The impact of endocrine disruptors on oocyte competence

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To date, approximately 60 chemicals have been identified as endocrine disruptors: exogenous agents that interfere with various aspects of natural hormone physiology. The potential reproductive and health hazards of these environmental chemicals have recently generated concern among the scientific community, policy makers and general public. The present review presents and discusses the available evidence that environmental chemicals are causing ovarian toxicity in various species, with particular attention to farm animals. The impact of chronic exposure to endocrine disruptors via food and drinking water cannot be neglected when studying fertility problems in these species. This review focuses attention on the superfamily of organochlorine chemicals, persistent organic pollutants (POPs), because of their persistence in the environment, ability to concentrate up the food chain, continued detection in environmental matrices and ability to be stored in the adipose tissue of animals and humans. Published data clearly indicate that POPs disrupt mammalian oocyte maturation and follicle physiology in every species studied so far, including farm animals. However, as most of the data available still derive from experiments performed on laboratory species or *in vitro* models, great care should be taken when extrapolations to other species or environmental situations are attempted.

Recently, there has been concern among the scientific community, policy makers and general public regarding the potential reproductive and health hazards of a range of environmental chemicals known as 'endocrine disruptors'. An endocrine disruptor is defined as an exogenous agent that interferes with the synthesis, secretion, transport, metabolism, binding, action or elimination of natural blood-borne hormones in the body that are responsible for homeostasis, reproduction and developmental processes (Kavlock and Ankley, 1996). Like hormones, small amounts of these chemicals (parts per trillion) are believed to affect the endocrine system of animals and humans.

Three main types of endocrine-disrupting mechanism have been identified:

- Mimics imitate naturally produced hormones such as oestrogen and testosterone. These chemicals can initiate chemical reactions in the body in the same way as naturally produced hormones.
- Hormone blockers 'lock up' a hormone receptor, preventing naturally produced hormones from entering the cell and performing their function.
- Triggers act through hormone-like pathways but initiate abnormal reactions in the cell that would not normally be produced by a hormone. The best-known triggers

are dioxin and dioxin-like chemicals. Dioxin acts through a hormone-like process to initiate entirely new responses.

To date, approximately 60 chemicals have been identified as endocrine disruptors. Chemicals with hormonal activity fall into three broad classes: (1) synthetic chemicals used in industry, agriculture and consumer products; (2) synthetic chemicals used as pharmaceutical drugs; and (3) natural chemicals found in human and animal food (phytoestrogens). Examples of these substances are provided (Box 1). About half of these compounds are chlorinated, including dioxins (PCDDs), polychlorinated biphenyls (PCBs) and organochlorine pesticides such as DDT, methoxychlor, dieldrin and hexachlorocyclohexane (HCH). Exposure to endocrine disruptors can occur in a variety of ways; humans and animals can be exposed involuntarily to endocrine disruptors as a result of drinking contaminated water, breathing contaminated air, ingesting food or coming into contact with contaminated soil.

Although endocrine disruptors have adverse effects on different hormone-dependent functions, for example, immune and thyroid dysfunction (Smialowicz *et al.*, 1989; Vos and van Loveren, 1995), studies have focused mainly on development and reproduction. The interference by endocrine disruptors of normal development of male and female reproductive organs as well as with reproductive functions in adulthood

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Box 1. Synthetic compounds known as or suspected to be endocrine disruptors
Herbicides and fungicides

- Dichlorophenoxyacetic acid (2,4-D)
- Trichlorophenoxyacetic acid (2,4,5-T)
- Alachlor
- Amitrole
- Atrazine
- Metribuzin
- Nitrofen
- Trifluralin

Insecticides

- Benzenhexachlorcyclohexane (B-HCH)
- Methoxychlor
- Toxaphene
- DDT and metabolites (DDE)
- Carbaryl
- Endosulfan
- Mirex
- Transnonachlor
- Chlordane

Nematocides

- Aldicarb

Industrial chemicals

- Dioxins
- Polychlorinated biphenyls (PCBs)
- Polybrominated biphenyls (PBBs), pentachlorophenol (PCP)

- Benomyl
- Mancozeb
- Zineb
- Metiram complex
- Maneb
- Ziram
- Tributyltin
- Hexachlorobenzene

- Oxychlordane
- Dicofol
- Heptachlor and heptachlor epoxide
- Dieldrin
- Parathion
- Methomyl
- Lindane (Y-HCH)
- Synthetic pyrethroids
- Chlordane (kepone)

- 1,2-Dibromo-3-chloropropane (DBCP)

- Penta- to nonyl phenols
- Phthalates
- Styrenes

has been well documented, in both wildlife and experimental animals (for reviews, see Colborn *et al.*, 1993 and Toppari *et al.*, 1996). In females, published data indicate that exposure to chemicals may cause alterations in reproductive behaviour and contribute to sub-fecundity, infertility, pregnancy loss, growth retardation, intrauterine fetal demise, birth defects and ovarian failure (for reviews, see Sharara *et al.*, 1998 and Bhatt, 2000).

