Review

# Use of ovary culture techniques in reproductive toxicology

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A B S T R A C T

There is increasing evidence to indicate that a substantial number of both man-made and naturally occurring chemicals are disruptive to human and wildlife reproductive health. Currently, reproductive toxicology testing is primarily carried out *in vivo*, however, in the past 50 years, various culture methods have been developed with the aim of growing ovarian follicles *in vitro*. These culture systems have become a widely used tool in reproductive biology and toxicology. In this review we describe how reproductive toxicology of the ovary is greatly enhanced by *in vitro* studies. Experiments using *in vitro* ovarian cultures to understand or detect damage to the ovary itself and to its specialised structures of the follicles and oocytes, allows for faster screening of potential developmental and/or reproductive toxicants.

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## 1. Introduction

### 1.1. The ovary

The ovary is central to female reproductive function, the site within which germ cells form follicles, develop and mature (Fig. 1). These cells communicate with each other and with ovarian stromal cells. Mammalian oocytes develop from primordial germ cells during gestation [1]. Following the proliferative stage, primordial germ cells then enter a pre-meiotic state of DNA replication before entering prophase I of meiosis. They then progress through the initial stages of meiosis, before entering meiotic arrest, around the time of follicle formation (Fig. 2) [2,3]. The oocytes remain in this meiotically arrested state throughout the phase of follicular development, thus the growing ovarian follicle contains an oocyte arrested in prophase I of meiosis, surrounded by somatic cells (granulosa and theca cells) as well as a basement membrane (BM). In the postpubertal ovary in particular, as an oocyte grows and matures, its follicle undergoes changes due to proliferation of the granulosa cells and formation of the fluid filled antral cavity, resulting in a dramatic increase in follicle size. Once it has reached full maturation, the pre-ovulatory, Graafian follicle expels its oocyte during ovulation, at which point the oocyte exits meiotic arrest and completes meiosis I.

The ovary is not only responsible for producing oocytes, but is also an important endocrine gland, the source of sex steroids which link reproductive and non-reproductive organs to the timing of the ovarian cycle. It is in the growing follicle that the majority of estrogens in the body are produced and once the oocyte has been ovulated, the remainder of the follicle becomes a corpus luteum (CL), a temporary endocrine structure secreting the progesterone critical for the establishment and initial maintenance of pregnancy. The ovary is responsive to hormones secreted from the anterior pituitary, in turn controlled by the hypothalamus, with which it is locked into a complex cyclical pattern of communication and feedback that underpins successful female reproduction. Ovarian follicles are dependent on both external and internal hormones for their growth, development, maturation and ovulation. Follicle growth through the primordial, primary and secondary stages is gonadotropin-independent, regulated primarily by oocyte-derived factors such as growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and local somatic-derived factors such as anti-Mullerian hormone (AMH) [4]. Once a follicle transits into the pre-antral stage, its growth to the early-antral stage is gonadotropin responsive, with further growth dependent on gonadotropins [5].

In recent years, both environmental and synthetic pharmaceutical compounds with endocrine-mimicking, -modulating or -inhibiting ability have become an increasing health concern. These compounds have reported harmful effects on gamete development and on the developing foetus and neonate [6]. Their relevance to human pregnancy has been identified in a recent opinion paper from the Royal College of Obstetricians and Gynaecologists (RCOG) which also highlighted the issue of exposure to multiple sources of chemicals. That paper outlined the potential reproductive hazards associated with exposure to the developing foetus of chemicals with the potential to interfere with foetal germ cells in the developing ovary, the effects of which would only manifest in the F2 generations, thus not becoming evident until decades later [7].

Various toxicological studies have been carried out on different animal models both *in vivo* and *in vitro* in order to investigate their effects on the female reproductive system [8]. Both types of toxicology testing have advantages and limitations, which must be carefully considered when planning a toxicological study. Here, we review the role of *in vitro* studies in the examination of toxicological effects on the ovary, comparing results from such methods to the effects of 'real-life', *in vivo*, exposure.

