SOD) react with superoxide anion radicals to form oxygen and H_2O_2. The three main SOD enzymes are copper-zinc SOD (CuZnSOD, SOD1, in cytoplasm), manganese SOD (MnSOD, SOD2, in mitochondria), and an extracellular form (ECSOD, SOD3) [17]. Catalase, various peroxidases and peroxiredoxins (PRDXs), including GSH peroxidases (GPXs) and some GSTs, can convert peroxides to water. Together, these interacting defense mechanisms permit cells to live in an oxidative environment, perform necessary biochemical processes, and even use these ROS/RNS as signaling molecules [6, 7]. For example, in the ovary there is a transient rise in ROS levels and decline in antioxidant expression after the preovulatory gonadotropin surge, and the rise in ROS is a necessary signal for ovulation [18–21].

EVIDENCE FOR A ROLE OF ANTIOXIDANT PROTECTIVE MECHANISMS IN OVARIAN FOLLICLE SURVIVAL

Numerous mouse models with deletions of antioxidant genes have been created in recent years and have been used to explore the regulation and function of redox control. With respect to reproductive function, normal fertility has been reported in mice that lack *Gpx1* [22] or catalase [23] or that bear an inactivating mutation in glutathione reductase [24–26]. However, detailed studies of ovarian function have not been conducted in these mice. Deletion of the entire *Gpx4* gene resulted in embryonic lethality [27], whereas deletion of the nuclear form [28] or of the mitochondrial form [29] reportedly had no effects on female fertility. This contrasts with the pronounced adverse effects of mitochondrial *Gpx4* deletion [29] or of overexpression of *Gpx4* [30] on male fertility and spermatogenesis.

Two groups have developed *Sod1* null mice, and both groups reported that the female mice were subfertile; however, the mechanistic basis for the reduced fertility of female *Sod1* null mice remains unclear. Matzuk et al. [31] reported that ovaries of adult female *Sod1* null mice had reduced numbers of preovulatory follicles and corpora lutea. They concluded that these mice were subfertile because of a defect in late follicular development or ovulation. In contrast, Ho et al. [17] reported that *Sod1* null female mice had normal ovarian histology and ovulated similar numbers of ova during a natural estrous cycle but displayed increased postimplantation embryonic lethality. Perhaps the different genetic backgrounds of these two *Sod1* knockout models accounts for these different findings. A study by Wong et al. [32] of copper chaperone for superoxide dismutase null mice, which have decreased ability to incorporate copper into SOD1, found a similar phenotype as Matzuk et al. [31], with abnormal development of antral follicles and no corpora lutea. Taken together, the evidence seems to support a role for SOD1 in antral follicle development. *Sod2* knockout is lethal prior to puberty. However, transplantation of ovaries from *Sod2* knockout juvenile mice to the ovarian bursa of wild-type mice, in which the ipsilateral ovaries had been removed and the contralateral oviducts had been cut, resulted in all stages of follicular development, ovulation, and fertility, suggesting that this enzyme is not critical for ovarian function [31].

Mice null for γ-glutamyl transpeptidase 1 (*Ggt1*) display a shortened life span, stunted growth, and a severe female reproductive phenotype, with complete infertility and lack of ovarian large antral follicles, corpora lutea, and ovulatory response to exogenous gonadotropins [33–35]. These mice have decreased ovarian cysteine concentrations but normal ovarian GSH concentrations compared to wild-type controls, and the female reproductive phenotype is completely rescued by cysteine replacement [33].

Glutamate cysteine ligase (GCL; formerly called gamma-glutamylcysteine synthetase), the rate-limiting enzyme in glutathione synthesis, is a heterodimer composed of a catalytic (GCLC) and a modifier (GCLM) subunit. *Gclc* knockout is embryonic lethal before Gestational Day 8.5 [36, 37]. *Gclm* null mice survive and reproduce [38, 39]; however, more recent detailed studies have revealed that female *Gclm* null mice have oocyte GSH concentrations less than 20% those of wild-type females and have markedly decreased fertility [40]. *Gclm*–/– females mated with wild-type males had smaller litter sizes and fewer uterine implantation sites compared to *Gclm*+/+ littermates [40]. This was due not to fewer ovulated oocytes but to decreased progression of zygotes to the two pronucleus stage at 0.5 days postcoitum and decreased progression to the blastocyst stage at 3.5 days postcoitum. In vitro fertilization and embryo culture studies showed that embryos derived from oocytes of *Gclm*–/– females fertilized with wild-type sperm had very low rates of progression to the blastocyst stage compared to those from *Gclm*+/+ females [40]. The latter results show that the decreased preimplantation development of embryos of *Gclm*–/– females is due mainly to low oocyte GSH

concentrations as opposed to low GSH concentrations in other parts of the female reproductive tract.

