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All Your Eggs in One Basket: Mechanisms of Xenobiotic Induced Female Reproductive Senescence

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1. Introduction

The irreplaceable mammalian primordial follicle represents the basic unit of female fertility, serving as the primary source of all developing oocytes in the ovary. These primordial follicles remain quiescent, often for decades, until recruited into the growing pool throughout a woman's adult reproductive years. Once recruited, <1% will reach ovulation, with the remainder undergoing an apoptotic process known as atresia (Hirshfield, 1991). Menopause, or ovarian senescence, occurs when the pool of primordial follicles becomes exhausted.

Pre-mature ovarian failure (POF; or early menopause) is an ovarian defect characterised by the premature loss of menstrual cyclicity before the age of 40, well below the median age of natural menopause (51 years). Approximately 1-4% of the female population suffers from this condition, making POF a significant contributor of female infertility (Coulam et al., 1986). There is now a growing body of evidence which suggests that foreign synthetic chemicals, also known as xenobiotics, are capable of causing POF by inducing premature follicular depletion. Indeed, exposure to pesticides, workplace chemicals, chemotherapeutic agents and cigarette smoke have all been associated with primordial follicle reduction resulting in premature ovarian senescence (Hoyer and Devine, 2001; Mattison et al., 1983a, 1983b; Sobinoff et al., 2010, 2011).

In addition to infertility, the loss of ovarian hormones which accompanies POF has been connected with an increased risk of early morbidity and mortality (Shuster et al., 2010). With current statistics indicating an increasing trend in western women opting to delay childbirth, xenobiotic exposure could have long lasting repercussions for both the fertility and long term health of these women. In this review we discuss the susceptible nature of primordial follicles and the consequences of xenobiotic induced POF. We then examine the mechanisms of ovotoxicity for environmental toxicants and xenobiotics known to target immature follicles, and discuss the development of novel methods of wildlife fertility control utilizing these ovotoxicants.

2. The primordial follicle: Precious and vulnerable

Oocyte development and maturation occurs within ovarian follicles. These follicles assemble when primary oocytes (arrested at meiosis prophase I) are enveloped by a single layer of flattened granulosa cells, forming the most immature stage of follicular development, the primordial follicle. The timing of this event is species-specific, but generally occurs in the primitive ovary during foetal development (McNatty et al., 2000). Due to the nature of follicular formation, the number of oocytes established around the time of birth is finite, and represents the total number of germ cells available to the mammalian female throughout her entire life (Edson et al., 2009). It is therefore the size and persistence of this primordial follicle pool which determines the female reproductive lifespan (Fig. 1).

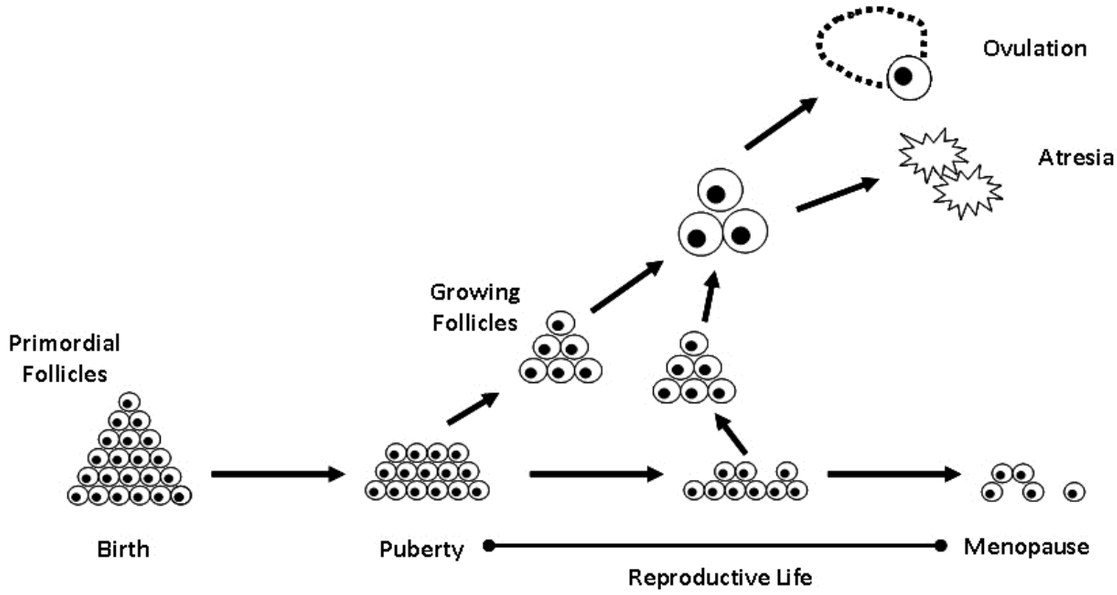

Fig. 1. Simple mechanistic diagram of the human female reproductive lifespan.

The first stage of folliculogenesis involves the recruitment of selected primordial follicles into the growing population. To prolong the length of the female reproductive lifespan, only a few primordial follicles are chosen for recruitment at any one time, with some follicles remaining in a quiescent (non-proliferative) state for months or years (Fig 1). This event occurs in regular waves, and is continuous from birth until ovarian senescence (McGee and Hsueh, 2000). Overall, only a few hundred of all the recruited follicles will complete folliculogenesis and undergo ovulation, with the vast majority being lost to atresia (Hirshfield, 1991). Atresia is thought to be an apoptotic process which selects the healthiest follicles for ovulation, although its mechanism of action is poorly understood. As virtually all follicles are lost during optimal follicular development, it is important that primordial follicles not only survive but also are maintained in a healthy state. Over-stimulation of primordial follicle activation and premature atresia results in the extensive depletion of the primordial follicle pool, resulting in premature ovarian senescence (Reddy et al., 2008).

There is now an increasing volume of studies which link primordial follicle depletion with xenobiotic exposure, suggesting that these irreplaceable follicles are highly sensitive to

cytotoxic insult (Hoyer and Devine, 2001; Mattison et al., 1983a, 1983b; Sobinoff et al., 2010, 2011). It is thought that this sensitivity may stem from the primordial follicles quiescent nature. For example, somatic cells which undergo regular rounds of proliferation constantly renew macromolecules and organelles by virtue of mitosis. However, the oocyte and granulosa cells of the primordial follicle are non-proliferative, and do not benefit from mitotic renewal, perhaps making them excessively vulnerable to xenobiotics which cause sub-lethal damage to mitochondria and other structures over time (Tarin, 1996). Similarly the location of the primordial follicle population within a poorly vascularised region of the ovarian cortex also makes them highly susceptible to toxins which damage ovarian blood vessels, with the resulting cortexual fibrosis destroying primordial follicle rich segments of the ovary (Guraya, 1985; Meirow et al., 2007; van Wezel and Rodgers, 1996).

In addition to direct primordial follicle injury, certain xenobiotics which target developing follicles have been shown to cause excessive primordial follicle activation (Keating, 2009; Sobinoff et al., 2010, 2011). This may be due to a homeostatic mechanism of follicular replacement, in which destroyed developing follicles result in primordial follicle activation to replace the developing pool. If the offending xenobiotic is not removed, this could potentially lead to a vicious cycle of primordial follicle depletion.