## 2. Toxicology and reproductive function

The environmental toxicants and pharmaceuticals of concern regarding reproductive function are from a broad spectrum of chemicals. One group in particular, endocrine disrupting compounds (EDCs), constitute a major focus. EDCs have been described by the United States Environmental Protection agency (USEPA) as agents that 'interfere with synthesis, secretion, transport, binding or elimination of natural hormones in the body that are responsible for maintenance of homeostasis, reproduction, development and/or behaviour' [9]. Pharmaceutical and chemical companies produce novel chemicals in the form of new drugs, and widely used industrial and agricultural compounds, which can, in some cases, act as EDCs [10]. Humans are exposed to thousands of these natural or man-made chemicals throughout their lifespan [6,10–12]. Some are ingested as drugs or absorbed through the skin *via* beauty products such as soaps and perfumes [10], whereas others can leach out of plastic or be inhaled from cigarette smoke, pollution or vehicle exhausts (Table 1).

There is increasing evidence suggesting that certain pharmaceuticals and EDCs have the potential to interfere with endocrine function, biosynthesis or homeostatic control, alter reproductive development and fertility and result in reproductive disorders [13,14]. Reproductive toxicants can interfere with endocrine mechanisms due to their weak intrinsic hormonal activity, most often by mimicking or inhibiting estrogens through binding to nuclear, membrane, neurotransmitter and/or orphan receptors.
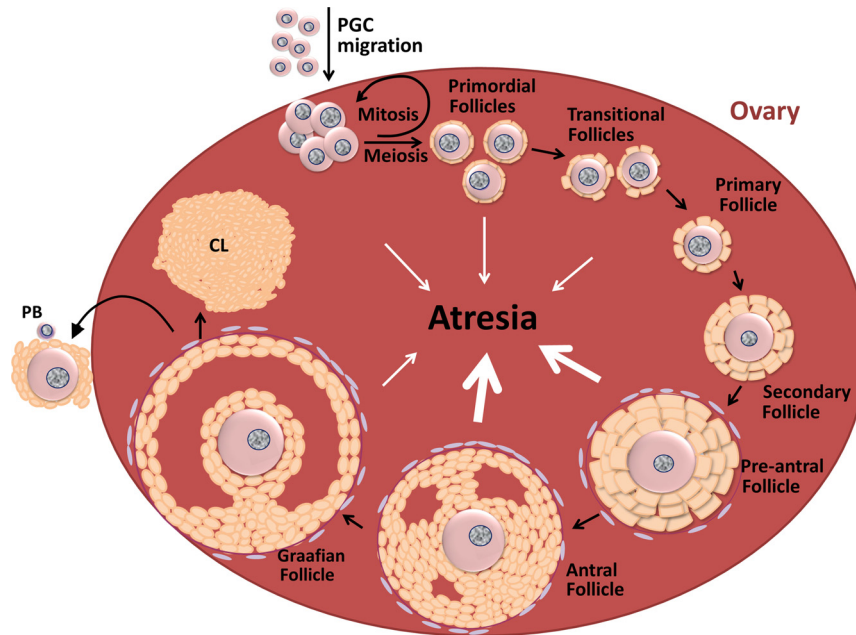
**Fig. 1.** Follicle growth in a human ovary. Proliferating primordial germ cells (PGCs) migrate to, and invade the developing ovary to form germ cell nests. They proliferate at a high rate, then enter meiosis and form primordial follicles as the germ cell nests break down. Throughout the reproductive lifespan, small cohorts of primordial follicles are continually released from dormancy as they enter the growing pool. The vast majority of growing follicles are lost to atresia, but beginning at puberty, a few follicles grow to the Graafian stage, normally resulting in the release of one oocyte each menstrual cycle. The remainder of the ovulatory follicle forms the corpus luteum (CL).
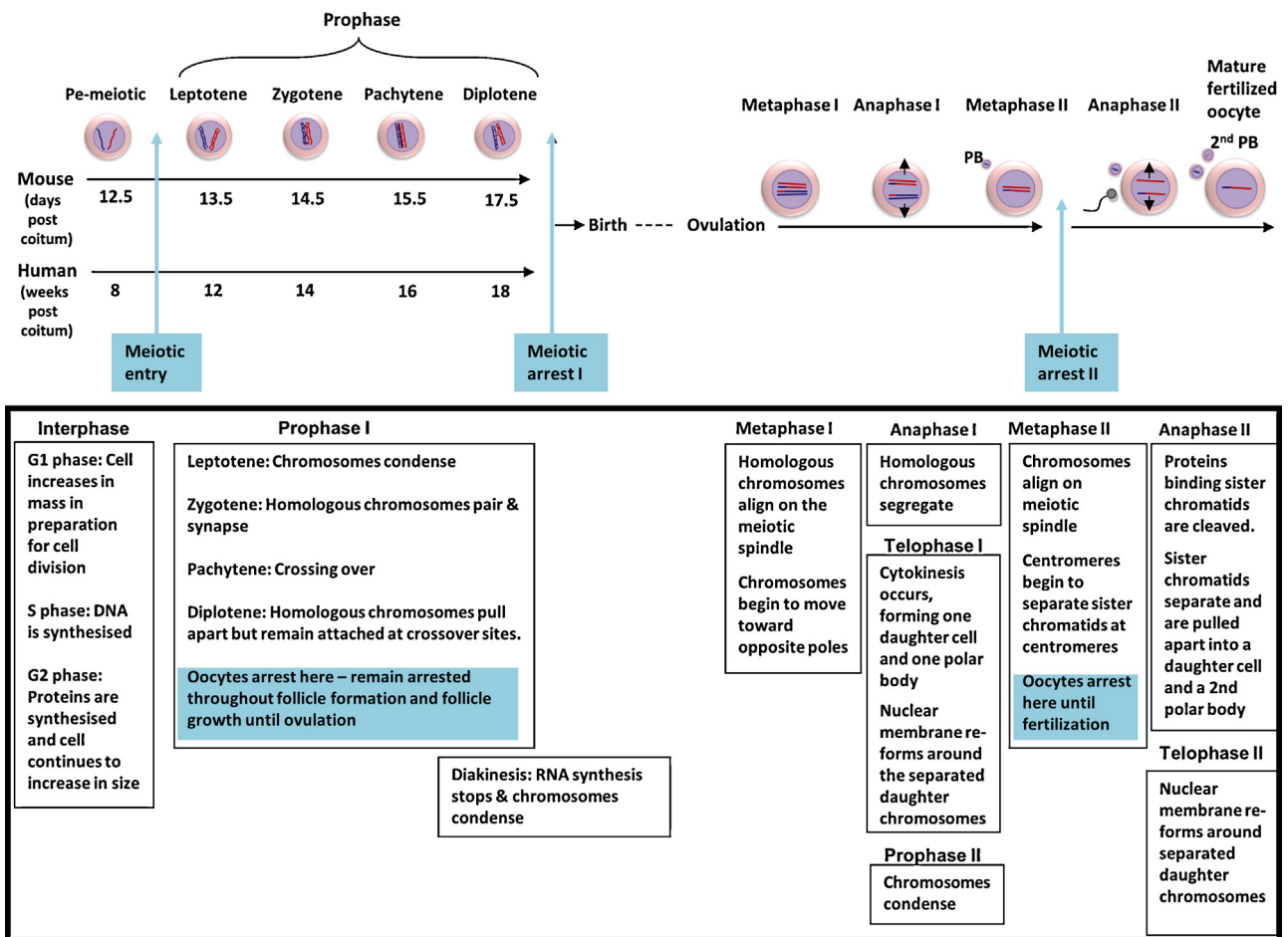


**Fig. 2.** Meiosis in oocytes. Stages and phases of mammalian meiosis, including two meiotic arrests during prophase I and metaphase II. The figure demonstrates chromosomal behaviour during meiotic progression in the mouse and human, with the time-course of events leading to the formation of an arrested oocyte within the growing follicle and ultimately, a mature fertilised oocyte.