In adult females, the reproductive cycle is a complex process characterized by the distinct phases of gametogenesis and embryogenesis. Each of these phases is under the control of steroid hormones (Knobil and Neill, 1988) and is therefore a potential target for endocrine disruptors. It has been demonstrated that increased concentrations of xenoestrogens may affect ovarian function through the disruption of feedback mechanisms in the hypothalamus–pituitary–gonadal axis (Crisp *et al.*, 1998). Moreover, the ovarian follicle has also been shown to be a direct target for xenoestrogens (Davis *et al.*, 1994; Hoyer, 1999; Wojtowicz *et al.*, 1999). Collectively, these studies show that endocrine disruptors can influence follicular growth and, through these effects, may also affect the oocyte, as has been shown in *in vitro* studies (Hafne *et al.*, 2000).

Environmental chemicals affect ovarian function in different ways

Female reproductive function can be compromised by exposure to toxic chemicals (Mattison, 1985) at a variety of sites, including the hypothalamus, pituitary gland, ovary and reproductive tract (Mattison, 1980; Thomas, 1993). Disruption of any of these sites can ultimately manifest as a disruption of ovarian function, resulting in infertility.

The ovary performs two important roles, delivery of the female gametes (oocytes) and production of ovarian hormones, for example oestrogen, progesterone and inhibin (Richards, 1980; Hirshfield, 1991). How reproductive toxicants can affect ovarian function is generally not well understood but the effects can be due to one of several possible mechanisms. Sites of action include the hypothalamus–hypophyseal system, resulting in disruption of the normal pattern of gonadotrophin secretion, and the ovary, resulting in direct destruction of the oocyte (ovotoxicity).

Oocyte destruction can result from a toxic chemical directly impairing oocyte viability. However, as oocytes at all stages of development are surrounded by follicular cells, these mechanisms might also be

indirect, involving alterations within the follicular wall, which compromise its ability to maintain oocyte viability (Buccione *et al.*, 1990). Extensive oocyte destruction damages ovarian follicles and, in turn, destroys steroid hormone production, which can result in ovarian failure. Therefore, oocyte destruction, ultimately, can disrupt the endocrine balance, causing a reduction in oestrogen and progesterone and an increase in FSH and LH. Finally, endocrine disruptors can affect other organs, leading indirectly to altered ovarian function, for example, through metabolic alterations that change the balance of feedback control of the hypothalamus–pituitary–ovarian system.

Susceptibility of the ovaries to the different classes of agent depends on the stage of development at which exposure occurs. For chemicals that destroy the oocytes, the stage of development at which the follicle is destroyed determines the impact that the exposure to the chemical will have on reproduction. Compounds that extensively destroy oocytes contained in primordial and primary follicles may have a delayed effect on reproduction until recruitment of growing and antral follicles can no longer be supported (Generoso *et al.*, 1971; Hooser *et al.*, 1994). Conversely, chemicals that selectively damage large growing or antral follicles generally interrupt reproductive function only temporarily because these follicles can be replaced by recruitment from the greater pool of primordial follicles. Thus, these chemicals produce a readily reversible infertility that is manifest relatively soon after exposure (Jarrell *et al.*, 1991; Davis *et al.*, 1994).

The concentration of endocrine disruptor required to produce ovarian damage is another factor that determines the final effect of the exposure to a reproductive toxicant. It is only under rare circumstances that individuals are exposed acutely to toxic concentrations of ovotoxic chemicals, and the effects can usually be detected and evaluated. However, the effects of chronic exposure to toxicants are more difficult to determine. Because of the insidious nature of toxicants, this type of exposure can cause 'silent' damage and is of the greatest concern.

Environmental chemicals can alter ovarian function acting both upstream and downstream from the ovary itself. Moreover, when the ovary is the target, consequences depend on the stage of follicular development, and the dose and duration of exposure.

The organochlorine chemicals superfamily

The present review discusses the evidence that environmental chemicals are causing ovarian toxicity in various species, with particular attention to farm animals. To date, the physiological consequences of the ingestion of endocrine disruptors by farm animals are largely unknown. However, the extent of exposure of domestic ruminants to these chemicals is such that

the impact of exposure to endocrine disruptors via food and drinking water cannot be neglected when studying fertility problems in these species. Indirect evidence of adverse effects is provided by a study that demonstrates an association between exposure to drinking water contaminated with sewage overflows and reduced reproductive performance of dairy cattle (Meijer *et al.*, 1997). Monitoring the adverse effect of endocrine disruptors on reproduction is important for the animal production industry, which is vulnerable to the inadvertent disposal of potentially dangerous chemicals. In particular, it is important to study the effect of endocrine disruptors on female reproductive physiology, as farm animal reproduction is based on the intensive use of a limited number of males, usually under strict management, and on the extensive use of a large number of females exposed to a wide range of different environmental conditions.

This review focuses attention on the superfamily of organochlorine chemicals (persistent organic pollutants, POPs) because of their persistence in the environment, ability to concentrate up the food chain, continued detection in environmental matrices and ability to be stored in the adipose tissue of animals and humans.

