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# Mechanisms of Ovarian Atresia Induced by Xenobiotic Exposures

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**Abstract**

The focus of this chapter is the mechanisms of apoptosis that occur during "normal" (physiologically-induced) or "abnormal" (chemical- or pathology-induced) attrition of ovarian oocytes. One of the primary functions of the ovary is development and maturation of oocytes, which occur within a follicular structure. During fetal development of the ovary, primordial germ cells (oogonia) are formed and become oocytes when they stop dividing, and are arrested at the diplotene stage (prophase) of the first meiotic division. At this stage the oocyte is surrounded by a single layer of flattened somatic cells (pre-granulosa cells) and a basement membrane to form primordial follicles (Hirshfield, 1991), the most immature follicular stage of development. As a result, the lifetime supply of oocytes is set at the time of birth, and is irreplaceable. Association of the granulosa cells with the oocyte is critical for maintenance of oocyte viability and follicle development (Buccione et al., 1990). Development and progression of a recruited follicle also requires the appropriate expression of numerous factors, including critical members of the transforming growth factor β superfamily, such as growth differentiation factor 9 (GDF9) and bone morphogenic protein (BMP15) (Paulini and Melo 2011). Characterization of GDF9 function has a demonstrated a required role in promoting somatic cells of the follicle to undergo mitosis and initiates paracrine signaling between the oocyte and the follicular cells surrounding it (Carabatsos et al. 1998; Hreinsson et al. 2002; Nilsson and Skinner 2002). The impaired fertility in GDF9 mice appears to primarily be a result of loss of function in somatic cells since oocytes of GDF knockout mice remain capable of undergoing in vitro maturation and progressing to metaphase II of meiosis (Carabatsos et al. 1998).

**Disciplines**

Animal Experimentation and Research | Animal Sciences | Medical Cell Biology | Medical Genetics | Reproductive and Urinary Physiology

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# 3

# Mechanisms of Ovarian Atresia Induced by Xenobiotic Exposures

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## 1. Introduction

The focus of this chapter is the mechanisms of apoptosis that occur during "normal" (physiologically-induced) or "abnormal" (chemical- or pathology-induced) attrition of ovarian oocytes. One of the primary functions of the ovary is development and maturation of oocytes, which occur within a follicular structure. During fetal development of the ovary, primordial germ cells (oogonia) are formed and become oocytes when they stop dividing, and are arrested at the diplotene stage (prophase) of the first meiotic division. At this stage the oocyte is surrounded by a single layer of flattened somatic cells (pre-granulosa cells) and a basement membrane to form primordial follicles (Hirshfield, 1991), the most immature follicular stage of development. As a result, the lifetime supply of oocytes is set at the time of birth, and is irreplaceable. Association of the granulosa cells with the oocyte is critical for maintenance of oocyte viability and follicle development (Buccione *et al*., 1990). Development and progression of a recruited follicle also requires the appropriate expression of numerous factors, including critical members of the transforming growth factor β superfamily, such as growth differentiation factor 9 (GDF9) and bone morphogenic protein (BMP15) (Paulini and Melo 2011). Characterization of GDF9 function has a demonstrated a required role in promoting somatic cells of the follicle to undergo mitosis and initiates paracrine signaling between the oocyte and the follicular cells surrounding it (Carabatsos et al. 1998; Hreinsson et al. 2002; Nilsson and Skinner 2002). The impaired fertility in GDF9 mice appears to primarily be a result of loss of function in somatic cells since oocytes of GDF knockout mice remain capable of undergoing *in vitro* maturation and progressing to metaphase II of meiosis (Carabatsos et al. 1998).

Puberty, the time after which oocytes are ovulated from the ovary and reproduction can occur, is generally between the ages of 9 and 16 in humans. Female puberty is identified as the first menstruation (menarche), which usually occurs prior to the first ovulation. From birth and throughout the prepubertal period, waves of follicular development in the ovary occur; however, all of these pre-pubertal follicles become atretic. This is important to note, because, dysregulated primordial follicle activation into the growing pool after puberty, also results in an atretic fate for these oocytes. In humans, 1 to 2 pre-ovulatory follicles develop approximately every 28 days, whereas in rodents, 6 to 12 follicles develop every 4 to 5 days (Richards, 1980). Depletion of all functional primordial follicles from the ovary is the underlying cause of ovarian failure. The average age of "natural ovarian failure" termed

menopause in the United States is 51 (Devine & Hoyer, 2005; Hoyer, 2005), however, premature ovarian failure (POF) is defined as loss of the ovarian reserve before age 40.