**Table 1**
Overview of some of the most common EDCs and their sources.

| EDCs | Exposures and uses | References |
| --- | --- | --- |
| Alkyl phenol | Detergents, cosmetics, paints | [15] |
| Bisphenol A (BPA) | Polyester–styrene resins, plastic food containers, baby bottles, eating utensils | [16] |
| Chlordane | Insecticide | [16] |
| Dioxins | Paper manufacturing, herbicides, incinerators | [16] |
| DDT and MXC | Organochlorine pesticides | [8] |
| Heavy metals (cadmium, lead, mercury) | Batteries, pigments, pharmaceuticals, plastic stabilisers, burning of fossil fuels and the incineration of municipal waste | [16,17] |
| Phthalates | Soft plastic chew toys, nail varnish, perfumes, flavourings and solvents | [16,18] |
| Phyto-estrogens | Soy, grains | [16] |
| Polybrominated diphenyl ethers (PBDEs) | Flame retardants | [16] |
| Polychlorinated biphenyls (PCBs) | Flame retardants, adhesives, electrical transformers | [15,16] |
| Polycyclic aromatic hydrocarbons (PAHs) | Industrial air pollution, coal and coke burners, tobacco tar | [16] |
| Trichlorfon (TCF) | Organophosphate insecticide | [19] |
| Triclosan | Antibacterial agent in personal care and household products | [20] |
| 4-Vinylcyclohexene diepoxide (VCD) | Occupational chemical, reactive diluent for diepoxides and epoxy resins | [21] |

## 2.1. Female reproductive toxicity

Given that reproductive toxicology research is carried out not only *in vivo*, but also using *in vitro* cultures [22], the main focus of this review is to examine and compare effects of compounds that have been tested both *in vivo* and *in vitro*. The majority of data reviewed will refer to EDCs since they have been more widely reported in female reproductive toxicology research in both *in vivo* and *in vitro* studies. In contrast, there is relatively little information on the effects of pharmaceutical compounds *in vitro*, other than in the examination of the effects of chemotherapy agents on the ovary. Chemotherapy agents can, none-the-less, have very important effects on the ovary [23,24].

Human epidemiological studies have shown an association between exposure to EDCs during development and adverse health outcomes in females [13,25–28]. For some chemicals, EDCs have the ability to disturb crucial pathways within the hypothalamic–pituitary–ovary axis at all stages of life: pre-natal, pre-pubertal and adulthood [13,22,29] (Table 2). However, certain periods of reproductive life are more susceptible to harm by EDC exposure than others. For example, foetuses exposed to estrogenic products through soy consumption of the mother and subsequently transferred *via* the placenta are more vulnerable than those exposed as adults, particularly if exposure to the estrogenic agents in soy products is at the critical period of germ cell nest breakdown and entry of PGCs into meiosis [30,31]. Between 1940 and 1970, pregnant women at risk of miscarriage were prescribed the xeno-oestrogen diethylstilbestrol (DES) [32]. Foetuses are exposed to endogenous oestrogen throughout pregnancy, but girls exposed to DES *in utero* during that critical period of reproductive tract development developed reproductive tract abnormalities and also had increased incidence of cervical–vaginal cancer later in life [33]. Any reproductive toxicant that has a direct effect on the ovary may also be able to alter epigenetic mechanisms in the oocyte, resulting in trans-generational epigenetic effects [31,34–37]. Therefore, subtle modifications to gene expression independent of gene mutations are entirely possible. An example of this type of germline transmission of an epigenetically modified trait is suggested in vinclozolin-treated rats, although the results are somewhat controversial [37–40].