POPs comprise a superfamily of chemicals persistent in the environment and with adverse effects on health and environment. Members of this superfamily include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzo furans (PCDFs), polychlorinated biphenyls (PCBs), polychlorinated diphenyl ethers (PCDEs) and polychlorinated naphthalenes (PCNs), as well as the polybrominated biphenyls (PBBs) and polybrominated diphenyl ethers (PBDEs). Each subfamily consists of many congeners that share the chemical backbone of the subfamily but have different numbers and positions of halogen substituents. The molecular structure of some of the most common POPs is shown (Fig. 1). Many of these compounds have four characteristics that make them intrinsically hazardous:

- Long environmental half-lives (persistent), resulting in a continued increase of the global inventory in the environment.
- Can be found at great distances from where they are used or released (Fig. 2).
- Characterized by low water solubility and high lipid solubility, leading to their bioaccumulation in fat tissues.
- Many of these compounds possess sex steroid activities, thereby causing endocrine disruption and a number of associated effects such as reproductive system dysfunction.

The stability and lipophilicity of POPs has led to increased concerns regarding the toxic effects that they can exert on a range of biota even at extremely low concentrations, in particular on species at the top of the food chain. Moreover, high concentrations of

Fig. 1. Molecular structure of some of the most common persistent organic pollutants. DDT: dichlorodiphenyltrichloroethane; PCBs: polychlorinated biphenyls; TCDD: tetrachlorodibenzo-*p*-dioxin.

Fig. 2. Schematic representation of the current theory on the global transport of persistent organic pollutants (POPs). Organochlorines tend to vaporize from soil and water in the mid-latitudes, travel about the earth and condense when they encounter colder air, returning to earth and working their way through food webs.

POPs are known to be present in sewage sludge from industrial, agricultural and domestic sources, which is spread on arable land and pasture as fertilizer (Wild and Jones, 1992), and are found in water (Fingler *et al.*, 1992; Abbassy *et al.*, 1999). Farm animals ingest these substances with food and drinking water and it is likely that the rate of ingestion will increase in the future as increased amounts of sewage sludge are recycled onto agricultural land (Wild and Jones, 1992). Rhind *et al.* (2002) investigated the accumulation of endocrine disruptors other than POPs (for example, phthalates and alkylphenols) on agricultural land after sewage sludge

treatment. Although the authors concluded that there is no significant long-term accumulation of the investigated endocrine disruptors in pastures after repeated sludge application, they also observed a relatively high concentration of endocrine disruptors in control areas. Therefore, the potential remains for the ingestion of endocrine disruptors by grazing animals to be biologically relevant, and a better understanding of the potential risk of sludge contamination on agricultural land will require the assessment of pasture accumulation rate for a broader range of chemicals, including POPs.

The present review covers the adverse effects on oocyte competence of only three POP subfamilies, that is PCDDs, PCBs and organochlorine pesticides. Although there may be concern about the reproductive toxicity of other classes of compound (for example, PBDEs) this study focuses attention on chemicals that have been quantified in reproductive tissues and in ovarian follicular fluid (Trapp *et al.*, 1984).

*Polychlorinated dibenzo-*p*-dioxins*

PCDDs are environmental contaminants, historically derived from industrial processes such as herbicide production, chlorination and combustion. PCDDs consist of up to 75 congeners (Safe, 1986) and can induce a diverse spectrum of biochemical and toxic responses in laboratory animals and mammalian cells in culture (Poland and Knutson, 1982). Among PCDDs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is considered the most toxic and one of the most potent environmental toxicants (DeVito and Birnbaum, 1995). The best-characterized mechanism for the action of TCDD is the

arylhydrocarbon receptor (AhR, Fig. 3; Burbach *et al.*, 1992), which is present in the cytoplasm bound with at least three additional proteins (Hankinson, 1995). These proteins are thought to keep the AhR in a state responsive to ligand binding. Regulatory proteins are displaced when ligand binding occurs, and the AhR enters the nucleus where it complexes with its nuclear partner AhR nuclear translocator (Arnt; Hoffman *et al.*, 1991). The newly formed heterodimer acquires the ability to bind specific DNA enhancer sequences known as xenobiotic responsive element (XRE; Denison and Wilkinson, 1985), acting as a transactivator of gene expression in a wide variety of species and tissues (Denison and Wilkinson, 1985; Dolwick *et al.*, 1993). Generally, products of these genes are of one or two broad categories: drug-metabolizing enzymes and growth-regulatory proteins. The most extensively studied AhR-target gene is cytochrome P450 1A1 (CYP 1A1), a protein involved in the metabolism of a large number of xenobiotics (Delescluse *et al.*, 2000), and which also plays an important role in oestrogen metabolism (Zhu and Conney, 1998). Changes in these metabolic enzymes do not appear to mediate directly the effects of the ligand. However, they play an important role, transforming 'pretoxicants' into their ultimate cytotoxic form or detoxifying potentially dangerous xenobiotics. Several genes that encode growth-regulatory proteins also appear to be responsive to AhR agonists. This group includes the epidermal growth factor receptor, the oestrogen receptor, interleukin 1 β and transforming growth factors α and β . There has been no direct demonstration that changes in expression of any of these genes are responsible for the toxic effects of TCDD-like compounds, but recent work indicates that some of the oestrogen-related genes and growth factors can play a critical role in the toxic action of TCDD (Yang *et al.*, 1999).