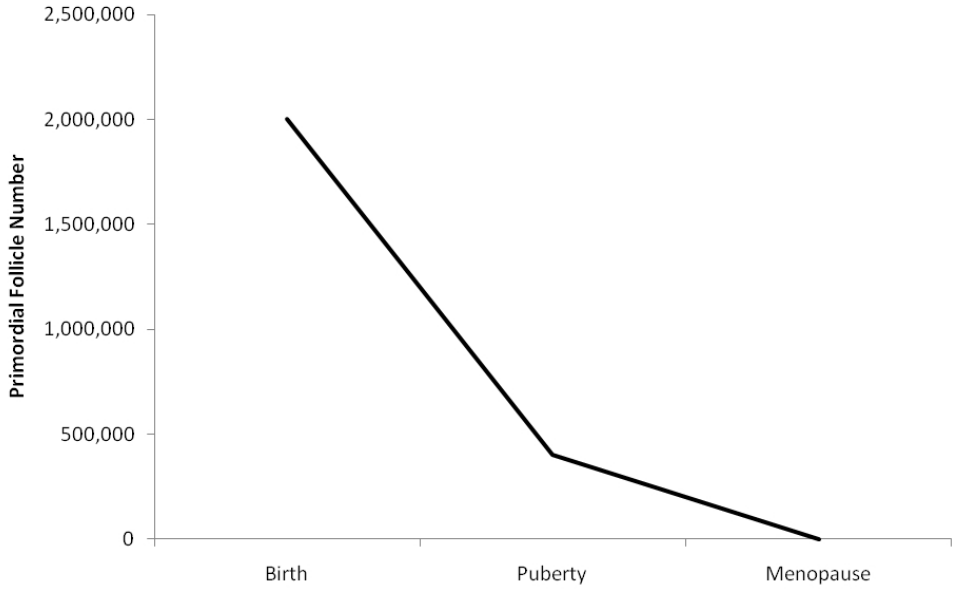


Fig. 1. Dynamics of primordial follicle atresia in humans. Primordial follicle loss at birth, puberty and menopause.

The number of oocytes present is dynamic and varies with age, with a peak in the total number of oocytes occurring during embryonic development. In humans, that number (about 7 million) occurs at five months gestation; at birth the number has dropped to 2 million; 250,000 to 400,000 at puberty; and no viable follicles remain at menopause (Hirshfield, 1991; Mattison & Schulman, 1980) (**Figure 1**). Pre-pubertal attrition of primordial follicles increases in the years leading up to puberty and the number of primordial follicles that remain at the time of first menarche define the ovarian reserve. During the lifetime of a woman, ovulation accounts for only 400 to 600 oocytes; the remainder have been lost at various stages of development by the process of atresia which occurs via programmed cell death (PCD; Tilly *et al.*, 1991). Therefore, **atresia is the natural fate of the vast majority of ovarian follicles (> 99%)** (Hirshfield, 1991).

## 2. Pathways involved in maintaining follicular viability

### 2.1 Phosphatidyinositol-3 kinase (PI3K)

The PI3K signaling pathway has recently been demonstrated to be critical for maintaining the viability of primordial follicles that comprise the ovarian reserve (Jagarlamudi *et al.*, 2009; Liu *et al.*, 2006; 2007; Rajareddy *et al.*, 2007; Reddy *et al.*, 2005; 2008; 2009; 2010). Kit Ligand (KL), a growth factor expressed in the granulosa cells of primordial follicles (Ismail *et al.*, 1996), binds to the oocyte-expressed receptor, c-KIT (Horie *et al.*, 1991; Manova *et al.*,

1990; Orr-Urtreger *et al.*, 1990). Once binding occurs, c-KIT becomes autophosphorylated, leading to activation of PI3K (Roskoski, 2005a; 2005b). PI3K are lipid kinases that phosphorylate the 3'-OH group on the inositol ring of inositol phospholipids. Activation of PI3K results in conversion of the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate ($PIP_2$) to phosphatidylinositol-3,4,5-triphosphate ($PIP_3$). $PIP_3$ can recruit proteins containing lipid-binding domains from the cytoplasm (Pawson & Nash, 2000) such as the serine/threonine kinases 3'-phosphoinositide-dependent kinase-1 (PDK1) and AKT (Cantley, 2002) to the plasma membrane where their proximity results in their phosphorylation. Once phosphorylated, AKT can translocate to the nucleus, where it regulates a number of cellular responses such as growth, survival and cell cycle entry (Datta *et al.*, 1999). Recently, the p110 β-subunit of class IA PI3K has also been implicated to promote autophagy (Dou *et al.*, 2010) in fibroblast cells (non ovarian origin).

Mice with oocyte-specific depletion of PDK1 have accelerated oocyte loss and were depleted of oocytes 8 weeks after birth (Reddy *et al.*, 2009). Follicle destruction was determined to occur at the primordial follicle stage, indicating a role for PDK1 in primordial follicle viability (Reddy *et al.*, 2009). Downstream of PDK1, AKT has also been determined to be involved in survival of the primordial follicle stage. Mice lacking oocyte specific expression of AKT were infertile with loss of primordial follicles by postnatal day (PND) 90 (Brown *et al.*, 2010).

This pathway also plays a "gatekeeper" role in determining the entry or the primordial oocyte into the growing follicular pool (Jagarlamudi *et al.*, 2009; Liu *et al.*, 2006; 2007; Rajareddy *et al.*, 2007; Reddy *et al.*, 2005; 2008; 2009; 2010). The forkhead transcription factor family member, FOXO3, is negatively regulated by AKT. Mice lacking FOXO3 expression experienced POF due to global activation of the primordial follicle pool (John *et al.*, 2008). In contrast, when FOXO3 was over expressed in an oocyte specific manner, mice were infertile due to lack of primordial follicle activation (John *et al.*, 2008). Thus, FOXO3 has been implicated as a critical regulator of primordial follicle activation. As mentioned earlier, it is noteworthy that when primordial follicle activation is over-stimulated, that those follicles do not go onto ovulation, but are destroyed by apoptosis.