SENSITIVITY OF OVARIAN FOLLICLES TO ROS

Antioxidant Depletion Increases and Antioxidant Supplementation Decreases Atresia of Antral Follicles

The tripeptide antioxidant GSH is present at moderate concentrations of 3–4 nmol/mg tissue or about 40–50 nmol/mg protein in the ovaries of adult rats and mice [41–44]. Treatment of adult cycling rats on estrus or proestrus with two doses of 5 mmol/kg buthionine sulfoximine (BSO; a specific inhibitor of GSH synthesis) administered 12 h apart suppressed ovarian GSH concentrations by greater than 50% compared to saline treated controls [41]. This acute depletion of ovarian GSH resulted in statistically significant increases in the percentage of atretic antral follicles and, though statistically nonsignificant, increases of a similar magnitude in the percentage of apoptotic antral follicles at 12 h after the second dose [41]. Further evidence that GSH may play a role in preventing antral follicle atresia comes from experiments using cultured rat antral follicles. Large (preovulatory) and small antral rat follicles spontaneously undergo apoptosis when cultured in the absence of gonadotropin hormone support, and treatment with follicle-stimulating hormone (FSH) prevents the initiation of apoptosis in these cultured follicles [45, 46]. A role for oxidative stress in the initiation of apoptosis by gonadotropin withdrawal was suggested by the observations that follicular ROS increased prior to any indicators of apoptosis and that an antiapoptotic FSH stimulus suppressed this ROS generation in cultured large antral follicles [47]. Moreover, in both small and large antral follicles, FSH treatment stimulated GSH synthesis [47, 48]. In large antral follicles, depletion of GSH with BSO in the presence of FSH partially but statistically significantly inhibited the antiapoptotic effect of FSH on granulosa cell apoptosis [47]. Moreover, each of the antioxidants ascorbic acid, *N*-acetylcysteine, SOD, and catalase protected against apoptosis of rat large antral follicles cultured without gonadotropin support [49].

H_2O_2

An ovarian culture system using Postnatal Day 4 (PND4) ovaries has been used to examine toxicity of chemicals on small ovarian follicles [50]. This system is quite useful because it contains a more concentrated population of small follicle stages, from primordial to small secondary follicles, than even young adult ovaries. PND4 mouse ovaries were exposed to multiple concentrations of H_2O_2 in culture for 8 days to determine their sensitivity to ROS. Following histological processing, the morphology of the ovaries was examined. Pyknotic cells were observed at concentrations of $\geq$3 mM (P. Devine, unpublished data). The lower concentrations affected cells around the periphery of the ovary. In spite of these signs of toxicity, primordial and small primary follicles maintained a normal appearance even in areas where other cells were pyknotic. At 6-mM concentrations of H_2O_2, morphological changes were observed throughout the ovary, except for a small amount of normal tissue in the center of the ovary. It was not until concentrations of 6 mM were used that small ovarian follicles demonstrated visible changes. Thus, primordial and small primary follicles seem to be more resistant to H_2O_2 than other ovarian cell types. Further work is needed to determine if comparable results would be produced by longer-lived or cell-permeable peroxides or oxidants.

4-Vinylcyclohexene Diepoxide

The chemical 4-vinylcyclohexene diepoxide (VCD) has been studied as a model compound that can accelerate ovarian follicle loss when given repeatedly to rodents [51, 52]. This metabolite of 4-vinylcyclohexene, a volatile by-product of chemical syntheses, had been identified by the National Toxicology Program to be a reproductive toxicant at high doses in rats and mice [53, 54]. VCD was found to specifically decrease numbers of primordial and smallest primary follicles [55, 56], with decreases in larger follicles occurring as a result of depletion of primordial follicles [57]. The mechanism of action by which VCD induces this effect has not been fully elucidated, although changes in apoptotic signaling [58–61], MAP kinase/AP-1 signaling [62], and KIT/KIT ligand signaling have been identified [63–65]. Further work is needed to better characterize VCD-induced changes in small ovarian follicles. The specificity of this ovotoxicant makes it a useful tool for studying small ovarian follicle susceptibility to chemical exposures.