3. Consequences of xenobiotic induced primordial follicle depletion

The overall impact of xenobiotic induced follicular depletion on female reproduction depends on the type of follicle targeted for destruction, dose, and duration of exposure (Hoyer and Sipes, 1996). For example, xenobiotics which target large developing follicles have an immediately noticeable effect on female fertility. Antral follicles are the primary producers of ovarian estrogen, and therefore play an important role in the FSH-LH negative feedback loop responsible for ovulation. Xenobiotics which selectively target antral follicles consequently have harmful effects on ovarian cyclicity, effectively acting as endocrine disruptors (Jarrell et al., 1991; Mattison and Schulman, 1980). Fortunately, both prolonged and acute exposure to these ovotoxic agents only causes temporary infertility, as these follicles can be replaced by the primordial follicle pool once the harmful xenobiotic is removed from the immediate environment.

Conversely, xenobiotics which target small pre-antral follicles have more permanent effects on female fertility which could potentially go unrecognised for years. Due to the non-renewing nature of primordial follicles, these xenobiotics are particularly damaging to female fertility, causing permanent infertility and premature ovarian senescence. What makes this type of ovotoxicity concerning is that it has a delayed effect on reproduction which is not made apparent until such a time that follicular recruitment cannot be supported (Hooser et al., 1994). Thus this extended period of time between cause and effect means that the detrimental action of xenobiotic contact often goes unnoticed, and consequently steps are not taken to minimise exposure until it is too late. Thus even a systemic low dose of xenobiotics may produce cumulative effects over time, resulting in the same consequences on female fertility as a large single exposure. With current statistics suggesting an increasing trend in developed countries of women opting to delay childbirth until late in their reproductive life (>30 years), accelerated follicle loss resulting from xenobiotic exposure can deprive these women of the chance to start a family in the conventional manner (Martin et al., 2003).

In addition to permanent infertility, the loss of ovarian hormones which accompanies early menopause has been associated with an increased risk for a variety of health problems. For example, estrogen deficiency (a consequence of menopause) is the most common cause of osteoporosis in humans (Cenci et al., 2003). Bone loss results from the absence of estrogen production by maturing ovarian follicles, which leads to a subsequent increase in FSH production due to the negative feedback of estrogen on pituitary gonadotropin secretion. In terms of bone remodelling, increased FSH production stimulates tumor necrosis factor (TNF) secretion, which in turn increases osteoclast formation and bone reabsorption (Cenci et al., 2003). Menopause induced estrogen withdrawal has also been associated with an increase in many traditional cardiovascular risk factors, including body fat redistribution, insulin resistance and high blood pressure, increased plasma triglyceride levels and high-density lipid cholesterol absorption (Bilianou, 2008; Rosano et al., 2007). Increased risk for Alzheimer's disease is also associated with the menopause induced loss of sex steroid hormones as evidenced by various epidemiological and experimental studies, although some clinical findings refute this evidence (Pike et al., 2009).

Over the course of the 20th century, the average life expectancy for women in the developing world has increased by ~40%, resulting in women now living up to a third of their lives in post menopausal years. Unfortunately, this means that women are now spending a larger proportion of their life with increased health risks brought about by the onset of menopause. In addition, increased risk resulting from xenobiotic induced premature menopause means an enhanced chance for problems. It is therefore important to understand the mechanisms behind xenobiotic induced primordial follicle depletion.

4. Mechanisms of xenobiotic induced primordial follicle depletion

4.1 The Aryl Hydrocarbon Receptor

The Aryl Hydrocarbon Receptor (Ahr) is a ligand activated transcription factor implicated in the regulation of a variety of physiological and developmental effects, including xenobiotic metabolism, cell cycle progression, apoptosis and oxidative stress (Denison and Heath-Pagliuso, 1998; Nebert et al., 2000). In its inactivated state, Ahr is found in the cytoplasm bound to a number of molecular chaperones including hsp90, Xap2, and p23 (Carlson and Perdew, 2002; Petrulis and Perdew, 2002). Ligand binding causes conformational changes which expose a nuclear import signal on the Ahr, resulting in its translocation into the nucleus (Pollenz et al., 1994). Once imported the Ahr-ligand receptor complex disassociates with its chaperones and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) to form an active transcription factor with high affinity to specific DNA sequences known as xenobiotic-response elements (XRE) within the promoter region of a variety of genes, inducing transcription (Fig.2) (Reyes et al., 1992).

The Ahr-ARNT ligand activated transcription factor is known to regulate the toxicity of various xenobiotic compounds such as polycyclic aromatic hydrocarbons, polychlorinated dibenzofurans and polychlorinated biphenyls which are found ubiquitously in the environment and are highly resistant to metabolic breakdown (Nguyen and Bradfield, 2007; Stapleton and Baker, 2003). In an adaptive response to their accumulation in the cell, Ahr induces the expression of a number of xenobiotic metabolising enzymes, including members of the cytochrome P450 A and B families which oxygenate the intruding xenobiotic as part

of a three tiered enzymatic detoxification mechanism (Conney, 1982). Unfortunately, this oxygenation often results in the bioactivation of the parent xenobiotic into a more reactive and therefore toxic metabolite (Harrigan et al., 2004; Melendez-Colon et al., 1999). Indeed, many of Ahr's known xenobiotic ligands, such as the polycyclic aromatic hydrocarbons benzo[a]pyrene (BaP), 9:10-dimethyl-1:2-benzanthracene (DMBA), and 3-methyl-cholanthrene (3-MC), cause primordial follicle destruction through Ahr initiated cytochrome P450 induced bioactivation (Borman et al., 2000; Mattison and Thorgeirsson, 1979). For example, BaP is initially metabolised by Ahr regulated cyp1A1 and cyp1B1 enzymes resulting in its biotransformation into 7,8-diol, and 9,10-diol macromolecular-adduct forming metabolites within the ovary. Inhibition of Ahr by α-naphthoflavone nullifies its effects on primordial follicle destruction (Bengtsson et al., 1983; Mattison et al., 1983a).