The effects of TCDD on sexual development and fertility have been extensively documented (Peterson *et al.*, 1993) and recent investigations have reported that TCDD can also compromise ovarian function. Human follicular fluid contains TCDD (Tsutsumi *et al.*, 1998) and the presence of a functional AhR has been shown in the ovarian tissues of several species, including rats (Son *et al.*, 1999), primates (Chaffin *et al.*, 1999), mice (Robles *et al.*, 2000) and cows (Fischer *et al.*, 2001). Exposure to TCDD is associated with significantly lower ovarian masses compared with controls in rats (Gao *et al.*, 1999; Son *et al.*, 1999), irregular oestrous cycle among rhesus monkeys (Allen *et al.*, 1977; Barsotti *et al.*, 1979) and loss of ovarian cyclicity in adult rats (Li *et al.*, 1995; Cummings *et al.*, 1996). Further investigations have shown that TCDD administration to rats before mating interrupts fertility via effects on ovulation (Giavini *et al.*, 1983; Chaffin *et al.*, 1996). Similar effects have been observed in hypophysectomized animals (Gao *et al.*, 1999) and after direct application of TCDD to the

Fig. 3. Outline of the arylhydrocarbon receptor (AhR) function as a ligand-activated transcription factor. (a) AhR resides in the cytoplasm (C) complexed with the AhR interaction factor (AIF) and two molecules of heat shock protein 90 (Hsp90). The ligand diffuses through the membrane from the extracellular side (E) and binds the AhR. (b) Ligand-bound AhR enters the nucleus (N) and dimerizes with the Ah receptor nuclear translocator (Arnt). (c) The AhR-Arnt complex binds the sequence GC/GTG specific for xenobiotic responsive element (XRE) and activates target gene transcription. Genes that are under control of the AhR belong to two major functional groups: (1) Genes coding for drug-metabolizing enzymes, the induction of which can alter the biotransformation activity for endogenous and exogenous substances, potentially leading to both beneficial and detrimental effects. (2) Aberrant regulation of genes that regulate cell proliferation and differentiation may underlie the toxic manifestations of many aromatic hydrocarbons.

ovary (Petroff *et al.*, 2000), indicating that the ovary can be a target organ for TCDD toxicity.

Polychlorinated biphenyls

PCBs are a group of halogenated aromatic hydrocarbons that consist of 209 isomers and congeners with

different numbers and positions of chlorine atoms substituted on the biphenyl moiety. PCBs were synthesized for approximately 60 years from the early 1920s until they were banned in many countries during the late 1970s. Uses of PCBs varied from closed-system applications in capacitors and transformers to open-system applications in the manufacture of adhesives, textiles and printing. Such a plethora of uses has facilitated the ubiquity of PCBs in the environment. It is estimated that 10^8 kg of PCBs still reside in the biosphere (Boyle *et al.*, 1992).

PCBs are small molecules (molecular weight 188–498) with a low solubility in water but high solubility in organic solvents, oils and fat (Ballschmiter *et al.*, 1989). As a result of this lipophilic nature, stability and resistance to degradation, PCB congeners can be found at all levels of the food chain and can accumulate in the animal and human body (Jones, 1988; McFarland and Clarke, 1989). Measurable concentrations of these compounds are found not only in the adipose tissue, but also in fluids of the female genital tract (Lindenau *et al.*, 1994). Trapp *et al.* (1984) demonstrated measurable concentrations of these chlorinated hydrocarbons in the follicular fluid of women and highlighted the potential threat to reproductive health posed by these contaminants. Measurable concentrations have been detected in human ovarian tissue (Mes *et al.*, 1990), embryos and fetuses (Nishimura *et al.*, 1977). It appears that PCBs have a systemic effect on reproduction with multiple targets, but the affected biological functions have not yet been identified precisely. Rodents exposed to PCBs experience a reduction in the number of germ cells, a decrease in weight of the reproductive organs, a reduced number of implantation sites, embryo toxicity and reduced litter size (d'Argy *et al.*, 1987; Linzey, 1988; Ronnback and de Rooij, 1994). Furthermore, a perturbing effect on the hypothalamic enzyme aromatase, involved in the control of sexual differentiation of the developing brain, has been found after prenatal exposure of rats to PCBs (Brevini-Gandolfi *et al.*, 1999a). Reproductive disorders have also been observed in non-human primates. Alterations of the menstrual cycle and increased incidence of abortions and embryo resorption are observed in monkeys exposed to Aroclor 1248 (Barsotti *et al.*, 1976). Some of these studies indicate direct and indirect effects of PCBs on ovarian function. For example, in rats exposed to the commercial mixture A-1242, a reduction in the number of follicles is observed (Jonsson *et al.*, 1975). In rhesus monkeys, administration of PCBs alters the menstrual cycle, induces amenorrhoea (Barsotti *et al.*, 1976) and inhibits ovulation in 50% of treated animals (Muller *et al.*, 1978).