### 2.1.1. Mechanisms of action of reproductive toxicants

Pre- and post-natal ovaries contain large numbers of germ cells and follicles at various stages of development. Pre-natally, this includes the rapidly proliferating primordial germ cells (PGCs) and oocytes undergoing the first meiotic division. Drug-induced damage at this time markedly reduces the post-natal follicle pool [41]. The mature ovary contains primordial follicles with all potentially viable oocytes in meiotic arrest, dividing granulosa cells and maturing oocytes of growing follicles, as well as ovulating oocytes resuming meiosis. The ever-changing environment must be carefully considered when studying the effects of environmental chemicals on the ovary, as follicles in different stages of growth may well vary in their susceptibility to different compounds.

Following follicle formation and growth initiation, ovarian follicles enter a period of continuous development until they either undergo atresia or develop to the Graafian stage, accompanied by rapid granulosa cell proliferation. That continual growth state, accompanied by the meiotic arrest of the oocyte for up to 45 years in humans, makes them particularly vulnerable targets for reproductive toxicants. The somatic cells and the BM of the follicle can be thought of as a protective sheath enclosing the oocyte, but this does not necessarily protect it from the effect of mutagens, directly or indirectly. The majority of toxic compounds are able to access the ovary *via* the circulation, but if these toxicants are able to pass through the BM, then the oocyte can also potentially be affected. Even if they directly affect only granulosa or thecal cells, they are still able to affect the oocyte indirectly. Once in the ovary, reproductive toxicants may be further metabolised, which may reduce or increase the risks, depending upon the metabolites generated [42].

Compounds targeting the primordial pool can have highly adverse effects on fertility, with broader consequences than those targeting growing follicles. If the primordial pool of follicles is damaged, future follicle growth and ovulation will be affected. At worst, a chemical that interferes markedly with the resting pool will likely result in premature ovarian insufficiency (POI) by depleting the ovarian reserve of primordial follicles [43]. In contrast, if a compound specifically targets growing or pre-antral follicles, perhaps by affecting dividing granulosa cells, these follicles will undergo atresia, resulting in cyclic disturbances for the few months following exposure to the compound. However, once the compound and its effects are removed, new follicles (from the unaffected resting pool) will begin growing and form normal ovulatory follicles, thus restoring fertility [44]. Effects on growing follicles can, though, have long-term consequences: studies into the effects of chemotherapy on the ovary are providing growing evidence that repeated damage to growing follicles can have a severe effect on the primordial pool, as the loss of growing follicles leads to premature activation of primordial follicles and consequently, a depletion of the primordial follicle pool [24,45–47].

There are several other ways in which chemicals can disrupt oocyte development. During meiotic progression of the oocyte, chromosomes utilise a bipolar spindle for their segregation during both meiotic divisions. If disturbed, this will likely lead to impairment in chromosome pairing or spindle formation, resulting in non-disjunction [48]. Regulation of progression of the cell cycle is tightly controlled by feedback mechanisms that sense disturbances