### 2.2 Pathways involved in follicular apoptosis and autophagy

Recently, it has been calculated that, in the pre-pubertal mouse ovary, 81 primordial follicles transition to the primary follicle stage per day, while 155 primordial follicles are lost to atresia daily (Tingen *et al.*, 2009). Thus, the majority of primordial follicles are undergoing PCD. To understand the process occurring, classic markers of apoptosis including caspase-3 and caspase-7 cleavage, PARP1 cleavage, and DNA fragmentation were investigated in mouse ovaries during the pre-pubertal transition period (PND(s) 7, 10, 13, 16, 19, 22, 26). Also, morphological examination of follicular structures was performed. These classical markers of apoptosis were detected in the secondary and antral follicles only – not in the primordial follicles. Also, no evidence of pyknosis in primordial follicles was noted. To determine if the primordial follicles were dying by apoptosis, but being cleared so quickly by phagocytic mechanisms that the investigators were missing them, ovaries were harvested every 3h across a 24h period on PND10 and stained for the presence of cleaved PARP1. Using this method, only 37 of the 155 follicles that die in a 24h period stained positive for PARP1. Another alternative theory investigated in this study was that primordial follicles

are "shed" into the bursal space. No primordial follicles were found in the intrabursal space in 11 ovaries examined, thus this possible route for primordial follicle loss was discounted. These data support that primordial follicles do not die by the mechanisms associated with classical apoptosis (Tingen *et al.*, 2009).

### 2.2.1 B cell lymphocytic-leukaemia protooncogene (Bcl-2) signaling

Following initial recruitment of a cohort of primordial follicles, specific mechanisms are required to promote development of preantral follicles to a tertiary stage of development and then onto an ovulatory follicle. The vast majority of recruited primordial follicles undergo some form of mechanized cell death prior to achieving ovulatory status. The mechanisms of cell death can vary but are primarily accomplished through apoptosis and/or autophagy. Apoptosis (aka PCD) occurs through multiple mechanisms and can be broadly broken into caspase-dependent or caspase-independent pathways. Caspase-dependent pathways result in the activation of initiator and effector caspases that proteolytically degrade cellular components. The regulation of caspase activation is largely controlled by members of the BCL2 family, of which numerous members are expressed in the growing follicle (Yang and Rajamahendran 2002; Yoon et al. 2009), and play critical roles in cell viability and onset of apoptosis. The anti-apoptotic members include BCL2, BCL-xL, MCL-1, BCL-2A1 and BCL-w, while the anti-apoptotic members include BIM, BAD, BAX, NOXA and PUMA. The ratio of anti-apoptotic BCL-2 members to pro-apoptotic members on the mitochondrial membrane determines the atretic fate of a cell. Should they become altered in favor of the pro-apoptotic proteins, the result will be pore formation in the mitochondrial membrane, followed by leakage of cytochrome c and other pro-apoptotic proteins (Krishna *et al.*, 2011). PI3K signaling can regulate BCL-2 mediated apoptotic events. Disrupted PI3K signaling can result in release of BAD from the cytoplasmic binding protein 14-3-3 and BAD movement to the mitochondrial membrane, where it induces pore formation, resulting to the leaking of mitochondrial components, an event that leads to cell death (She *et al.*, 2005).

Characterization of Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) protein expression in bovine oocytes of variable quality revealed that while BAX expression was present in all grades of oocytes, the expression of Bcl-2 was much greater in high quality oocytes compared to fragmented and low quality oocytes (Yang and Rajamahendran 2002). This data underlines the importance of the BCL-2:BAX ratio in regulating apoptosis. To determine if BCL-2 over-expression is beneficial to oocyte growth and development, Guthrie and coworkers (Guthrie et al. 2005) created a transgenic pig that expressed Bcl-2 cDNA driven by the inhibin alpha subunit protein. Transgene expression was more prevalent in follicular cells of healthy (86%) follicles compared to follicular cells of atretic follicles (54%). Despite the positive correlation of the BCL2 transgene expression with healthy follicles, the rate of atresia or ovulation rate did not differ between transgenic and wild type pigs(Guthrie et al. 2005), suggesting Bcl2 expression alone is either not sufficient to dramatically influence caspase mediated apoptosis or that additional apoptotic pathways are contributing to cell death.

### 2.2.2 Mitogen Activated Protein Kinase (MAPK)

There are three major MAPK members; extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. The three are regulated independently, and control many processes involved in cell survival, growth, and death (Ip & Davis, 1998; Marshall,

1995; Whitmarsh & Davis, 1996). The ERK pathway is generally involved in cell growth and proliferation (Hill & Treisman, 1995), while JNK and p38 mediate the stress response and apoptosis (Johnson *et al*., 1996; Rincon *et al*., 1998; Xia *et al*., 1995; Zanke *et al*., 1996). The transcription factor complex of the AP-1 consensus site is composed of Jun, Fos and other subunits and is downstream of MAPK signaling molecules (Foletta *et al*., 1998). As detailed in the section on 4-vinylcyclohexene, the MAPK family play important roles in ovarian follicle viability.