Organochlorine pesticides

Organochlorine chemicals that have been identified as possible or definite endocrine disruptors are pesti-

cides such as DDT, lindane and methoxychlor (MXC). Generally, the chemical structure of organochlorine pesticides is based on a benzene ring with one or more chloride atoms attached. Like the other POPs described, the organochlorine pesticides are harmful to all living systems, due to their high affinity for fat tissue, and to their persistence in the environment. Their half-life is at least 20 years in both soil and water, and some soils retain up to 38% of the amount originally applied (Martijn *et al.*, 1993). This group of pesticides can bioaccumulate in the food chain due to their fat solubility (Clarkson, 1995). In the 1970s most industrialized countries banned compounds like DDT because of their ability to accumulate. However, in developing countries some of these pesticides are still in use, particularly for mosquito control, but also in general agriculture, due to high efficiency and relatively low cost. Environmental concentrations of residues were reduced after organochlorine pesticides were banned in industrialized countries, until an equilibrium was reached in the 1980s. These compounds are still present in the environment due to their resistance to biodegradation: the half-life of DDT and some of its metabolites can be more than 50 years. Body fat concentrations recorded in humans are still fairly high, particularly in developing countries (up to approximately 60 mg g^{-1} ; Rivero-Rodriguez *et al.*, 1997).

The adverse effects on reproduction of organochlorine pesticides are well documented. The effects of DDT in experimental animals include decreased fertility, abortion and stillbirths. Multigenerational studies in rodents show that DDT decreases general fertility and gonadal mass, increases the duration of the oestrous cycle, decreases the number of implantations, increases the rate of embryo mortality, decreases litter size and increases the duration of gestation (Coulston, 1985). MXC was developed to replace DDT and it is considered to be less toxic. However, reports demonstrate that it can influence the function of the reproductive system (Bal, 1984). For example, reduced litter size and increased rate of abortion is observed in rabbits and rats orally exposed to MXC (Khera *et al.*, 1978). Furthermore, long-term oral exposure of rats to MXC reduces maternal fertility and increases fetotoxicity, as well as reducing fertility in offspring (Cummings and Gray, 1987). Finally, female rabbits treated with lindane show a significantly reduced ovulation rate (Lindenau *et al.*, 1994). Observations indicate that these three toxicants are interfering mainly with oestrogen-mediated activities and recent studies indicate a direct effect on the ovary and oocyte.

On the basis of the studies described above, it is clear that POPs present a real danger and have a well-demonstrated negative effect on female reproductive efficiency. The specific effects that these chemicals exert on the oocyte will now be discussed.

Sites of ovotoxicity

Ovarian development

An important factor related to the effect of exposure to a reproductive toxicant is the developmental stages in the reproductive lifespan at the time of exposure. Temporary infertility may be manifest in adult cyclic females, but sterility might be produced by a chemical-induced destruction of germ cells during fetal development. The developing embryo and fetus, in fact, appear to be more sensitive than adults to endocrine disruption, and alterations in reproductive development are normally observed at a concentration much lower than that able to induce toxicity in adult animals. Rapidly dividing primordial germ cells and oogonia present during fetal development in all species are highly sensitive to destruction by a variety of environmental chemicals (Hoyer and Sipes, 1996). Ironically, it is the fetus that receives significant exposure or the greatest body burden of environmental POPs. As evidence of fetal exposure, offspring at birth have concentrations of TCDD that are up to 25% of maternal concentrations (Masuda *et al.*, 1978; Jacobson *et al.*, 1984; Koppe *et al.*, 1992). This uptake of polyhalogenated POPs by the fetus raises concerns about the potential for adverse health outcomes. To date, few studies have investigated the effects of prenatal exposure to persistent organic pollutants on ovarian development. *In utero* exposure to TCDD adversely affects reproductive function and anatomy in female rodent offspring, resulting in permanently reduced ovarian mass, decrease in the number of corpora lutea, premature ovarian senescence and early decline in fertility and fecundity (Silbergeld and Mattison, 1987; Gray *et al.*, 1997; Wolf *et al.*, 1999). Prenatal exposure to dioxin-like PCBs has been shown to reduce the number of germ cells in the ovaries by 40–50%. This decrease appears in all stages of oocytes and follicles and leads to premature reproductive ageing (Ronnbeck, 1991). The similarity between the effects noted after exposure to dioxin-like PCB congeners or TCDD (Smits-van Prooije *et al.*, 1992; Gray and Kelce, 1996) indicates that the arylhydrocarbon receptor might be involved in the reproductive effects observed. Matikainen *et al.* (2002) hypothesized that AhR has a prominent role in regulating apoptosis during female gametogenesis and is a possible mediator of fetal ovarian germ loss.