Oxidative stress was examined as another possible mechanism underlying VCD-induced ovarian follicle loss. Polar conjugates in urine of VCD-exposed rats and mice suggested that GSH might be involved in detoxifying VCD (Salyers and Sipes, unpublished data). GSH levels were measured in liver and ovary following single or repeated (15 days) exposures of Fischer 344 rats to 80 mg/kg VCD, a dose and duration known to specifically reduce primordial and primary follicle numbers [66]. Hepatic GSH was significantly decreased 2 h after a single i.p. exposure to VCD, with recovery occurring within 6 h (Fig. 2A). In contrast, a specific inhibitor of the rate-limiting step in GSH synthesis, BSO (450 mg/kg; 2 mmol/kg), caused a more persistent decrease in hepatic GSH levels (2–6 h) that returned to normal by 26 h. VCD alone had no effect on ovarian GSH levels, and BSO reduced ovarian GSH by only approximately 25% and only at 6 h after exposures (Fig. 2B). No increases in lipid peroxidation products, measured as thiobarbituric acid-reactive substances, were observed in either ovarian or hepatic tissues 2–24 h following single VCD exposures. Furthermore, 15 days of i.p. injections with BSO did not affect small ovarian follicle numbers, even though it did significantly reduce both hepatic and ovarian GSH levels [66]. Overall, these results suggest that VCD does not cause overall changes in ovarian GSH levels. It is possible that VCD causes only localized changes in redox status in target follicles, but other methods would need to be used to better characterize such changes.

Subsequent experiments tested whether antioxidants could protect against VCD-induced follicle loss in vitro [67]. Cultured PND4 rat ovaries exposed to 30 μM VCD for 15 days exhibited 90% fewer primordial follicles. No significant protection was provided by including the antioxidants vitamin E, vitamin C, or GSH (1 mM) in the culture medium with or without VCD. However, because GSH does not easily penetrate cells, a more cell-permeable form should be tested.

17β-Estradiol (E_2) has been reported to have antioxidant properties at pharmacologic levels [68]. In contrast to the antioxidants used above, exogenous E_2 was found to have protective effects against VCD in vivo. Thompson et al. [69] demonstrated that daily dosing of 0.1 mg/kg E_2 or genistein alone to adult rats did not affect small follicle populations but did protect primary follicles against the effects of 15 days of dosing with 80 mg/kg VCD. There was no protective effect on primordial follicles. Binding to the receptor seemed to be involved because cotreatment with E_2, VCD, and the estrogen receptor antagonist 4-hydroxytamoxifen blocked the protective

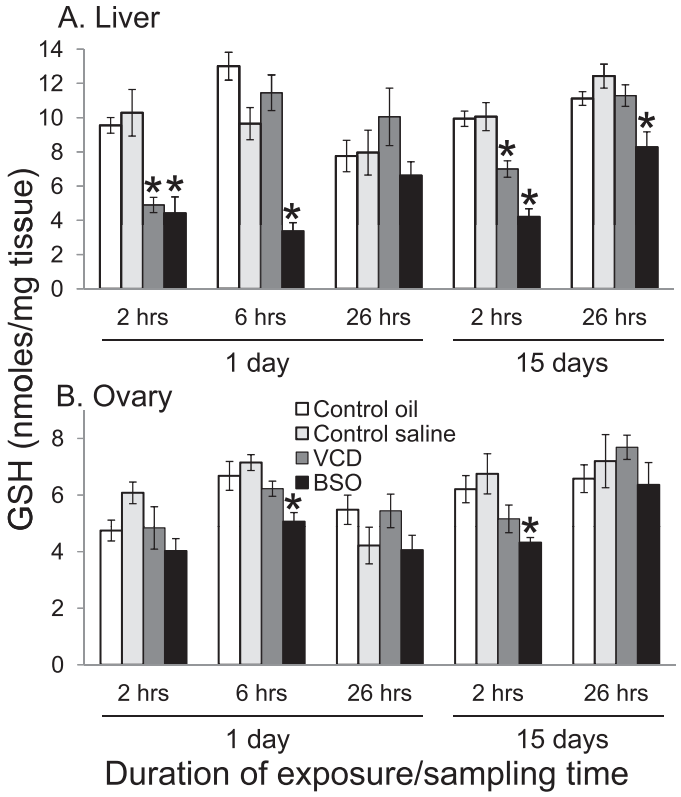

FIG. 2. VCD-induced reductions in hepatic (**A**) but not ovarian (**B**) GSH levels. GSH levels were measured by HPLC in tissue homogenates of female Fischer 344 rats 2, 6, or 26 h after a single i.p. dose or 2 or 26 h following 15 days of dosing of 80 mg/kg VCD in sesame oil, 450 mg/kg (2 mmol/kg) BSO in saline, or vehicle control (sesame oil or saline). Values represent means ± SEM; n = 8–12 animals per group. *Significantly different from control, $P < 0.05$. Modified from Devine et al. [66] with permission of the Society of Toxicology and Oxford University Press.

effect of E_2 on VCD-induced primary follicle loss [69]. Whether this effect of E_2 or genistein on the ovarian toxicity of VCD involves protection against ROS is not known. Overall, results with VCD do not suggest a strong mechanistic link between VCD-induced follicle loss and oxidative stress or ROS.