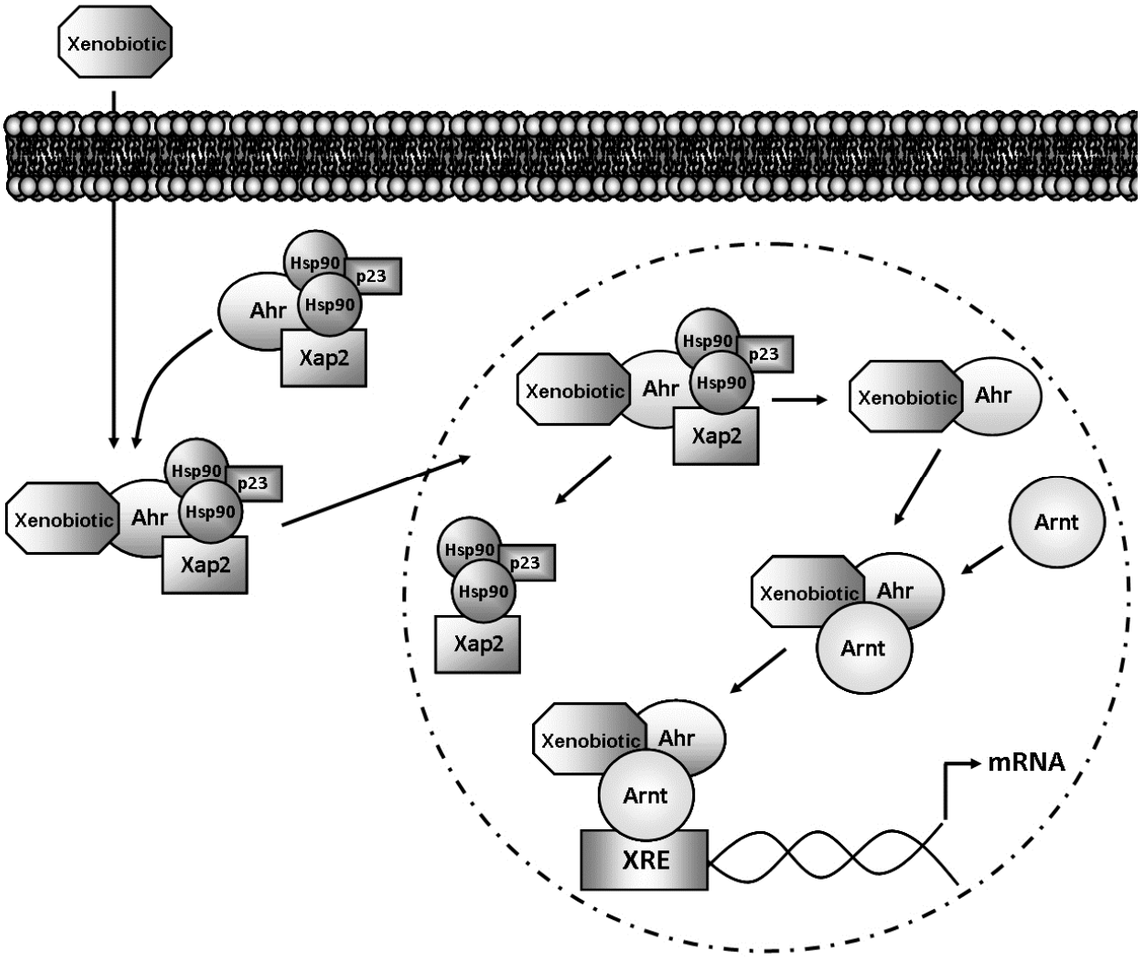

Fig. 2. Molecular mechanism of Arh-Arnt directed gene expression in response to xenobiotic exposure.

In addition to detoxification, the development of Ahr-deficient mice has revealed a physiological role for Ahr in regulating reproduction, growth and development (Benedict et al., 2000, 2003; Nebert et al., 1984; Robles et al., 2000; Schmidt et al., 1996). Benedict et al.

(2000) demonstrated that ovaries from mice deficient for Ahr expression contained significantly more fully formed primordial follicles compared to wild type mice on PND2-3. Robles et al (2000) found similar results, identifying more than a two-fold significant increase in the primordial follicle pool of Ahr deficient PND4 mice compared to wild type mice. These results suggest a developmental role for Ahr in regulating primordial follicle formation and atresia in the mouse. Although the exact details of Ahr role in the regulation of the primordial follicle pool have yet to be determined, given that Ahr xenobiotic ligands cause primordial follicle depletion, we hypothesise that part of these ovotoxic compounds method of ovotoxicity may involve perturbed AhR developmental signalling, inducing premature primordial follicle atresia.

4.2 Bioactivation

Humans come into contact with a variety of xenobiotics over the course of their lifetime, and have evolved a number of physiological mechanisms designed to remove their harmful influence from within the body. Hydrophilic xenobiotics tend to be less toxic, as the body is able to directly excrete them relatively unchanged. However, if the xenobiotic is lipophilic, it will need to be modified by a series of biochemical reactions before it can be eliminated (Pavek and Dvorak, 2008). This series of biochemical reactions is termed biotransformation, and can be divided into two phases. Phase I metabolism involves the introduction or exposure of a reactive polar group on the xenobiotic via oxidation, resulting in a more reactive/water soluble metabolite to facilitate excretion and/or the induction of phase II metabolism. The cytochrome p450 super family of oxidases catalyse the majority of these reactions, although other oxidases, esterases, amidases, and monooxygenases can also be involved (Schroer et al., 2010). Phase II metabolism involves the conjugation of charged species such as glutathione, sulphate, glycine or glucuronic acid to the phase I metabolite to increase its water solubility (Kohalmy and Vrzal, 2011). The addition of these large anionic groups detoxifies reactive electrophiles, resulting in a more polar metabolite which can be actively transported out of the cell. These reactions are carried out by a broad range of transferases, such as glutathione S-transferase, UDP-glucuronosyltransferases, sulfotransferases, N-acetyltransferases, and methyltransferases (Jancovaa et al., 2010).

Unfortunately, phase I metabolism of xenobiotics by the liver and other tissues occasionally results in the production of a more cytotoxic metabolite, a process known as bioactivation (Dekant, 2009). These highly reactive metabolites are electrophilic, and are capable of forming covalent bonds (or adducts) with the nucleophilic centers of cellular macromolecules, such as proteins, DNA, and RNA. Cellular toxicity occurs when these adducts disrupt the normal structure and/or function of these macromolecules, resulting in apoptosis, necrosis or carcinogenesis. The main site of xenobiotic biotransformation within the body is the liver, although the ovary is capable of both phase I and phase II metabolism (Igawa et al., 2009; Rajapaksa et al., 2007a, 2007b; Shimada et al., 2003). Therefore, there is potential for the vulnerable primordial follicle to come into contact with bioactivated ovotoxic metabolites via several routes of exposure. Bioactivated metabolites produced by the liver maybe stable enough to diffuse back into the venous circulatory system, resulting in direct ovarian exposure. Additionally, as the primordial follicle is capable of expressing xenobiotic metabolising enzymes itself, oocytes may be exposed to localised bioactivation. Finally, the xenobiotic may be bioactivated locally into its ovotoxic metabolite by

neighbouring somatic ovarian cells and taken up by the primordial oocyte, contributing to localised bioactivation.

A number of studies performed *in vitro* have revealed that the ovary is capable of the localised bioactivation of a number of xenobiotics into ovotoxic intermediates which target primordial follicles for destruction (Rajapaksa et al., 2007a, 2007b). An example of this localised bioactivation is the reported metabolism of the polycyclic aromatic hydrocarbon DMBA (Fig.3). Ovarian exposure to DMBA disrupts folliculogenesis, resulting in the destruction of all follicle populations leading to POF in rodents, although recent evidence suggests an alternate mechanism of ovotoxicity resulting in primordial follicle depletion in the mouse (Mattison and Schulman, 1980; Sobinoff et al., 2011). This toxicity has been attributed to the bioactivation of DMBA into its ultimate DNA-adduct forming intermediate DMBA-3,4-diol-1,2-epoxide (Shiromizu and Mattison, 1985). DMBA is bioactivated by Cyp1B1 to a 3,4-epoxide which is then converted into a 3,4-diol by the microsomal epoxide hydrolase (MeH) phase II enzyme. This intermediate is then further modified by either Cyp1A1 or Cyp1B1 to form the ultimate ovotoxicant DMBA-3,4-diol-1,2-epoxide (Shimada and Fujii Kuriyama, 2004; Shimada et al., 2001). These three enzymes required for DMBA's biotransformation are all expressed and induced by DMBA exposure in the murine ovary (Igawa et al., 2009; Rajapaksa et al., 2007b; Shimada et al., 2003). In further support of localised DMBA bioactivation, inhibition of MeH in cultured rat ovaries inhibited DMBA induced ovotoxicity, while ovarian culture in the presence of DMBA-3,4-diol induced significantly more primordial follicle depletion than DMBA alone (Igawa et al., 2009; Rajapaksa et al., 2007b).