Oocyte maturation

Oocyte maturation is a critical prerequisite for subsequent fertilization and development. Thus, disruption of this process has considerable potential to impair female reproduction. Oocyte destruction by environmental chemicals requires that these compounds reach the ovary. After Trapp *et al.* (1984) reported

that various organochlorine persistent chemicals such as PCBs, PCDDs and DDT are present in the follicular fluid, the presence of PCBs was confirmed at a concentration ranging from 4.7 to 27 ng ml⁻¹ (Kholkute *et al.*, 1994) and similar concentrations have been found in human plasma (Swain, 1991; Ayotte *et al.*, 1997). Therefore, ovaries and follicles are exposed to environmental organochlorines as are most other organs in the body.

The ovotoxic potential of PCBs has been studied in different species using *in vivo* and *in vitro* models. However, very few studies have been conducted on the possible toxic effects of PCBs on mammalian oocytes. Preliminary observations indicate that human embryos derived from oocytes contained in follicles with increased pollutant concentrations have low cleavage rates after IVF (Trapp *et al.*, 1984).

Kholkute *et al.* (1994, 1997) tested the effects of PCB mixtures, such as A-1254 and 1268, on the *in vitro* maturation and fertilization of mouse oocytes. The results indicate that the addition of PCB mixtures to the maturation medium, at concentrations ranging from 0.01 to 10 µg ml⁻¹, affect the fertilizing capability of the oocytes. Exposure to A-1254 at a concentration of 10 µg ml⁻¹ failed to reveal any significant effect on the viability of the oocytes, although morphology and structural changes were not evaluated (Kholkute *et al.*, 1994). Furthermore, Greenfield *et al.* (1998) observed that A-1254 at the same concentrations did not affect the fertilization competence of exposed cumulus-free mouse oocytes, indicating a possible role of cumulus cells in PCB-induced toxicity.

At present, only two main studies have been conducted to evaluate the effects of PCBs on bovine oocytes. Krogenaes *et al.* (1998) demonstrated that the addition of PCBs 153 (non-coplanar) or 126 (coplanar) to the maturation medium has adverse effects on bovine oocytes. In particular, PCB 153 has no effect on maturation but reduces the percentage of oocytes able to complete the first mitosis after fertilization, whereas PCB 126 shows adverse effects on maturation only at the highest concentrations, but affects the subsequent embryo development also at lower levels of exposure. Pocar *et al.* (2001a) investigated the adverse effects of exposure of bovine oocytes during the maturation process to A-1254. This is a technical mixture of PCBs, the composition of which is considered to be environmentally relevant. A-1254 significantly decreases the percentage of oocytes that can reach metaphase II at concentrations as low as 0.01 µg ml⁻¹. This effect is probably caused by a block or a delay of the maturation process, as a significant percentage of the exposed oocytes is arrested at metaphase I. Furthermore, exposure to PCBs during maturation significantly decreases the fertilization ability of oocytes while increasing polyspermy. The negative effect of the addition of A-1254 to the maturation medium appears

not to be limited to maturation and fertilization, but includes embryonic development, as there is a significant decrease in the proportion of cleaved embryos reaching the blastocyst stage. Consistent with the results of Krogenaes *et al.* (1998), fertilization and embryonic development are affected by a PCB concentration ($0.001 \mu\text{g ml}^{-1}$) lower than that required for inducing the reduction of maturation rate. It is important to note that the concentration range used in these studies is comparable to that observed in the serum of non-exposed women (0.001 and 0.4 ng g^{-1} for PCBs 126 and 153, respectively; Johansen *et al.*, 1994), which, in turn, is similar to the concentrations of persistent organic pollutants in follicular fluid (Kimbrough, 1995). Moreover, PCB 153, as a major and very stable PCB congener, has been shown to correlate directly with the total amounts of PCBs (Pauwels *et al.*, 1999). However, at present, no data are available on the PCB concentrations present either in cattle serum or follicular fluid. Such concentrations, in fact, could be significantly different from those recorded in humans as a result of the difference of diet and digestive physiology between the two species. However, PCBs are not the only organochlorine compounds that induce oocyte toxicity in farm animals. Alm *et al.* (1998) studied the effects of *in vitro* exposure of bovine oocytes to DDT, lindane (hexachlorocyclohexane or gamma HCH) and MXC. All of these pesticides affect maturation rate in a dose-dependent manner, but to different extents. The higher concentrations of pesticides are associated with higher rates of chromatin degeneration, up to 50%. Furthermore, DDT and gamma HCH concentrations that do not decrease fertilization rates induce a significant reduction of embryos developed to the morula and blastocyst stage by days 7 and 8 after IVF, the commonly used parameter for normal development *in vitro*. This result is similar to that observed in the experiments with PCBs but did not occur with MXC.

The observation that for PCBs, DDT and gamma HCH, a low-dose effect on oocyte developmental competence is delayed and visible only at the later stages of embryo preimplantation development is of particular significance because it indicates that the analysis of all phases of development is necessary for the full assessment of the risk posed by these compounds.