Cyclophosphamide

Alkylating chemotherapeutic drugs and radiation are used in treating cancers, and their activity is thought to be through induction of DNA damage to tumor cells. These treatments, although necessary for survival of the patient, often have negative side effects, including detrimental effects on the reproductive system [70–72]. Depletion of the ovarian follicle pool and subsequent permanent infertility have been reported [71, 73], suggesting that human primordial follicles are sensitive to at least some types of treatments. Also, the rapid but temporary loss of cyclicity in some patients suggests toxicity to antral follicles from certain drugs [71, 73, 74]. The use of mixed exposures (multiple drugs and radiation) complicates the characterization of potential reproductive toxicity of each drug.

Cyclophosphamide (CP), an alkylating chemotherapeutic drug used often against cancers and autoimmune disorders, has been known for decades to cause temporary or permanent amenorrhea, reduced fertility, or infertility [73, 75–78]. Destruction of follicles at all stages of development has been reported in rodents and humans [79–83]. Dose-dependent decreases in primordial follicles were induced by CP in vivo in mice [83, 84]. Oxidative stress has been implicated in CP-induced toxicity to granulosa cells of antral follicles (see below), but the mechanism underlying small ovarian follicle loss remains unknown. CP requires metabolic activation via oxidation by cytochrome P450 enzymes to 4-hydroxycyclophosphamide, which then undergoes ring opening to aldophosphamide, which spontaneously decomposes to the reactive metabolite phosphoramide mustard (PM) [85–87]. PM is thought to be the active metabolite responsible for CP's anticancer activity as well as its ovarian toxicity [84, 87].

To evaluate CP's mode of action more specifically, the role of DNA damage in CP-induced ovarian follicle loss was examined [88]. Primordial ovarian follicle loss has been reported in multiple strains of mice. Rapid primordial and primary follicle loss were characterized in CD-1 mice at $\geq$150 mg CP/kg. Furthermore, primordial follicle loss occurred in both cultured PND4 mouse and rat ovaries at concentrations of $\geq$3 or 30 μM, respectively, of PM [88, 89]). Also, in mice, small primary follicles were sensitive to concentrations as low as 0.1 μM PM [88]. Loss of primordial and primary follicles was independent of caspase activation, as culture with the pan-caspase inhibitor z-VAD-fmk did not prevent small follicle degeneration [89]. Under the same conditions, a marker for DNA double-strand breaks was detected in oocytes of cultured ovaries. The histone H2AFX is incorporated into nucleosomes throughout the chromatin of all organisms, and this protein becomes phosphorylated at sites of DNA double-strand breaks. This occurs above background levels before morphological changes are observed (24–48 h after exposures) at approximately the same PM concentrations that cause significant follicle loss [88]. Although DNA double-strand breaks have been observed in response to oxidative stress, further work is needed to provide evidence for or against the involvement of ROS in PM-induced DNA damage [88].

Although as discussed above, CP targets primordial and primary follicles in mice, in adult rats a single injection of 200 mg/kg CP destroyed secondary and antral follicles but not primary and primordial follicles [79]. This destruction of secondary and antral follicles was shown to be caused by the dose-dependent induction of apoptosis in granulosa cells of these follicle types at 24 h after a single injection of 50 or 300 mg/kg CP [41]. Subsequent studies revealed that induction of apoptosis by CP in granulosa cells of ovarian follicles was associated with activation of caspase 9 and caspase 3 (Fig. 3). This contrasts with the absence of caspase activation during primordial follicle death induced by CP [89]. Treatment with BSO, a specific inhibitor of GSH synthesis, 2 h before administration of CP did not enhance the induction of apoptosis in growing follicles by CP. However, the low solubility of BSO limited the dose of BSO that could be administered, and therefore the maximal depletion of ovarian GSH concentrations achievable in vivo with BSO was limited to about 50% of control levels at 12 h after injection with levels recovering thereafter [41]; it is possible that more complete depletion of GSH would have potentiated the effects of CP.