### 2.2.3 Autophagy

Autophagy is a cellular process utilizing lysosomal machinery to degrade cellular components. While not always terminal, autophagy represents another cellular mechanism of programmed cell death in addition to classical apoptotic pathways (Tsujimoto and Shimizu 2005). Screening of prepubertal rat oocytes demonstrated that while some oocytes were only positive for markers of apoptosis or autophagy, most were positive for both. Beclin I (BECNI), a Bcl-2-binding protein (Liang *et al*., 1999), is a central protein in the autophagy-promoting complex, and has been demonstrated to be located at all stages of follicular development in the mouse, with highest expression at the primordial stage (Gawriluk *et al*., 2011). BECN1 protein expression has been reported in the theca interna and corpus luteum in human ovaries (Gaytan *et al*., 2008). Oocyte-specific deletion of BecnI in mice caused premature depletion of primordial germ cells and increased numbers of atretic granulosa cells. Another study found the cigarette-smoke induced follicle loss was also associated with an increase in markers of autophagy (Gannon *et al*., 2011).

## 3. MicroRNA regulation of cell death

MicroRNA (miRNA) are endogenously synthesized non-coding RNAs (ncRNA) whose functional size is approximately 18-24 nucleotides long (Bartel 2004). The mature sequence confers significant biological impact on the cells in which they are synthesized and processed through perfect or imperfect pairing to the 3' untranslated region (UTR) of a target mRNA. The binding of a miRNA to its target mRNA 3'UTR generally results in posttranscriptional gene silencing (PTGS) through the action of several mechanisms, including translation inhibition, target transcript degradation and chromatin silencing via methylation (Bartel 2004; Chen and Meister 2005). miRNA are involved with numerous cell processes, including apoptosis (Carletti et al. 2010; Huang et al. 2011). Human glioblastoma cells have a 5- to 100-fold increase in miR21 and inhibition of miR21 increased apoptotic activity in these cells as measured by TUNEL staining (Chan et al. 2005). The ability of miR21 to promote cellular proliferation and inhibit cell death is a result of its interaction with several important mRNA targets such as programmed cell death 4 (PDCD4), myristoylated alanine-rich protein kinase c substrate (MARCKS) and tumor suppressor gene tropomyosin 1 (TPM1) (Asangani et al. 2008; Chen et al. 2008; Frankel et al. 2008; Zhu et al. 2007; Zhu et al. 2008). Of these three genes, all are expressed in the porcine oocyte (Whitworth et al. 2005). In mice, it has recently been demonstration that luteinizing hormone is capable of upregulating miR21 expression in granulosa cells in mice and that *in vivo* inhibition of miR21 significantly reduces ovulation rate (Carletti et al. 2010). Additionally, significant upregulation of miR21 in prepubertal pig ovaries following the development of their first cohort of antral follicles has been demonstrated along with

validation of miR21 upregulation in the oocyte during *in vitro* maturation and its ability to influence PDCD4 protein expression in metaphase II arrested oocytes (Ross *et al*., unpublished data).

## 4. Impact of environmental chemical exposures on follicular atresia

Exposure to environmental or occupational chemicals can disrupt female reproductive function (Mattison, 1985). A number of studies have shown that exposure to ovarian toxicants can lead to oocyte destruction (Hoyer and Sipes, 1996; Krarup *et al*., 1967; 1969; 1970; Maronpot, 1987; Melnick *et al*., 1990). How these effects are produced is becoming better understood and detailed for a number of chemical classes discussed herein. While outside of the scope of this chapter, it is important to note that the ovary has the capability to biotransform chemicals to more or less toxic metabolites, and these metabolism processes are highly active in ovarian tissues (Igawa *et al*., 2009; Keating *et al*., 2008a; 2008b; 2010; Rajapaksa *et al*., 2007a; Rajapaksa *et al*., 2007b) and contribute to the extent of ovotoxicity observed.

The stage of development at which the follicle is lost determines the reproductive impact. If the large or antral follicles are depleted temporary interruptions to reproductive function are observed since these follicles can be replaced by recruitment from the finite pool of primordial follicles (Hoyer & Sipes, 1996). Due to the irreplaceable nature of the ovarian reserve, chemicals that destroy oocytes contained in primordial follicles can lead to permanent infertility and POF. Also, the level and duration of exposure to an environmental toxicant can influence the reproductive impact. Chronic, low dose exposures, likely to be environmental in nature, are difficult to identify because their ovarian impact may go unrecognized for years. Ongoing selective damage of small pre-antral follicles may not initially raise concern until the onset of POF that will eventually result. Further, the age at which exposure occurs can impact the outcome. As noted earlier, pre-pubertal exposure may not cause the same extent of follicle loss as that post-pubertal, due to the higher number of follicles present during childhood. However, damage to oocytes by chemical exposures *in utero* and/or during childhood present a concern, which would not be detected until the reproductive years.

### 4.1 Chemotherapeutics

Concerns over side-effects of anti-neoplastic chemotherapy have increased as survival rates of cancer patients improve; currently ~ 56% overall survival rate; (Byrne, 1990). Thus, the toxic effects of chemotherapeutic drugs in women cancer survivors have become an important issue. Since the beginning of their use, the ability of these agents to produce POF has been documented. These effects have been described in patients treated with cyclophosphamide (CPA), nitrogen mustard, chlorambucil, cisplatin or vinblastine (Chapman *et al*., 1983; Damewood and Groschow, 1986; Dnistrian *et al*., 1983; Koyama *et al*., 1977; Miller & Cole, 1970; Miller *et al*, 1971; Sobrinho *et al*., 1971; Warne *et al*., 1973). Effects of chemotherapeutic exposures on reproductive function are primarily a concern for those under the age of 40 who may wish to have children. Children exposed to chemotherapy prior to puberty are less likely to become permanently infertile than adults (Blumenfeld, 2002), however, the Childhood Cancer Survivor Study reported that 6.3% of girls will

undergo acute ovarian failure, and 8% will experience pre-mature menopause (Garcia, 2007).