**Table 2**
Overview of associations between EDCs and female reproductive dysfunction.

| Chemical | Effect | Species | References |
|---|---|---|---|
| Bisphenol A (BPA) | Disturbances in spindle formation and chromosome alignment | Mouse | [51,53] |
| | Multioocyte follicles | Mouse | [57] |
| | Increased length of estrous cycle | Mouse | [68] |
| | Cessation of estrous cycle | Rat | [69] |
| Cyclophosphamide (Cy) | Disrupted ovarian folliculogenesis and altered steroid production | Rat | [70] |
| | Decreased E2 levels in plasma and reduced uterine weight | Mouse | [71] |
| | Destruction of antral follicles | Mouse | [72] |
| | Reduced ovarian follicle numbers | Mouse | [41] |
| Cadmium | Reduced germ cell population, retarded germ cell migration and poor gonadal development | Mouse | [73] |
| Diethylstilbestrol (DES) estrogenic | Girls exposed *in utero* had increased cervico-vaginal cancers, decreased fertility and POI. | Human | [74–76] |
| | Meiotic spindle defect in Meiosis II | Mouse | [50] |
| | Neonatal exposure results in the absence of corpora luta, hypertrophy of interstitial ovarian tissue and haemorrhagic cysts | Mouse | [77] |
| | Perinatal exposure induces polyovular follicles | Mouse | [78] |
| Genistein | Neonatal exposure causes prolonged and abnormal cycles in adult animals | Mouse | [79] |
| | Neonatal exposure causes dysfunction of post-pubertal reproductive performance and abnormal development of female gonads | Rat | [56] |
| | Reduced progesterone synthesis | Porcine | [80] |
| Methoxychlor (MXC) | Inhibition of folliculogenesis and increased AMH production in ovary | Rat | [81] |
| | Inhibited implantation and delayed embryogenesis | Mouse | [82] |
| | Suppressed LH surge and irregular estrous cycles | Rat | [83] |
| | Decreased sex steroid hormone levels | Mouse | [84] |
| Phthalates (DEHP/MEHP/BBP) | Prolonged estrous cycles, reduced serum estradiol levels and absence of ovulation | Rat | [85] |
| | Increased estradiol and progesterone production, leading to mid-gestation abortions | Rat | [86] |
| Polychlorinated biphenyls (PCBs) | Increased blastocyst loss | Rabbit | [87] |
| Trichlorfon (TCF) | Meiotic delay, nondisjunction and polyploidy | Mouse | [19,88] |

and by checkpoint controls that protect the cell from such errors and ensure that aneuploidy is prevented [49]. Failure during these meiotic checkpoints can result in meiotic errors, and the resulting mutations introduced to the genetic material have the potential to be passed on to the subsequent generation. Studies have been carried out on the possible effect of environmental chemicals on different stages of meiosis, including chromosome synapsis, recombination events and chromosome segregation [50,51]. These studies illustrated that environmentally relevant doses of bisphenol A (BPA) and DES have the ability to interfere with the actions of oestrogen receptors (ERs) [52] and cause abnormalities in the alignment of chromosomes and spindle formation [50,53]. Estradiol (E2) inhibits germ cell nest breakdown and protects oocytes from programmed cell death. Germ cell nest breakdown is an important process and may contribute to elimination of germ cells with genetic anomalies [54]. Endocrine disrupting chemicals are likely to interfere with this process, and studies looking at the actions of E2, progesterone and genistein on the newborn rodent ovary found that they all inhibit cyst breakdown, with binuclear oocytes and multioocyte follicles (MOFs) reported as a result of EDC exposure [55]. MOFs are often used as an indicator of an adverse effect, as they are considered a likely result of disruption to germ cell nest breakdown [31,55–60]. Although the vast majority of oocytes affected by chemicals are likely to end up becoming atretic [61], some might form aneuploid ovulated oocytes, with the consequent potential of an aneuploid embryo and likely miscarriage.

Reproductive toxicants might either target the oocyte specifically, or have more general effects on the surrounding somatic cells. In either scenario, reproductive disorders can occur. The follicle is a complex structure relying on interactions between the oocyte and its somatic cells [62–66]. Granulosa and theca cells are responsible for hormone production within the ovary as well as controlling the release of oocytes throughout the adult reproductive lifespan. This is mainly regulated through the expression of autocrine and paracrine factors, creating intricate feedback loops within the follicle that are essential for normal follicle development

and for meiotic competence of the oocyte. Due to this complex communication network formed by the oocyte, granulosa cells and theca cells, together driving follicle development, a chemical affecting any one of these components will affect the whole follicle [64]. These chemicals can also interfere with the feedback loop between the ovaries and pituitary gland to perturb the balance of the hypothalamic–pituitary–gonadal axis. Disruption can, therefore, not only have negative effects on follicle development, oocyte maturation and ovulation, but also significantly impair fertility by affecting the production of ovarian hormones [67].