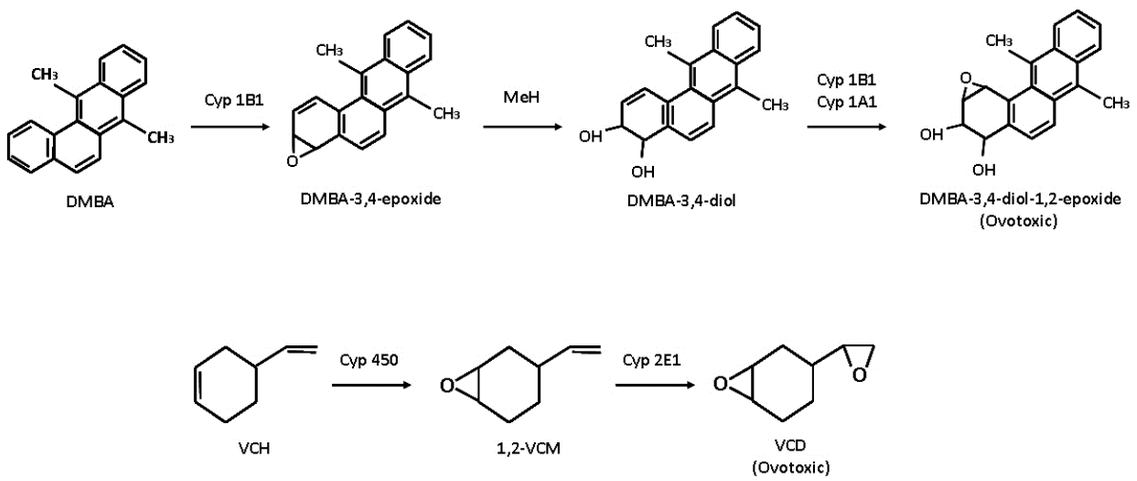

Fig. 3. Metabolism of DMBA and VCD into their ovotoxic metabolites.

Another example of localised bioactivation is the conversion of the industrial chemical 4-Vinylcyclohexene (VCH) into VCH diepoxide (VCD) (Fig. 3). VCH is metabolised by cytochrome P450 phase I enzymes to form VCM-monoepoxide (VCM), which is then converted into VCD. Studies have shown VCD to be the ultimate ovotoxicant, targeting both primordial and primary follicles for depletion (Hu et al., 2001; Smith et al., 1990; Sobinoff et al., 2010). As demonstrated *in vivo* and *in vitro* via knockout studies, VCH/VCM is bioactivated into VCD exclusively by the cyp2e1 isoform in the ovary (Rajapaksa et al.,

2007a). Rajapaksa et al (2007a) cultured neonatal ovaries from both cyp2e1$^{+/+}$ and cyp2e1$^{-/-}$ neonatal mice in VCM and VCD containing media. Both VCH metabolites caused primordial follicle depletion in cyp2e1$^{+/+}$ cultured ovaries. However, unlike VCD, VCM did not produce an ovotoxic affect in cyp2e1$^{-/-}$ cultured ovaries, thus demonstrating its role in VCH induced bioactivation.

4.3 Xenobiotic induced reactive oxygen species generation

Reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide and the highly toxic hydroxyl free radical, are highly reactive oxygen-containing molecules which are produced naturally as a consequence of oxidative energy metabolism (Valko et al., 2007). These short lived ROS play an important role in regulating signal transduction, selectively oxidizing cysteine residues on proteins resulting in a variety of reversible molecular interactions (Janssen-Heininger et al., 2008). However, in excess these highly unstable molecules may lead to perturbed signal transduction and/or oxidative damage to cellular macromolecules, inducing DNA mutations, lipid peroxidation and premature protein degradation. These molecular lesions coupled with perturbed signal transduction can ultimately result in abnormal cellular function, apoptosis and necrosis (Valko et al., 2006, 2007; Wells et al., 2009).

The ovary is a highly redox sensitive organ, with oocytes themselves being particularly vulnerable to excess ROS exposure due to the low rates of oxidative repair in post-mitotic cells (Cadenas and Davies, 2000; Terman et al., 2006). According to the free radical hypothesis of ageing, non-renewing primordial follicles, which can remain quiescent for many years, gradually produce ROS through electron leakage from the mitochondrial electron transport chain (Tarin, 1996). Over time this excess ROS damages the mitochondrial membranes, leading to more electron leakage and further ROS production. Given the redox sensitive nature of primordial follicles, it is reasonable to assume that the generation of xenobiotic induced ROS formed through detoxification may exacerbate this process, contributing to primordial follicle loss (Bondy and Naderi, 1994; Danielson, 2002; Wells et al., 2009).

Xenobiotic enhanced ROS formation may occur via several mechanisms in the primordial follicle (Fig.4). If the ovotoxic xenobiotic contains a quinone-like structure, it may undergo redox cycling with the corresponding semiquinone radical to produce superoxide anions. Further enzymatic and/or spontaneous dismutation of the superoxide anions produces hydrogen peroxide, which can further react with trace amounts of iron or other transition metals to form hydroxyl free radicals (Bolton et al., 2000). Given the futile cyclical nature of redox cycling, this would allow a relatively small concentration of quinone-like xenobiotics to generate an amplified production of ROS in the ovary (Park et al., 2005). For example, menadione (MEN), a synthetic vitamin K with a quinone-like structure, is a potent toxicant which exerts its cytotoxic affect via quinone cycling (Thor et al., 1982). Recently, we examined the effects of MEN on folliculogenesis in neonatal mouse ovaries *in vitro* (Sobinoff et al., 2010). This study found that MEN caused wide spread oxidative stress and DNA damage resulting in primordial and small developing follicle destruction, as evidenced by the detection of increased levels of the hydroxyl radical–induced mutagenic DNA lesion 8-

hydroxyguanine, and Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) analysis (Klaunig and Kamendulis, 2004).