In order to further study the mechanism by which CP induces apoptosis in granulosa cells and how this is modulated by GSH, a human granulosa cell tumor line, COV434 cells, was used [90]. This cell line was chosen because the cells possess many of the characteristics of normal granulosa cells, including expressing FSH receptors and synthesizing E_2 in response to FSH and androstenedione [90]. A preactivated form of CP, 4-hydroperoxycyclophosphamide (4HC), which spontaneously breaks down in solution to 4-hydroxycyclo-

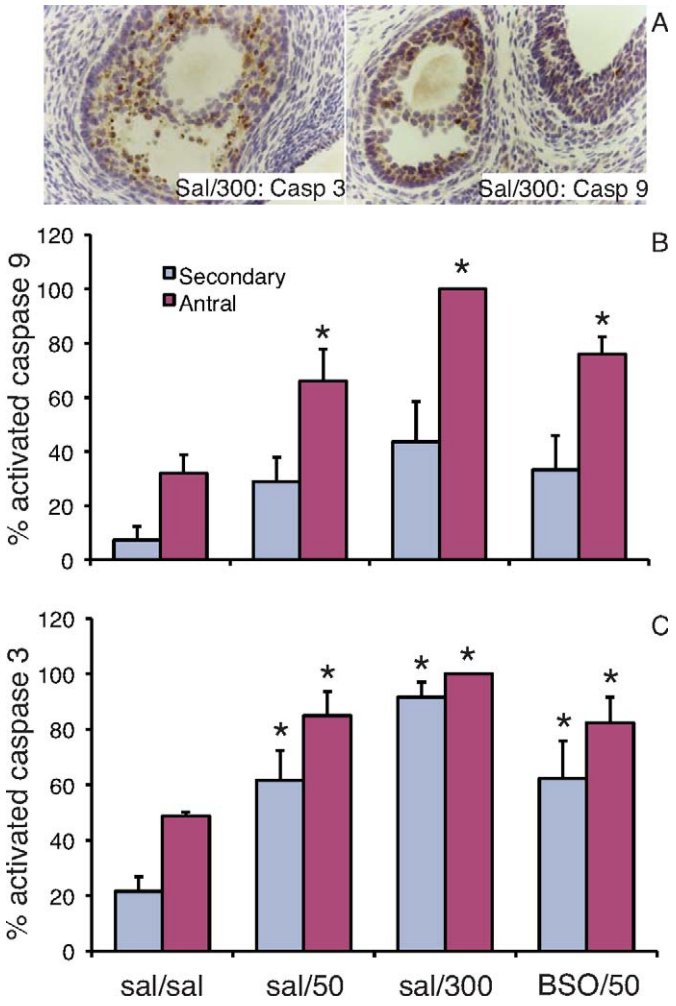

FIG. 3. Cyclophosphamide (CP)-induced apoptosis in granulosa cells of ovarian follicles involves activation of caspases 9 and 3. Proestrous female rats received sequential i.p. injections 2 h apart: normal saline followed by normal saline with 10% DMSO (sal/sal), saline followed by 50 mg/kg CP in DMSO (sal/50), saline followed by 300 mg/kg CP (sal/300), or 5 mmol/kg buthionine sulfoximine followed by 50 mg/kg CP (BSO/50) [41]. The animals were euthanized 24 h later and ovaries (n = 3 per group) were processed for immunostaining with antibodies to activated (cleaved) caspase 9 or activated caspase 3 (both from Cell Signaling Technologies, Beverly, MA) using the Vectastain ABC Kit. Secondary and antral follicles with more than three positively stained granulosa cells per largest cross section were counted in 16 sections per ovary blind to treatment group. **A**) Representative images of antral follicles with positively stained (brown) granulosa cells in ovaries from rats treated with 300 mg/kg CP. The graphs show the mean ± SEM percentages of secondary and antral follicles that stained positively for activated caspase 9 (**B**) and activated caspase 3 (**C**). *Significantly different from respective sal/sal control, $P < 0.05$. (U. Luderer, previously unpublished data obtained on same ovaries as TUNEL data published in Lopez and Luderer [41]).

phosphamide [87, 91], was used to study the effects of CP in COV434 cells. Treatment of these cells with 4HC at concentrations of 1–50 μM rapidly and dose dependently depleted intracellular GSH with a concomitant rapid rise in ROS, followed by initiation of apoptosis as measured by activation of caspase 3, TUNEL, and Hoechst 33342 staining [92]. Depletion of GSH with BSO enhanced and supplementation of GSH with GSH ethyl ester or supplementation with other antioxidants prevented the initiation of apoptosis by 4HC [92]. Taken together, these findings demonstrate that the rise in ROS mediates 4HC-induced apoptosis in granulosa cells [92].