All of the studies described evaluated the toxic effects of only one class of POPs at a time, an experimental approach essential for understanding the mechanism of action of each POP. However, Campagna *et al.* (2001) used an environmentally relevant mixture of more than 15 organochlorines (including PCBs, DDT, its metabolite DDE, and lindane) to investigate the effect of these chemicals on *in vitro* maturation of pig oocytes. This study has a particular ecological significance as mixtures, not individual compounds, have been used industrially and have accumulated in the environment. An environmentally relevant mixture provides a better representation

of conditions because it contains components that can interact with each other in an additive or non-additive fashion (synergistic or antagonistic), and can interact with different receptors and molecular pathways. Campagna *et al.* (2001) demonstrated that exposing pig oocytes to an organochlorine mixture during *in vitro* maturation negatively affects the maturation rate, without increasing degeneration rate. Developmental competence of the exposed oocytes is also affected, reducing blastocyst rate and quality in a dose-dependent manner. Furthermore, there is a decrease in the quality and viability of cumulus cells, which may account for reduced maturation and developmental competence.

Mechanisms of ovotoxicity

The intracellular mechanisms involved in organochlorine ovotoxicity are only beginning to be understood. In the studies described, maturation stages were determined by the evaluation of nuclear morphology. However, this evaluation did not allow for assessment of cytoplasmic maturation, a process through which immature oocytes acquire the competence to be fertilized and to sustain embryo development. Cytoplasmic maturation is characterized by ultrastructural and spatial rearrangements of the ooplasm as well as by chemical changes of the molecules stored therein (Hyttel *et al.*, 1997; Brevini-Gandolfi and Gandolfi, 2001). For this reason, a set of experiments was designed to examine the effects of the exposure of bovine oocytes to a dose of A-1254 known to be detrimental to nuclear maturation and embryo development, on two important aspects of cytoplasmic maturation: modulation of maternal mRNA polyadenylation (Brevini-Gandolfi *et al.*, 1999b) and migration and exocytosis of cortical granules (Damiani *et al.*, 1996).

Alteration of mRNA stored in the ooplasm

It is generally accepted that mRNA and protein molecules synthesized during oocyte growth and maturation contribute to early development before embryonic genome activation (Telford *et al.*, 1990). The storage of mRNA takes place during oocyte growth and the extent of poly(A) tail at the 3' end of the transcripts (Fig. 4) has emerged as an important regulatory element for determining stability (Richter, 1996) and represents a key regulatory step for early embryonic development (Vassalli and Stutz, 1995; Brevini-Gandolfi *et al.*, 1999b). Results show that A-1254 induces changes to the polyadenylation pattern of five out of ten genes examined, indicating a perturbing effect exerted by this contaminant on the translational regulation of these transcripts. However, PCB action on polyadenylation seems to be different from that of other factors affecting oocyte competence. For instance, when reduction of developmental competence is induced by removing gonadotrophins

Fig. 4. Post-transcriptional regulation of gene expression relies on two main modulatory systems: targeted degradation of mRNA and control of translation initiation and activity. The half-life of individual mRNAs may vary from a few minutes to many days and depends on degradation by specific exonucleases. RNA molecules are protected from the attack of these enzymes by a 5' methylguanosine cap structure (5' Cap) and a 3' polyA tail of adenosine residues (3'AAAAAAn), which together interact in controlling mRNA stability and decay. Shortening of the polyA tail and 5' decapping allows exonucleolytic degradation of the mRNA and is followed by rapid decay of RNA molecules. PolyA tail elongation, by contrast, prevents enzymatic degradation and increases mRNA stability. 5' and 3' ends are also involved in the regulation of translation: mRNAs with high base homology at the two ends form stable secondary structures that prevent access to the translation start site, causing very inefficient translation. Translational activity is conversely enhanced by polyA tail elongation that causes binding of a polyadenylation binding protein PABP to a specific translation factor eIF4F.

from the maturation medium, only those transcripts that would normally undergo deadenylation during the maturation process display an alteration of the poly(A) tail. Transcripts that either become adenylated or do not change during maturation are not affected by the absence of gonadotrophins (Brevini-Gandolfi *et al.*, 1999c). On the contrary, exposure of oocytes to A-1254 during *in vitro* maturation induces changes in polyadenylation in a more varied way: as described for gonadotrophins, PCBs induce a more pronounced deadenylation of some of the genes that would deadenylate in control conditions (that is, glucose transporter type 1, connexin-43 and plakoglobin); however, at the same time, a longer poly(A) tail is observed at the 3'-end of connexin-32, a gene that normally readenylates during maturation. Finally, another pattern has been observed for heat shock protein 70, which instead of undergoing a deadenylation process as in control conditions, shows an extension of the tail at the end of *in vitro* maturation (Pocar *et al.*, 2001b).