A time- and dose-dependent relationship between CPA exposure and ovarian toxicity in mice has been confirmed (Plowchalk and Mattison, 1992). Additionally, growing follicles have been reported to be depleted by CPA in rhesus monkeys (Ataya et al., 1989). In rats, CPA destroyed antral follicles at doses that did not affect primordial follicles (Jarrell et al., 1987). In contrast, in mice under conditions that completely destroyed primordial follicles, only partial destruction of antral follicles was observed (Plowchalk and Mattison, 1992).

Phosphoramide mustard (PM) has been determined to be the anti-neoplastic and ovotoxic form of this chemical (Plowchalk and Mattison, 1992; Desmeules and Devine, 2006). Increased TUNEL staining was observed in cultured mouse ovaries following 24 hours of *in vitro* incubation with PM (Desmeules and Devine, 2006). There was no increased staining, however, for cleaved caspase-3 in response to PM exposure, and caspase inhibition had no effect on PM-induced ovotoxicity (Desmeules and Devine, 2006). Additionally, PM induces DNA damage in ovaries of mice and rats, as detected by the appearance of the DNA repair protein, γH2AX, in the oocyte of primordial follicles, even at concentrations at which follicle loss is not observed (Petrillo *et al*., 2011).

Exposure of PND5 mouse ovaries to cisplatin increased oocyte p63, c-ABL protein expression and TUNEL staining, all of which preceded oocyte death (Gonfloni *et al*., 2009). p63 is a homolog of p53 that is expressed in mouse oocytes around the time of birth and has been proposed as a "germline guardian" (Suh *et al*., 2006; Livera *et al*., 2008). c-Abl, a tyrosine kinase receptor regulates cell proliferation, cytoskeletal rearrangement, survival and stress responses (Pendergast, 2002). Co-treatment with the c-Abl inhibitor, imatinib, prevented *c-Abl* and *p63* oocyte mRNA accumulation and further prevented follicle destruction both *in vitro* and *in vivo*, supporting a role for altered *p63* and *c-Abl* during cisplatin-induced oocyte death. Further, cisplatin increased mRNA levels of pro-apoptotic *p38 Mapk*, along with the BCL-2 pathway members, *Noxa* and *Puma* (Gonfloni *et al*., 2009).

### 4.2 Polycyclic aromatic hydrocarbon

Polycyclic aromatic hydrocarbons (PAH's) are widespread in the environment from various combustion processes, including automobile exhaust and cigarette smoke. A positive connection between smoking and POF has been established (Jick and Porter, 1977). Three PAH's have been shown to be ovotoxic; benzo(a)pyrene (BaP), 3-methylcholanthrene (3-MC) and 7,12-dimethylbenz(a)anthracene (DMBA; Mattison and Thorgeirsson, 1979; Vahakangas *et al*., 1985). These three compounds destroy oocytes in small follicles in rats and mice within 14 days following a single dose (Mattison and Thorgeirsson, 1979). A direct relationship between the dose of PAH's and destruction of primordial follicles has been shown in the mouse ovary (Mattison and Thorgeirsson, 1979).

The involvement of GSH and generation of reactive oxygen species (ROS) during DMBA-induced preovulatory follicle destruction has been evaluated. DMBA exposure increased ROS generation but did not alter concentrations of total GSH. However, GSH depletion prior to DMBA exposure resulted in increased apoptosis and cleaved caspase-3 positive follicles (Tsai-Turton *et al*., 2006). Additionally, DMBA increases mRNA and protein

expression of GST isoform pi in cultured rat ovaries (Bhattacharya and Keating, 2011), potentially as a protective measure to counteract ROS generation by DMBA.