Ionizing Radiation

Women treated with ionizing radiation to the pelvis often suffer from amenorrhea or premature ovarian failure [73]. Treatment of nonovarian cells with ionizing radiation has been shown to result in the generation of ROS [5, 93]. In vivo studies show that ionizing radiation destroys small follicles as well as antral follicles in rats and mice [71, 94, 95] and rhesus monkeys [96]. High doses of gamma irradiation (8.3 Gy) to Postnatal Day (PND) 21 mice caused significant and rapid increases in degenerating primordial and primary follicles, as determined by morphological changes at 2, 8, and 14 h after irradiation [95, 97]. Pretreatment with 100 μg melatonin, which is a good antioxidant, partially and significantly protected against radiation-induced primordial follicle loss at all time points; 10 μg melatonin was less protective [95]. Melatonin pretreatment provided less consistent protective effects for primary and secondary/antral follicles [95].

In women, temporary amenorrhea, with eventual resumption of menstrual cycling, may be caused by destruction of growing follicles by ionizing radiation without complete destruction of the primordial follicle pool. Studies in COV434 granulosa tumor cells showed that gamma irradiation caused a rapid (within 30 min) and sustained increase in ROS that was followed by apoptotic death at 6 h [98]. COV434 cells that were stably transfected with constructs directing overexpression of one or both subunits of the rate-limiting enzyme in GSH synthesis, glutamate cysteine ligase, had increased levels of GSH and were protected against the generation of ROS and the initiation of apoptosis by gamma irradiation [98].

Chromium

The role of oxidative stress in the ovarian toxicity of the heavy metal chromium (Cr) has been examined. This metal can be present naturally, leaching into drinking water supplies as chromium (CrVI) [99]. Occupational exposures are also possible since Cr is used in multiple industrial applications, such as leather tanning, electroplating, and wood preservatives, and in the production of steel alloys used in orthopedic implants and other applications [100]. Cr induces follicular atresia in adult mice when given in drinking water [101]. The mechanism of action is thought to involve ROS as the metal cycles through various oxidation states within cells, and treatment with ascorbate has been shown to protect against reproductive toxicity in male monkeys [102]. Banu et al. [103] examined the effects of in vivo lactational exposures to chromium on female reproduction and the ovary. From birth until weaning on PND 21, mothers of pups were given 200 mg/L potassium chromate with or without 500 mg/L ascorbate in their drinking water. Pups thus exposed to Cr via lactation had delayed puberty (PND 55 versus PND 33 in controls) and longer estrous cycles, and ascorbate protected against these effects [103]. In these animals, numbers of each follicle type were significantly reduced in Cr-treated rats at PND 21 and PND 45, but secondary and antral follicles recovered by PND 65. Serum E_2, testosterone, progesterone, growth hormone, and prolactin were reduced at all time points examined, and FSH was increased (PND 21 and PND 45) with Cr treatments. The mechanism of follicular destruction was examined in cultured rat granulosa cells; Cr increased expression of proapoptotic BCL2 family proteins, decreased expression of antiapoptotic BCL2 family proteins and AKT, increased phosphorylation of TRP53 and MAPK3/1, caused cleavage of caspase 3 and PARP, and induced apoptotic cell death in a time-dependent manner [104]. Ascorbate prevented these effects both in vivo and in vitro [103, 104]. Lower doses must be examined, as well

as the possible mechanisms by which Cr can have such effects, but oxidative stress is likely involved. Primordial and small primary follicles do appear targeted by these exposures, although not necessarily specifically. The most sensitive follicle type may be identified when reduced exposures are tested.