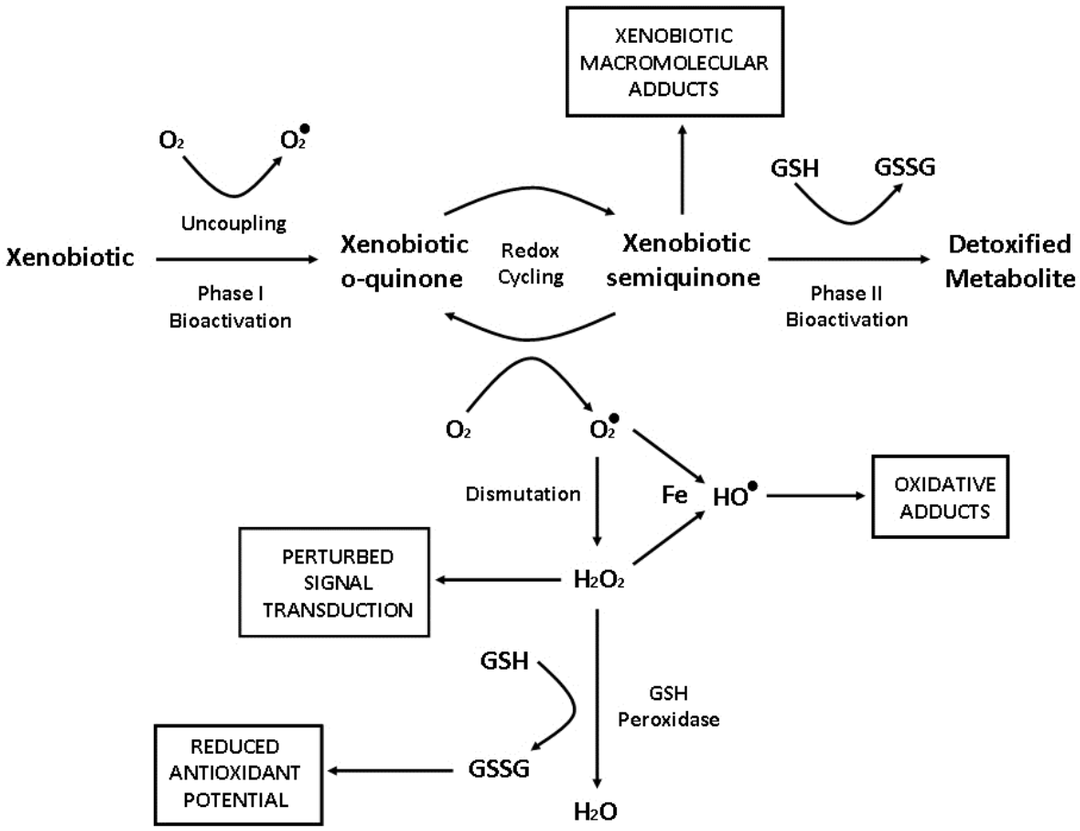

Fig. 4. Biochemical pathways outlining the mechanisms of xenobiotic induced ROS production which may contribute to primordial follicle depletion. Abbreviations: Fe, iron; $O_2^{\bullet}$, superoxide; H_2O_2, hydrogen peroxide; $HO^{\bullet}$, hydroxyl radical.

Another mechanism of xenobiotic induced ROS formation is the phase I bioactivation of the offending xenobiotic into reactive and redox active o-quinone metabolites. As mentioned previously, the PAH BaP is converted into 7,8-diol, and 9,10-diol by Ahr induced cyp1A1 and cyp1B1 enzymes in the ovary (Bengtsson et al., 1983). Studies have also shown that cyp1A1 is also capable of converting BaP into the BaP o-quinones benzo[a]pyrene-3,6-dione and benzo[a]pyrene-6,12-dion (Schwarz et al., 2001). Additionally, cyp 1A1 bioactivated BaP 7,8-diol can be further metabolised via $NAD(P)^+$-dependent oxidation by the phase I dihydrodiol dehydrogenase Akr1c1 enzyme, resulting in the formation of a ketol. This ketol then undergoes tautomerisation to form catechol 7,8-dihydroxybenzo[a]pyrene. Two subsequent 1-electron auto-oxidation events produce a *o*-semiquinone anion, followed by the formation of the o-quinone benzo[a]pyrene-7,8-dione (Trevor et al., 1996). Given the increases observed in cyp 1A1 expression in the ovary in response to BaP exposure, and the relatively high level of dihydrodiol dehydrogenase expression in the ovary compared to the

liver, it is reasonable to assume BaP may be exerting part of its ovotoxic affect through o-quinone formation (Hou et al., 1994). Indeed, both benzo[a]pyrene-3,6-dione and benzo[a]pyrene-6,12-dion were detected in rat ovaries after a single dose exposure of BaP in rodents (Ramesh et al., 2010).

The un-natural "uncoupling" of phase I cytochrome P450 enzymes may also contribute to xenobiotic induced ovotoxicity via ROS production. Cytochrome P450 enzymes use H^+ obtained from NADPH to reduce O_2, which leads to the production of hydrogen peroxide and/or superoxide anion radicals as part of phase I oxygenation. Unfortunately, the P450 catalytic cycle can be uncoupled, resulting in the release of the reactive hydrogen peroxide and/or superoxide anion radical from the enzyme substrate complex (Meunier et al., 2004). Although all cytochrome P450 enzymes experience uncoupling, cyp 2E1 experiences a high rate of the phenomenon (Caro and Cederbaum, 2004). Even in the absence of substrate, cyp 2E1 undergoes un-natural "uncoupling" due to its NADPH oxidase activity independent of phase I metabolism (Ekstrom and Ingelman-Sundberg, 1989). As described previously, VCH is exclusively bioactivated by cyp 2E1 to produce the ovotoxic metabolite VCD. It is therefore possible that VCH may partially cause primordial follicle depletion via excess ROS production. Indeed, studies conducted in our laboratory have demonstrated VCD itself, along with the pesticide methoxychlor (MXC) and MEN, is capable of inducing cyp 2E1 expression and oxidative stress in the form of 8-hydroxyguanine adduct formation in primordial follicles (Sobinoff et al., 2010).

Another mechanism by which ovotoxic xenobiotics may cause oxidative stress is through the depletion of glutathione peroxidase (GSH) via detoxification. GSH is the body's most abundant antioxidant, providing protection against all forms of oxidative stress by scavenging ROS by virtue of its reducing thiol group, forming oxidised glutathione disulfide (GSSG) (Kidd, 1997). The glutathione system (GSH/GSSG ratio) acts as a homoeostatic redox buffer that contributes to maintenance of the cellular redox balance, with a reduction in the GSH/GSSG ratio indicating oxidative stress (Schafer and Buettner, 2001). In addition to its function as a ROS scavenger, GSH is also employed in the phase II metabolism of many ovotoxic xenobiotics (Keating et al., 2010; Tsai-Turton et al., 2007; Wu and Berger, 2008). For example, VCD is conjugated to GSH by the glutathione S-transferase Gst isoform pi (Gstp) as part of phase II detoxification in the ovary (Keating et al., 2010).