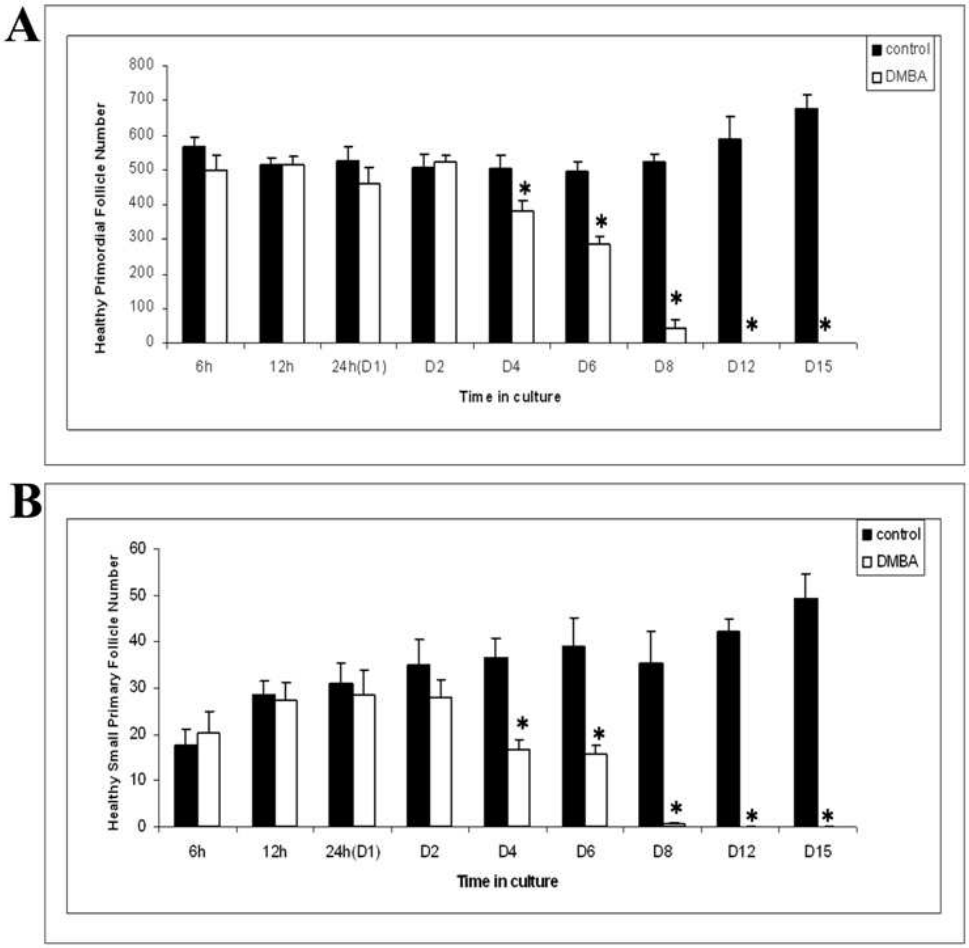


Fig. 2. Temporal pattern of DMBA-induced primordial and small primary follicles loss. Postnatal day 4 Fisher 344 rat ovaries were cultured in media containing vehicle control or DMBA (1 μM) for 2-15 days. Following culture, ovaries were histologically evaluated and follicles classified and counted. Data represent mean healthy follicle number ± SE; n = 5 ovaries per treatment; * Different from control, $P < 0.05$. Adapted from Igawa *et al.*, 2009 with journal permission.

Follicle destruction by DMBA is driven by oocyte apoptosis, followed by death of the somatic cells (Morita and Tilly, 1999). DMBA depletes primordial and small primary follicles in a dose- and time-dependent manner in cultured postnatal day 4 (PND4) rat ovaries (**Figure 2**). Up-regulation of the pro-apoptotic protein, BAX, in primordial follicle oocytes

from cultured PND4 mouse ovaries is induced by DMBA exposure (Matikainen *et al*., 2001). DMBA exposed isolated follicles were shown by immunocytochemistry to have increased staining for BAX after 24 hours, followed by increased TUNEL and cleaved caspase 3 staining after 48 hours of exposure (Tsai-Turton *et al*., 2006). Also, ovaries from Bax-deficient mice are resistant to DMBA-induced primordial follicle destruction (Tsai-Turton *et al*., 2006). Thus, the effects of DMBA are mediated (at least partially) by the pro-apoptotic branch of the BCL-2 family of proto-oncogenes.

Use of an apoptotic gene array coupled with Northern blot analysis identified 16 genes that were increased in mouse ovaries 12 hours after DMBA exposure (50 mg/Kg), including *p53*, receptors *Tnfrsf10B*, *11A*, *12* and *21* and pro-apoptotic *Bax* (Pru *et al*., 2009). Immunohistochemical staining demonstrated that TNFRSF12 protein was upregulated in oocytes of follicles at all stages in response to DMBA. Some of the genes that were identified as responsive to DMBA exposure are targets of p53 transcription factor signaling. In addition, p53-deficient mice are resistant to the ovotoxic effects of DMBA (Pru *et al*., 2009).

DMBA-induced follicle destruction is accelerated when PI3K signaling is inhibited (Keating et al., 2009). Recently, it has been determined that DMBA-induced apoptosis is mediated through down regulation of the PI3K signaling pathway (Sobinoff et al., 2011). DMBA caused a decrease in the downstream members of PI3K, Foxo3a and phosphorylated mTOR (Sobrinoff et al., 2011). Thus, DMBA induced ovotoxicity is a consequence of a number of pathways working together.

### 4.3 4-vinylcyclohexene

The dimerization of Butadiene forms 4-vinylcyclohexene (VCH), released at low concentrations during the manufacture of rubber tires, plasticizers and pesticides (IARC, 1994). The diepoxide metabolite of VCH, VCD, is the ovotoxic form and selectively destroys primordial and primary follicles, thus, damage caused by VCD would go unnoticed in exposed individuals. Mechanistic studies in rats have determined that VCD accelerates the natural process of atresia (apoptosis; Springer *et al*., 1996; Borman *et al*., 1999).

Pro-apoptotic signaling events in the BCL-2 and MAPK families have been shown to be selectively activated in fractions of small pre-antral follicles (targets for VCD; Hu *et al*., 2001a; 2001b). Expression of pro-apoptotic *bax* mRNA along with total and phosphorylated BAD protein were increased in isolated small pre-antral follicles following *in vivo* dosing of rats with VCD (Hu *et al*., 2001a). VCD also caused a translocation of BCL-$_{XL}$ from the mitochondria to the cytoplasm resulting in an increased mitochondrial ratio of BAX/BCL-$_{XL}$ in the target follicle population (pro-apoptotic event; Hu *et al*., 2001a). In addition, VCD increased cytochrome c leakage from the mitochondria and activation of caspase 3 in targeted follicles (Hu *et al*., 2001b). Collectively, these findings demonstrated a molecular mechanism by which VCD causes follicular atresia via the pro-apoptotic branch of the BCL-2 proto-oncogene family.