9,10-Dimethyl-1,2-Benzanthracene

The polycyclic aromatic hydrocarbons 9,10-dimethyl-1,2-benzanthracene (DMBA), benzo[*a*]pyrene, and 3-methylcholanthrene are well known to destroy primordial and primary follicles following in vivo treatment of mice and rats [105–107]. Early studies also noted that DMBA treatment destroyed antral follicles [108]. A more recent study investigated the mechanism by which DMBA destroys antral follicles. Culture of rat large antral follicles with DMBA at concentrations greater than or equal to 1 μM in the presence of an antiapoptotic concentration of FSH resulted in increased expression of the proapoptotic protein BAX in granulosa cells by 24 h, followed by increased caspase 3 activation and DNA fragmentation detected by TUNEL staining by 48 h [109]. ROS were already significantly increased relative to follicles treated with FSH alone by 12 h of DMBA treatment and remained elevated through 48 h, but GSH concentrations were not decreased. Depletion of GSH with BSO enhanced the initiation of apoptosis by DMBA [109]. Supplementation with GSH ethyl ester but not with the antioxidants butylated hydroxytoluene and dithiothreitol prevented the initiation of apoptosis by DMBA [109]. These results are consistent with a role for ROS in the initiation of apoptosis by DMBA in granulosa cells of antral follicles and demonstrate an antiapoptic effect of GSH.

Methoxychlor

In vivo treatment of mice with the organochlorine insecticide methoxychlor at $\geq$32 mg/kg for 20 days was reported to induce atresia of antral follicles but not of primordial, primary, or secondary follicles [110]. At these doses, ovaries of mice treated with methoxychlor for 20 days had elevated levels of H_2O_2 and of oxidative protein and DNA damage assessed via nitrotyrosine and 8-hydroxy-2′-deoxyguanosine immunostaining, respectively [111]. Ovarian enzymatic activity and mRNA levels of SOD1, GPX1, and catalase were also significantly decreased in ovaries after 20 days of treatment with 32 or 64 mg/kg methoxychlor [111]. Similarly, antral follicles cultured with 10 or 100 μg/ml but not 1 μg/ml methoxychlor underwent atresia by 72 h of treatment, which was preceded by a rise in mRNA levels of *Bax* after 48 h and which was prevented by supplementation with the GSH precursor *N*-acetylcysteine [112, 113]. Follicular expression of the antioxidant genes *Sod1*, *Gpx2*, and catalase were unaltered after 24 h of methoxychlor treatment in vitro and were significantly decreased in all concentration groups when atresia was already well advanced at 96 h [112]. At 48 h, *Sod1* mRNA levels were significantly decreased at the 1- and 10-μg/ml concentrations of methoxychlor, and glutathione peroxidase 1 and catalase were significantly increased at the 100-μg/ml methoxychlor concentration [112]. These observations provide support for a role of oxidative stress in the initiation of antral follicle atresia by methoxychlor.

Conclusions on Sensitivity of Small Follicles to Oxidative Stress

Overall, evidence regarding the roles of oxidative stress and ovarian antioxidant status in toxicant-induced destruction of small ovarian follicles is mixed. H_2O_2 did not induce atresia of small follicles in cultured neonatal ovaries, and antioxidants were not protective against the primordial follicle toxicity of VCD [67]. In contrast, oxidative stress may play a role in the destruction of primordial follicles by exposure to the heavy metal Cr or exposure to ionizing radiation since treatment with antioxidants was protective [95, 103, 104]. Future studies could involve altering expression of protective antioxidant defense mechanisms specifically in small follicles or oocytes using available transgenic mice expressing Cre recombinase only at certain follicle stages. Further research measuring the effects of toxicant exposure on ROS generation and the modulation of toxicant effects by antioxidant supplementation or depletion in isolated cultured follicles and ovaries would also help in characterizing small follicle sensitivity.

Conclusions on Sensitivity of Antral Follicles to Oxidative Stress

Antral follicles appear to be highly sensitive to oxidative stress-induced apoptosis of granulosa cells. The observations that GSH depletion enhances and antioxidant supplementation inhibits the initiation of apoptosis in antral follicles and granulosa cells by a variety of toxicants and by ionizing radiation support a role for ROS in this process. Direct measurements of rising ROS levels prior to any increases in markers of apoptosis further support the contention that ROS are involved in the initiation of apoptosis by various toxicants and ionizing radiation in antral follicles and granulosa cells. Because glutathione-*S*-transferase-catalyzed conjugation with GSH is an important phase II detoxification pathway for active metabolites of DMBA and CP [91, 114, 115], it is also possible that GSH depletion enhances toxicity by preventing the detoxification of these metabolites and that GSH supplementation enhances detoxification of these metabolites. Arguing against the latter explanation for CP is the observation that supplementation with other antioxidants besides GSH ethyl ester was also protective [92]. For DMBA, GSH supplementation was protective, but supplementation with butylated hydroxytoluene or dithiothreitol was not [109]. Therefore, additional studies are needed to clarify the role of ROS in the initiation of apoptosis in antral follicles by DMBA.