Fig. 5. The cortical granule reaction. Cortical granules are specialized membrane-bound, secretory granules responsible for the slow block of polyspermy in mammalian zygotes. The content of cortical granules makes the zona pellucida 'refractory' to sperm penetration. During oocyte maturation and fertilization, cortical granules follow a characteristic pattern of migration that has been considered as a valuable index of the cytoplasmic maturation and developmental competence. (a) Initially, cortical granules form big clusters distributed throughout the cytoplasm. (b) Upon the resumption of meiosis, the granules become localized in small clusters at the periphery of the ooplasm, until (c) they form a layer of single granules just under the membrane, at the time of ovulation. (d) Finally, the exocytosis of the granules occurs after interaction with the spermatozoa. (Reproduced with permission from Pocar *et al.*, 2001b.)

Disruption of cortical granule redistribution

A common attribute of cytoplasmic maturation is the migration and redistribution of organelles, including cortical granules, in temporal co-ordination with the nuclear stages (Fig. 5). Most oocytes exposed to A-1254 exhibit delayed migration and dispersal of cortical granules, indicating impaired cytoplasmic maturation. In addition, a significantly higher percentage of fertilized oocytes fail to release cortical granules after sperm penetration and present multiple fertilization after IVF (Pocar *et al.*, 2001b). A similar study conducted on cumulus-free mouse oocytes found that exposure to A-1254 during maturation does not allow spontaneous exocytosis of cortical granules (Greenfeld *et al.*, 1998). It is possible that PCBs block the molecular pathways that trigger exocytosis of cortical granules and consequentially cause polyploidy, as the slow block to polyspermy is not created.

Recent *in vitro* studies have demonstrated that POPs can affect viability and steroid production of follicular

cells. As oocyte development and maturation occur within the follicle, endocrine disruptors interfering with steroid production can interfere with ovulation.

TCDD is capable of disrupting human ovarian steroid production by acting directly on the follicular granulosa cells, reducing oestradiol secretion and increasing apoptosis. Furthermore, TCDD may alter cell membrane permeability, disrupting steroid secretion (Heimler *et al.*, 1998). Hirakawa *et al.* (2000) reported that TCDD, at concentrations considered environmentally relevant (10 pM), significantly downregulates the FSH receptor in rat granulosa cells, indicating that the effects of TCDD on steroidogenesis may be attributable to downregulation of the gonadotrophin receptor. Recent data on farm animals show that TCDD decreases both progesterone and oestradiol secretion by granulosa cells in pigs, in a dose-dependent manner, by altering the activity of enzymes involved in the steroid biosynthesis cascade (Gregoraszcuk, 2002). The same study indicates the presence of cross-talk between the AhR and the oestrogen receptor pathways in follicular cells in pigs. Two recent studies investigating the effects of PCBs in follicular cells in pigs have revealed that PCBs 126 and 153 have dioxin-like and non-dioxin-like activity, respectively (Wojtowicz *et al.*, 2000, 2001). Both PCBs 153 and 126 alter steroid secretion in follicular cells. PCB 153 decreases oestradiol secretion and increases progesterone secretion, indicating that it causes the disruption of the aromatization process. In contrast, PCB 126 is able to induce only the increase of progesterone with no effect on oestradiol secretion by granulosa cells. This finding differs from that previously observed for TCDD, indicating the presence of a different mechanism of action, at least in part. In agreement with this hypothesis, recent investigations indicate that AhR is not directly involved in the effects exerted by PCB mixtures on bovine cumulus-oocyte complexes (Fischer *et al.*, 2001). Despite cumulus granulosa cells expressing the AhR signalling components, exposure to A-1254 during the maturation period is unable to activate the AhR signal transduction pathway. These findings are consistent with previous observations in mice demonstrating that *in vivo* exposure to different well-known activators of the AhR (the polycyclic aromatic hydrocarbons, benzo(a)pyrene, 3-methylcholantrene and 7,12-dimethylbenz(a)anthracene) induces oocyte destruction in the ovary of both Ah-responsive and Ah-non-responsive strains (Mattison and Nightingale, 1982).

Conclusions and future directions

The published data clearly indicate that POPs (singly or in combination) disrupt mammalian oocyte maturation and follicle physiology even at very low concentrations. However, most of the data presently available derive from experiments performed on laboratory species or *in vitro* models; therefore, extrapolations to other species

or situations should be done with caution. The lack of information on the metabolism and tissue distribution of these chemicals, which greatly depend on species physiology, concentrations and duration of exposure, as well as interactions between single components of the complex mixtures present in the environment, means care should be taken before final conclusions are drawn. Moreover, the specific cellular pathways activated by these compounds are still unclear. Therefore, the search for the specific mechanisms involved in the ovotoxicity and metabolism of POPs should be the focus of further research in this field. In addition, future studies should systematically identify environmental chemicals that can disrupt normal development and function of the reproductive system. Currently, only about 60 environmental pollutants have been identified as endocrine disruptors (Colborn *et al.*, 1993; Toppari *et al.*, 1996) and most of these have been identified accidentally, rather than as a result of an exhaustive screening process. Such widespread screening of all potentially toxic compounds will require the identification of appropriate biomarkers to be used for risk assessment in mammals, and the development of relevant *in vitro* markers of reproductive toxicity.

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