The mammalian ovary itself is highly redox sensitive, with maturing oocytes containing the highest concentration of GSH compared to any other cell type in the body (Calvin et al., 1986; Clague et al., 1992; Luderer et al., 2001). It is therefore likely that ovarian somatic and germ cell GSH plays an important role in protecting ovarian follicles from damage by ovotoxic xenobiotics. This is especially evident in primordial follicles, where a natural decrease in the GSH/GSSG ratio with advancing reproductive age increases primordial follicle susceptibility to xenobiotic induced destruction (Mattison et al., 1983b). Therefore, we hypothesise that the detoxification of ovotoxic xenobiotics via GSH conjugation reduces the GSH/GSSG ratio in primordial follicles, leaving them vulnerable to oxidative stress and primordial follicle depletion. Indeed, DMBA detoxification involves GSH conjugation, and its ovotoxic ROS production can be reduced through the addition of GSH, curbing its ovotoxicity (Tsai-Turton et al., 2007). There is controversial evidence for this mechanism of ovotoxicity in VCD induced primordial follicle loss. Rodent exposure to VCD was shown to reduce GSH concentrations by 25% and 55% in rat and mouse ovaries 2 hours after VCD

administration (Bhattacharya and Keating, 2011). Additionally, rodents given the same dose of VCD over a period of several days caused specific primordial follicle depletion only after 15 days of continual dosing (Springer et al., 1996). The significant decrease in GSH concentrations almost immediately after exposure, coupled with the delayed loss of follicles following chronic exposure suggest that GSH reduction over time due to VCD detoxification could leave the susceptible primordial follicle vulnerable to increasing concentrations of xenobiotic induced ROS, resulting in primordial follicle destruction. It is pertinent that a single dose of a higher concentration of VCD (320 mg/kg) causes significant primordial follicle depletion 6 days after exposure, but is not specific to the primordial follicle pool (Devine et al., 2004). Additionally, VCD *in vitro* culture assays have linked an increase in Gstp expression with the first signs of primordial follicle loss after 6 days of exposure in neonatal rat ovaries (Bhattacharya and Keating, 2011; Keating et al., 2010). As Gstp catalyses VCD-GSH conjugation, the increase in enzymatic expression and therefore activity could have contributed to the observed primordial follicle loss due to a reduction in GSH/GSSG oxidative buffer. Conversely, substituting VCD culture media with antioxidant such as GSH does not prevent primordial follicle depletion, suggesting it is not the ultimate cause of depletion (Devine et al., 2004).

4.4 Xenobiotic induced primordial follicle activation

Traditionally, studies attempting to identify the molecular mechanisms behind xenobiotic induced POF have focused on premature follicular atresia as the main source of primordial follicle depletion. However, there is now a growing body of evidence which suggests that xenobiotics cause primordial follicle depletion through accelerated primordial follicle activation (Keating, 2009, 2011; Sobinoff et al., 2010, 2011). A study of VCD and MXC induced primordial follicle depletion has revealed a selective mechanism of pre-antral ovotoxicity involving small developing follicle atresia and primordial follicle activation both *in vitro* and *in vivo* (Sobinoff et al., 2010). Extracted neonatal mouse ovaries cultured in either VCD or MXC were immunopositive for the apoptotic markers caspase 2, caspase 3, and TUNEL in small developing follicles from the primary stage onward, but were absent in primordial follicles (Fig.5). In addition, the primordial follicles in VCD and MXC cultured ovaries expressed proliferating cell nuclear antigen (PCNA), a marker of primordial follicle activation (Picut et al., 2008; Tománek and Chronowska, 2006). VCD and MXC exposure also induces primordial follicle activation and developing follicle atresia *in vivo* as evidenced by increased primordial follicle PCNA expression and histomorphological analysis (Sobinoff et al., 2010). Microarray analysis confirmed via qPCR also showed VCD and MXC up-regulated PI3K/Akt and mTOR signalling, two synergistic pathways intimately associated with primordial follicle activation (Reddy et al., 2010). Further evidence for PI3K/Akt signalling in VCD induced primordial follicle activation comes from a study conducted by Hoyer et al (2009), in which LY294002, an inhibitor of PI3K, prevented primordial follicle depletion in cultured rat ovaries (Vlahos et al., 1994).

The polycyclic aromatic hydrocarbon DMBA, which was previously thought to cause indiscriminate follicular destruction, has also been shown to cause pre-antral ovotoxicity through selective immature follicle destruction and primordial follicle activation (Mattison and Schulman, 1980; Sobinoff et al., 2011). In addition to showing signs of maturing follicle atresia (caspase 2, caspase 3, TUNEL) and primordial follicle activation (PCNA), DMBA

induced Akt1 phosphorylation, mTOR activation, and decreased FOXO3a expression in DMBA cultured primordial oocytes. All of these events occur downstream of the PI3K/Akt and mTOR signalling pathways, providing evidence for these pathways involvement in xenobiotic induced primordial follicle depletion (Reddy et al., 2010). Unlike VCD however, PI3K/Akt inhibitor studies utilising LY294002 in DMBA cultured rat ovaries caused accelerated primordial follicle depletion (Keating, 2009). In addition to its role in primordial follicle activation, PI3k/Akt signalling is also responsible for augmenting cellular survival by inhibiting the activation of proapoptotic proteins and transcription factors (Blume-Jensen et al., 1998; Testa and Bellacosa, 2001). Therefore, in addition to acting synergistically with mTOR signalling to cause primordial follicle activation, PI3k/Akt signalling may help preserve the primordial follicle pool in times of cytotoxic stress. Interestingly however, mTOR signalling does not require PI3k/Akt signalling to induce primordial follicle activation, and in fact may be the sole driver of DMBA induced primordial follicle activation (Adhikari et al., 2010).

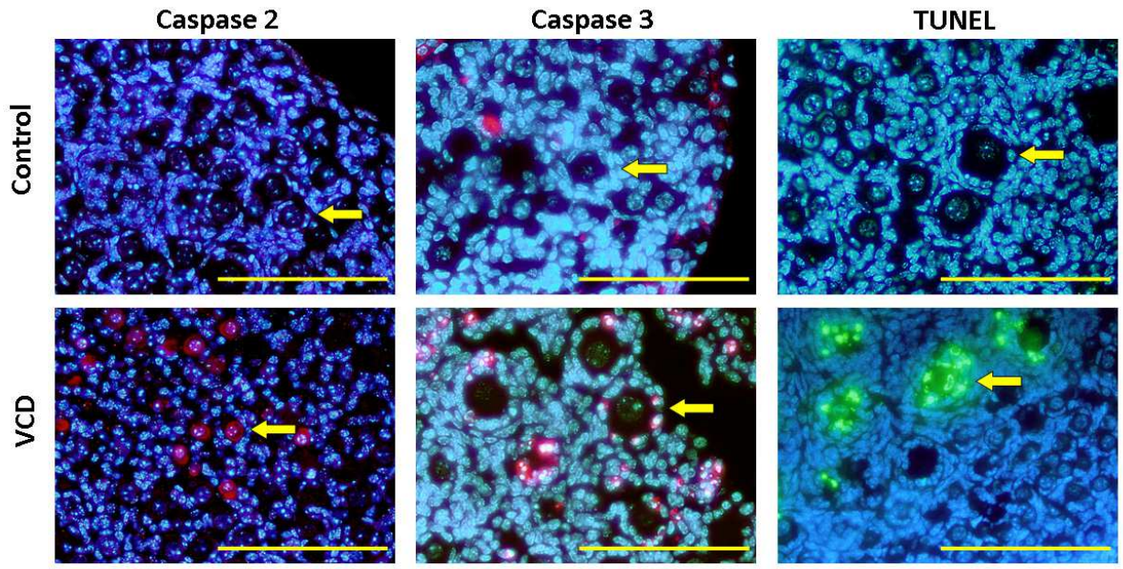

Fig. 5. Immunohistological staining of apoptotic markers in VCD exposed neonatal mouse ovaries. Blue staining (DAPI) represents nuclear staining; red staining (Cy-5) represents specific staining for the protein of interest; green staining (Fluorescein) represents specific staining for degraded DNA (TUNEL). Thin arrow=developing follicle; scale bar is equal to 50μm.