Activation of the pro-apoptotic branch of the MAPK family by VCD has also been demonstrated. VCD induced both JNK and p38 MAPK protein in small pre-antral follicles isolated from rats following *in vivo* dosing (Hu *et al*., 2001b). In addition, VCD also caused an increase in phosphorylated c-JUN (p-c-JUN) protein and a decrease in nuclear protein

binding to the AP-1 consensus site in the target follicle population (Hu *et al*., 2002). Furthermore, a role for the Glutathione S-transferase isoform pi in inhibition of JNK action has been demonstrated in ovaries from PND4 rats treated with VCD. In the presence of VCD, there was an increase in the amount of JNK that was bound to GSTpi, with a corresponding decrease in the level of the JNK target, p-c-JUN (Keating *et al*., 2010).

VCD at a concentration of 30 μM induces significant loss of primordial and small primary follicles following 6 days of exposure in cultured PND 4 rat ovaries (**Figure 3**; Devine *et al*., 2002; Keating *et al*., 2009). A key gene identified to be altered by VCD in a microarray study was the PI3K pathway member, *c-Kit*. When PI3K was inhibited in PND4 ovaries exposed to VCD, primordial follicles were protected from VCD-induced ovotoxicity (Keating *et al*., 2009). It was subsequently demonstrated that VCD induces a decrease in c-KIT autophosphorylation (Mark-Kappeler *et al*., 2011) and AKT phosphorylation (Keating *et al*., 2011) after two days followed by a decrease in *c-Kit* mRNA expression on day 4, prior to an increase in *KL* mRNA and follicle loss on day 6 of exposure (Fernandez *et al*., 2008). Further, exogenous KL in culture partially attenuated VCD-induced follicle loss, while there was no effect of exogeneous BMP15 or GDF9 (Fernandez *et al*., 2008). Thus, the initial ovarian target of VCD is the oocyte, and VCD-induced inhibition of the PI3K pathway is thought to be an early initiating ovotoxic event, which precedes the activation of the classical apoptotic pathways. Additionally, it is hypothesized the VCD accelerates the entry of primordial follicles into the growing follicular pool, at which point they undergo apoptosis (Keating et al., 2009).

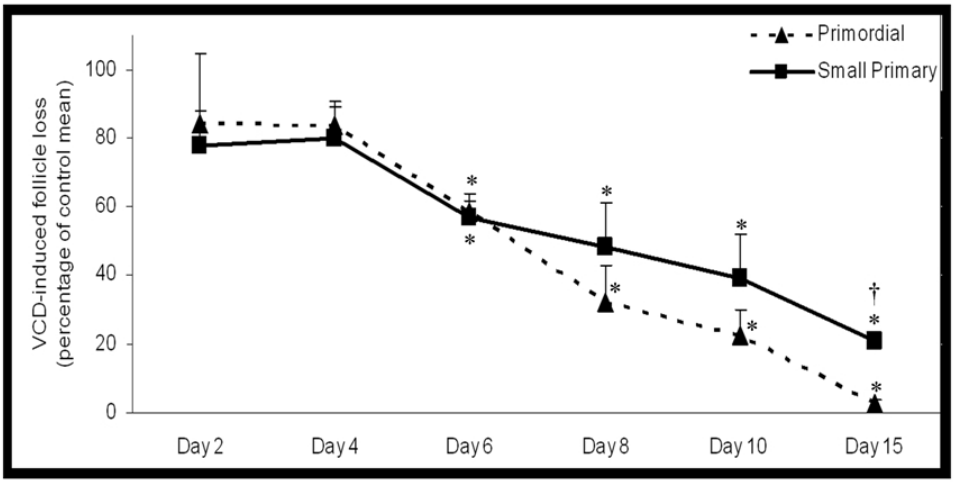


Fig. 3. Temporal pattern of VCD-induced primordial and small primary follicles loss. Postnatal day 4 Fisher 344 rat ovaries were cultured in media containing vehicle control or VCD (30 μM) for 2-15 days. Following culture, ovaries were histologically evaluated and follicles classified and counted. Data represent mean healthy follicle number ± SE; n = 5 ovaries per treatment; * Different from control, $P < 0.05$. Adapted from Keating *et al*., 2009 with journal permission.

### 4.4 Methoxychlor

The organochlorine pesticide Methoxychlor (MXC) is used on agricultural crops as a replacement for DDT (Murono and Derk, 2005). Early studies reported that MXC induces ovarian atrophy in mice (Eroschenko *et al*., 1995) and decreases steroidogenesis in rat ovarian cells (Bal *et al*., 1984). Following MXC exposure in mice, the oocyte in the antral follicles becomes separated from the cumulus granulosa cell layer, which becomes disorganized followed by the appearance of pyknotic bodies (Borgeest *et al*., 2002). Mechanistic investigations have indicated that MXC accelerates atresia via increased apoptosis involving the BCL-2 proto-oncogene family (**Figure 4**) (Borgeest *et al*., 2002, Miller *et al*., 2005) in isolated mouse antral follicles. Immunohistochemical analysis demonstrated increased pro-apoptotic BAX protein staining in MXC-treated antral follicles (Borgeest *et al*., 2004). MXC increased *Bax* mRNA expression in cultured mouse antral follicles *in vitro* after 48, 72 and 96 hours, but decreased mRNA expression of *Bcl-2* only after 96 hours (Miller *et al*., 2005). MXC took a longer time to inhibit growth of antral follicles from both Bax deficient and Bcl-2 over expressing mice (96 hours) when compared to the wild type controls (72 hours; Miller *et al*., 2005). Interestingly, anti-apoptotic Bcl-2 over-expressing mice were shown to have more healthy antral follicles following MXC treatment when compared to MXC-treated wild type controls following 20 days (Borgeest *et al*., 2004).