EFFECTS OF OXIDATIVE STRESS ON OOCYTES AND PREIMPLANTATION EMBRYONIC DEVELOPMENT

Oocyte GSH concentrations increase rapidly after the preovulatory gonadotropin surge, with maximal levels in ovulated MII oocytes, and oocyte GSH is important during fertilization and early embryonic development [116–124]. Biochemical depletion of GSH with BSO during oocyte maturation in vitro [116, 123] or in vivo [124] prevented sperm chromatin decondensation and formation of the male pronucleus after in vitro fertilization. The observation that the majority of zygotes of *Gclm*–/– female mice fail to form the second, male pronucleus at 0.5 days postcoitum in vivo [40] provides further evidence of the importance of oocyte GSH in formation of the male pronucleus. These effects of GSH depletion and GSH deficiency are believed to be due to the requirement for reduction of protamine disulfide bonds in the sperm nucleus for sperm nuclear reactivation to occur [116, 125]. Brief (and reversible) depletion of GSH following exposure to an oxidant (diamide) in ovulated hamster oocytes prior to in vitro fertilization disrupted hamster oocyte meiotic spindles, as well as sperm chromatin decondensation, leading to the formation of abnormal female pronuclei and suggesting

that altered redox status can impact zygote formation in several ways [117]. In contrast, no spindle abnormalities were reported when bovine oocytes were depleted of GSH using BSO [123]. These results suggest that there may be a species difference in the importance of GSH for meiotic spindle function, that the presence of small amounts of reduced GSH in BSO-treated oocytes may be sufficient to support normal meiotic spindles under normal conditions when GSH turnover from the oxidized form occurs quickly, or that meiotic spindle function may be sensitive to oxidative stress but not to GSH depletion per se.

In hamsters, embryonic GSH concentrations decline from highest levels in the metaphase II oocyte to low levels in blastocysts [118], and similar declines are observed in mouse embryos [120, 126]. Two-cell mouse embryos do not normally express *Gclc* or synthesize GSH de novo, whereas blastocysts express *Gclc* and synthesize GSH [119–121]. Moreover, although two-cell embryos upregulated *Gclc* expression in response to an oxidant stimulus that depleted GSH, *tert*-butyl hydroperoxide, they were unable to do so in response to depletion of GSH by an electrophilic toxicant, diethylmaleate, and GSH depletion with BSO for 45 h beginning at the two-cell stage inhibited development to the blastocyst stage [119–121]. In vivo treatment with BSO beginning prior to initiation of a superovulation protocol significantly diminished concentrations of GSH in oocytes and increased the percentage of degenerating embryos retrieved at 1.5 days postcoitum [126]. In contrast, BSO injections beginning at 0.5 days postcoitum significantly decreased GSH concentrations in oviductal and uterine secretions but did not decrease zygote GSH concentrations or affect embryonic development at 1.5 or 2.5 days postcoitum [126]. Superovulated oocytes from *Gclm−/−* females have GSH concentrations less than 20% of wild-type oocytes [40]. Embryos derived from oocytes of *Gclm−/−* females and fertilized in vitro with sperm from wild-type males developed to the blastocyst stage in culture at very low rates compared to embryos derived from oocytes of *Gclm*+/+ females [40]. Together, these findings support the conclusion that GSH present in the oocyte is critical for normal zygote formation and preimplantation development, whereas GSH in reproductive tract secretions appears to play a lesser, supportive role.

CONCLUSIONS

In recent years, there has been growing interest in the roles of ROS and oxidative stress in female reproduction. Endogenous ROS have been shown to play important roles as signaling molecules, for example, during ovulation. Accumulating evidence demonstrates that ROS are key signals in the initiation of apoptosis in antral follicles and granulosa cells of antral follicles by diverse stimuli, such as gonadotropin withdrawal, exposure to exogenous toxicants, and exposure to ionizing radiation, and that antioxidants protect against these stimuli. Studies have also demonstrated that the high concentrations of the antioxidant GSH in oocytes are necessary for normal fertilization and subsequent preimplantation embryonic development. These studies suggest that antral follicles and fertilization and early embryonic development may be particularly sensitive to exposures to environmental stressors and chemical toxicants that induce oxidative stress. In contrast, the current evidence is less consistent regarding the sensitivity of primordial and primary follicles to oxidative stress or changes in antioxidant status. Future studies should aim to clarify the roles of antioxidant defense mechanisms and ROS in the development and survival of small follicles and further explore the involvement of ROS and antioxidants in toxicant-induced destruction of small follicles. Future studies should also test the possible protective effects of in vivo antioxidant supplementation on female fertility.

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