As xenobiotic induced primordial follicle activation is reportedly accompanied by small pre-antral follicular destruction, it has been hypothesised that xenobiotic induced primordial follicle depletion is the result of a homeostatic mechanism of follicular replacement (Keating, 2009; Sobinoff et al., 2010). In this hypothesis, the ovotoxic xenobiotic targets and destroys developing follicles, leading to increased primordial follicle recruitment to maintain the developing pool (Fig. 6). Although the developing pool may be maintained for some time, eventually the rate of developing follicle destruction will exceed the dwindling primordial follicle pools rate of replacement, resulting in POF. Indeed, it is well known that

rapidly dividing cells, such as the granulosa cells of developing follicles, are highly susceptible to the action of cytotoxic xenobiotics (Blumenfeld and Haim, 1997; Hirshfield, 1991). Therefore, if the xenobiotic targeted these proliferating granulosa cells for destruction, the entire follicular structure would demise (Hughes and Gorospe, 1991). Even given the vulnerable nature of the primordial follicle explained earlier in this review, the primordial follicles quiescent nature may reduce their susceptibility to certain xenobiotics, and are only destroyed once a commitment to activation/recruitment has been made.

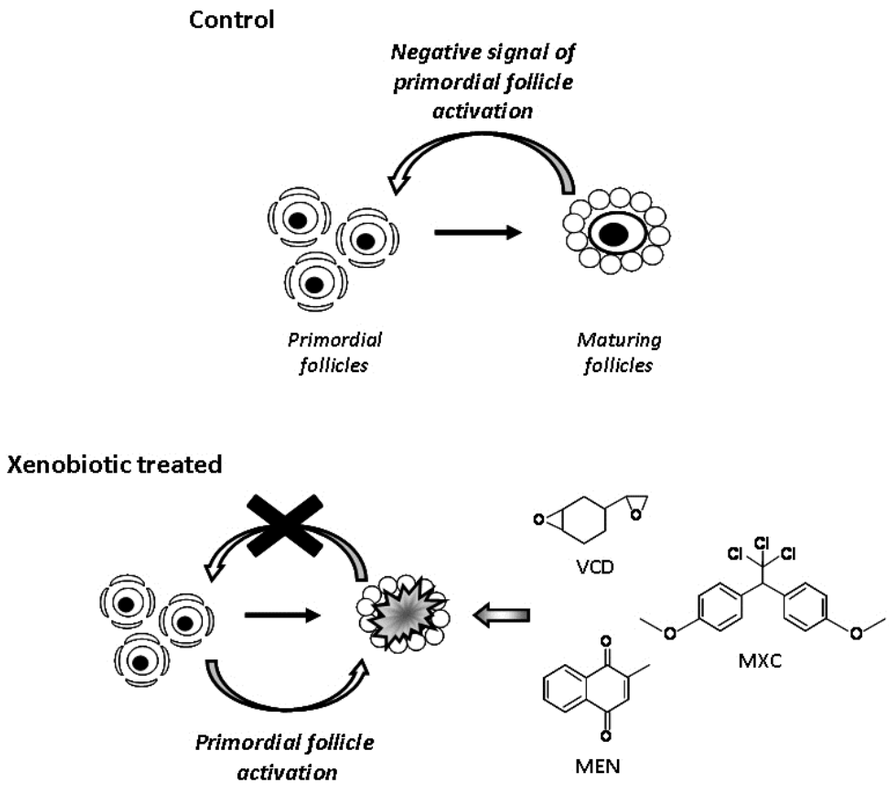

Fig. 6. Homeostatic mechanism of follicular replacement hypothesis. Under control conditions, premature primordial follicle activation is prevented by negative cytokine signals excreted from the developing pool of follicles. Xenobiotic exposure results in the destruction of this developing pool, removing these negative signals causing primordial follicle activation.

Another proposed mechanism of xenobiotic induced primordial follicle activation may involve perturbed signal transduction caused by oxidative stress. As described previously, ROS play a physiological role in regulating signal transduction by selectively oxidising cysteine residues on proteins resulting in a variety of reversible molecular interactions (Wells et al., 2009). It is therefore conceivable that increased levels of xenobiotic induced ROS could lead to abnormal cysteine oxidation and consequently dysregulated signal transduction. For example, the PI3K/Akt signalling pathway has been shown to be up-regulated by increased levels of ROS through the H_2O_2 oxidation of phosphatases which

negatively regulate the pathway (Kim et al., 2005; Naughton et al., 2009). Given the PI3K/Akt pathway's role in the regulation of primordial follicle recruitment, increased ROS production could potentially cause primordial follicle activation in xenobiotic treated ovaries. Indeed, all three xenobiotics which have thus far been reported to induce primordial follicle activation also cause oxidative stress and induce the expression/activation of members of the PI3K/Akt signalling pathway in the ovary (Sobinoff et al., 2010, 2011; Tsai-Turton et al., 2007).

Xenobiotic induced primordial follicle activation may also be the result of abnormal cross-talk between signalling pathways. For example, DMBA exposure was shown to induce *Dnajb6* expression, a heat shock protein whose expression is normally induced by Nrf2 and Hsf1 in response to oxidative stress (Sobinoff et al., 2011; Thimmulappa et al., 2002; Wang, K. et al., 2009). Dnajb6 responds to stress by inhibiting nuclear factor of activated T cells (NFAT) transcriptional activity through the recruitment of class II histone deacetylase (Dai et al., 2005). In turn NFAT positively regulates PTEN expression, a known inhibitor of Akt1 phosphorylation. Therefore DMBA induced Dnajb6 expression may inhibit NFAT transcriptional activity, reducing PTEN expression and stimulating Akt1 phosphorylation, resulting in primordial follicle activation (Baksh et al., 2002; Reddy et al., 2010; Wang, Q. et al.).

4.5 Xenobiotic induced cell death

Ovarian follicles undergo physiological cell death via the apoptotic process of atresia, which is thought to select dysfunctional follicles and thus reserving the healthiest follicles for ovulation (Tilly et al., 1991). A number of studies have concluded that ovotoxic xenobiotics which target primordial follicles for destruction do so by inducing premature follicular atresia (Hu et al., 2001; Matikainen et al., 2001; Tilly and Robles, 1999). In this review we have already discussed the mechanisms by which ovotoxic xenobiotics may induce follicular atresia in primordial follicles (Ahr activation, Bioactivation, and ROS generation). However, other forms of cell death have been reportedly induced by xenobiotic exposure. Cell death by necrosis usually occurs in response to tissue injury, and elicits an inflammatory response in the surrounding tissue. Necrosis can be distinguished from apoptosis via histomorphological and ultrastructural analysis (Gobe and Harmon, 2001). In a study by Mattison (1980), the three PAHs BaP, 3-MC, and DMBA were shown to cause morphological changes in mouse primordial follicle oocytes which were consistent with necrosis (Mattison, 1980). The alkylating chemotherapeutic agent cyclophosphamide was also shown to cause necrotic damage in mouse primordial follicle oocytes three days after a single i.p injection (Plowchalk and Mattison, 1992). However, lower doses of cyclophosphamide produced atretic changes in primordial follicle oocytes, suggesting the type of cell death (apoptosis/necrosis) caused by xenobiotic exposure depends upon the dose given, and the duration of exposure. Therefore, concentrations of xenobiotic which cause mild cellular damage may result in active cell death, or apoptosis, while concentration which result in severe damage will result in passive cell death, or necrosis (Raffray and Gerald, 1997).