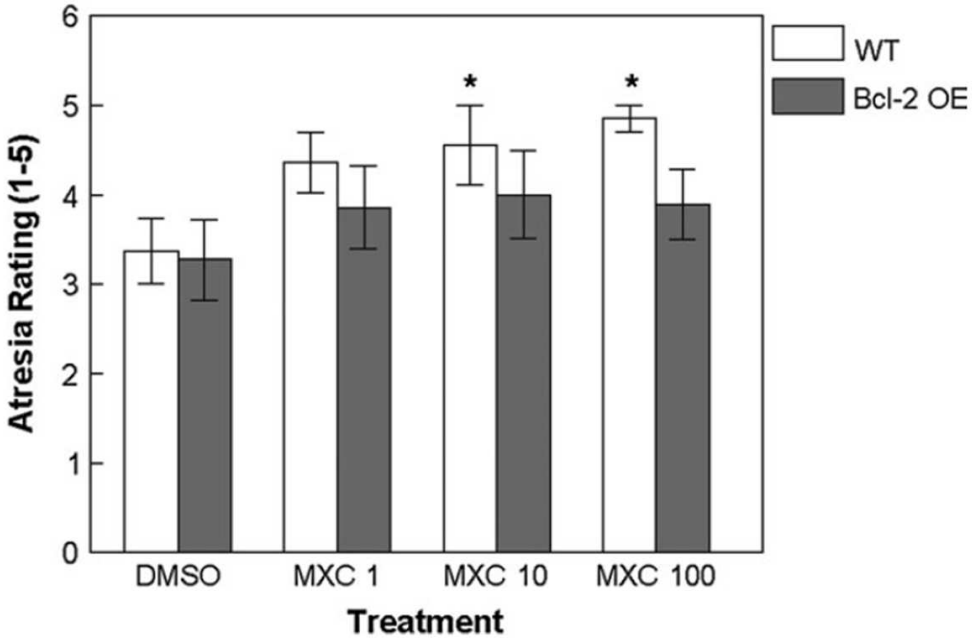


Fig. 4. Effect of *in vitro* MXC exposure on antral follicle atresia in wild type versus Bcl-2 over expressing mice. Large antral follicles were exposed in vitro to 1-100 µg/ml MXC for 96h (n = 5-11 follicles per treatment). DMSO = dimethylsulfoxide vehicle control. Graph represent means ± SE from three separate experiments; * $P < 0.05$; Adapted from Miller *et al*., 2005 with journal permission.

Incubation of antral follicles with 17β-estradiol protected against atresia induced by MXC or the MXC metabolite, HPTE (Miller *et al*., 2006). Furthermore, atresia was enhanced in antral follicles from MXC-exposed ERβ-overexpressing mice compared with their wild type counterparts (Tomic *et al*., 2006). Thus, it appears that ER-mediated pathways may mediate the ovarian toxicity of MXC.

MXC has been shown to increase oxidative stress-induced mitochondrial damage (Gupta *et al*., 2006a; 2006b). MXC-treated mouse ovaries had decreased *Sod1* mRNA and increased *Gpx* and *Cat* mRNA expression after 48 hours of MXC treatment, however, at the time of MXC-induced antral follicle atresia (96 hours), there was a decrease in SOD1, GPA and CAT (Gupta *et al*., 2006b). At that time, MXC treatment also increased $H_2O_2$ levels. N-acetyl cysteine (NAC), an antioxidant, prevented the detrimental effects of MXC on antral follicle growth and atresia and the MXC-induced changes in SOD1, GPX and CAT (Gupta *et al*., 2006b). Further, treatment with Vitamin E prevented MXC-induced OSE damage (Symonds *et al*., 2008). Thus, the mechanism(s) by which MXC is ovotoxic appears to include an increase in production of reactive oxygen species as well as reduced capacity for antral follicles to sustain a response to oxidative stress.

## 5. Summary

A number of key pathways are altered during chemical-induced ovarian follicle loss as detailed in this chapter. To date, primordial follicle loss remains ill-understood however advances are being made in increasing our knowledge of how follicle atresia occurs, and the regulatory molecules involved. The delicate balance between maintaining the primordial follicle pool in a dormant state, along with controlling apoptosis and activation into the growing pool is beginning to become better appreciated. There remains, however, a dearth of information regarding which follicular/ovarian compartment that apoptotic processes are initiated within, and whether these locations change as a response to different follicle depleting stimuli.

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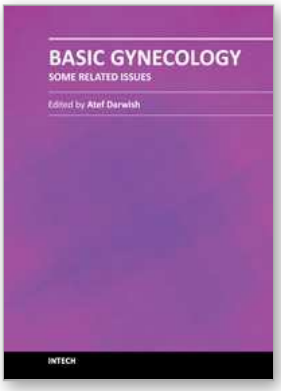


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