Autophagy or "self eating" is another possible non-apoptotic mechanism of cell death which may result in primordial follicle depletion. This conserved catabolic process involves the lysosomal-dependant turnover of cytoplasmic organelles and proteins during times of

starvation or nutrient deficiency, allowing the regeneration of metabolic precursor molecules to ensure survival (Levine and Klionsky, 2004). Increased incidences of autophagy have also been observed in response to other environmental stresses, including hypoxia, oxidative stress, and xenobiotic exposure (Kiffin et al., 2006; Kondo et al., 2005). Under these conditions autophagy may renew damaged or dysfunctional organelles, thereby maintaining a healthy cell population. Although the activation of autophagy in response to cell stress may be a cellular adaptation to promote survival, excessive activation beyond a key threshold may result in cellular collapse and atrophy, a process known as autophagic cell death (Galluzzi et al., 2008). While debatable whether autophagic cell death is independent from apoptosis, it has been almost universally accepted that excess autophagy can induce apoptosis (Levine and Yuan, 2005; Maiuri et al., 2007). Recent studies have suggested autophagy as an alternate form of programmed cell death in the ovary, with evidence indicating it is the main mechanism by which oogonia are lost prior to primordial follicle formation (Duerrschmidt et al., 2006; Lobascio et al., 2007; Rodrigues et al., 2009). Thus prolonged xenobiotic exposure resulting in organelle damage may induce autophagic cell death in primordial follicles, resulting in depletion. Indeed, proteins responsible for regulating apoptosis, such as members of the Bcl2 family, have also been found to regulate autophagy (Maiuri et al., 2007; Shimizu et al., 2004). Therefore, gene expression studies in which these pathways have been thought to induce xenobiotic atresia could be inducing primordial follicle destruction by apoptotic independent or dependent autophagy (Flaws et al., 2006).

5. Ovotoxic xenobiotics as agents for wildlife fertility control

Population control of native and exotic pest species is necessary to prevent environmental degradation, competition and predation of native wildlife, the spread of pathogenic diseases, and conflicts with humans over food production. Traditionally, population control has involved the elimination of the target species through poisoning, trapping and shooting (McAlpine et al., 2007). Although effective immediately, these methods are seen as inhuman, unsustainable, and ineffective over the long term. Manipulating the reproductive rate, particularly in females, instead of increasing the mortality rate is potentially more humane, species specific, and effective at curtailing populations (Kirkpatrick, 2007). The use of ovotoxic xenobiotics as agents of contraception/sterilisation represents a novel approach to fertility control. Of particular interest are xenobiotics which have been shown to cause POF by specifically targeting the primordial follicle population for degradation (Hoyer and Devine, 2001; Sobinoff et al., 2010), thus causing permanent sterility.

To achieve widespread efficacy ovotoxic xenobiotics in fertility control must be delivered via single or minimal oral administration. To be successful an oral agent must also have permanent or very long lasting effects, be specific for the target pest species and be humane/environmentally safe (Castle and Dean, 1996). Rodents such as the rice-field rat represent a serious pest in cereal agriculture, accounting for an average annual loss of between 5-10% of rice crops in Asia, 17% of rice crops in Indonesia, between 15-100% of maize in Africa, and between 5-90% of total crop production in South America (Geddes, 1992; Mwanjabe and Leirs, 1997; Rodríguez and Jaime, 1993; Singleton, 2003; Taylor, 1968).

VCD represents an ideal fertility control agent due to its ability to induce rapid small follicle depletion resulting in POF in rodents at concentrations which do not cause widespread

cytotoxicity (Springer et al., 1996). Additionally, VCD metabolism in the liver and hepatic tissue of rodents results in the production and excretion of the inert compound from the body, potentially reducing its effects on predators and its bioaccumulation in the environment (Flaws et al., 1994; Keating et al., 2010; Rajapaksa et al., 2007a). However, VCD does have disadvantages which make it fall short of the ideal fertility control agent. As described previously, VCD requires multiple doses to cause complete infertility in the rodent model (Springer et al., 1996). In addition, a VCD containing bait would need to be both attractive and palatable to the pest species, but not palatable or accessible to non-pest species. Currently, VCD is being trialled as an oral fertility control agent in the rice-field rat *Rattus argentiventer*. Registered by SenesTech Inc. as ContraPest®, the company website suggests the formulated bait is palatable, causes complete sterility within one month's ingestion, and does not adversely affect the animal's health and well being (http://www.senestech.com/). The use of other ovotoxicants as oral fertility control agents has been less successful. In a study by Sanders et al (2011) ERL-4221, a less toxic diepoxide, cycloaliphatic epoxide resin, which recently replaced VCD in industry, was investigated as a possible fertility control agent for pigs. A 20 day treatment period using palatable bait containing 16.0 mg ERL-4221 kg–1 bodyweight failed to produce any difference in follicular composition compared to control treated animals (Sanders et al., 2011). In summary, ovotoxicants represent potential fertility control agents, provided the xenobiotic delivers significant follicle depletion without side effects, and does not adversely affect the environment or food chain.

6. Conclusions

Ovotoxic xenobiotics cause primordial follicle depletion via several mechanisms which ultimately lead to their destruction or activation. These chemicals are rarely ovotoxic by themselves, and require hepatic or ovarian metabolism to exert their destructive effects on reproduction. This type of ovotoxicity is insidious in its nature, and is not usually detected until the primordial follicle pool has become severely depleted, resulting in premature reproductive senescence. Besides a loss in fertility, reproductive senescence is also associated with an increased incidence of a variety of health problems. Despite the negatives associated with ovotoxic xenobiotics, there is potential to use their destructive nature for wildlife control and agricultural gain. It is a form of poetic justice that ovotoxic xenobiotics which prevent women from conceiving may be used to combat one of the biggest causes of death in the third world, starvation. Future research should be aimed at further elaborating the specific mechanisms of primordial follicle ovotoxicity, improving our ability to predict/detect human risk from environmental exposure, and investigating the possibility of using these ovotoxicants for the environmental control of pest species.

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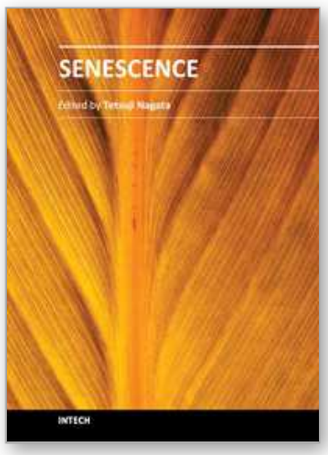

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The book "Senescence" is aimed to describe all the phenomena related to aging and senescence of all forms of life on Earth, i.e. plants, animals and the human beings. The book contains 36 carefully reviewed chapters written by different authors, aiming to describe the aging and senescent changes of living creatures, i.e. plants and animals.

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