## 3. *In vitro* culture systems as a tool for studying reproductive toxicology

Over the last half century, various culture methods have been developed with the aim of growing follicles from an immature state to fully mature, fertilizable oocytes [89–98]. These cultures have become a widely used tool to study the development of follicles in reproductive biology and toxicology and have been successfully established in mouse, rat, cattle, sheep, pig, primates and humans [99–104]. In 1989, the first successful *in vitro* pre-antral follicle culture in rodents was carried out, leading to the birth of live pups from these cultured pre-antral follicles [90]. Subsequently, many *in vitro* cultures have been designed with the aim of growing individual follicles, or whole ovaries, at varying stages of development. These cultures include short- and long-term methods, individual pre-antral follicle cultures, granulosa cell–oocyte complexes, co-cultures and whole ovary cultures, depending on the endpoints required for the study [90,105]. Cultures spanning earlier stages of ovary development have proven more challenging, and although a few studies have managed to create the culture conditions necessary to produce live pups from immature cultured follicles [91,92,94,95,98,106], achieving this using cultured oocytes from a pre-meiotic stage has, to-date, been unsuccessful without major manipulation of the follicles [94]. The details of each culture system are outside the scope of this review, but for extensive

reviews on commonly used culture systems see for example one of: Hartshorne [107], Murray and Spears [105], Devine et al. [108], or Picton et al. [109].

*In vitro* ovary and follicle culture models allow for the possibility of varying culture parameters in a highly controlled manner, and thus have the potential to allow a more thorough evaluation for reproductive toxicity studies than do *in vivo* studies alone. Potential endpoints include studying the mechanisms of action of toxicants and how they contribute to oocyte or somatic cell damage, analysis of oocyte quality, effects on the establishment of the primordial follicle pool, and paracrine interactions [110]. Culture systems have the potential to reveal whether the effects of the toxicants directly affect the ovary, with effects observed *in vivo* but not *in vitro* are presumably indirect. The cultures can also reveal whether compounds target follicles at specific stages of development and enable deeper insight into the way toxicants might affect the chromosomal integrity of the oocyte, or if they have the ability to alter hormonal signalling within and/or between follicles.

The culture methods available for toxicological research vary in terms of species, follicle stage, time-course, and composition of culture media. Each culture system has its own pros and cons, all requiring careful consideration before choosing the best method for a toxicology study.

### 3.1. Embryonic ovary culture

The majority of available culture systems involve the post-natal ovary and germ cells that have already entered meiotic arrest, but the foetal stage of reproductive development is a crucial and sensitive period. It includes migration of germline stem cells, their proliferation, and subsequent entry into the first meiotic division, together with proliferation and differentiation of the somatic compartment and the interaction between the germ and somatic cells. Pre-meiotic germ cells, not enclosed in follicles may well be directly responsive to chemicals, making foetal development a particularly vulnerable period [41,111]. A culture system whereby embryonic mouse ovaries, containing oogonia undergoing the first meiotic division, can be cultured intact would have the potential to provide an important contribution to the field of reproductive toxicology, particularly as aneuploidy predominantly occurs during the first meiotic division of oocytes [112–114]. Although the development of rodent oocytes from pre-meiotic foetal germ cells has been attempted [3,89,93,115–118], there has been relatively little success in culturing them through both meiotic divisions to produce mature oocytes, other than through the use of invasive techniques such as nuclear transfer [94]. An embryonic culture technique has the potential to be a great contribution to the field of reproductive toxicology testing, due its capacity to demonstrate direct effects of compounds on the foetal ovary, proliferation, entry into meiosis and the establishment of primordial follicle pool and early stages of developing follicles.

### 3.2. Rodent neonatal ovary culture

In rodent ovaries, primordial follicle assembly occurs around the time of birth. Abnormalities in primordial follicle formation and growth could potentially lead to reproductive problems later in life, such as POF and infertility. The process of follicle formation and the subsequent initiation of follicle growth are two separate processes both covered by the culture of neonatal rodent ovaries [108]. The neonatal ovary culture involves culturing of a whole rodent ovary from after birth for up to 20 days [91,92,95,119,120]. It is an appropriate culture system for studying the biology of primordial follicle assembly and the primordial-to-primary follicle transition, and is

thus a valuable asset to toxicological research to identify potentially hazardous compounds that could interfere with these processes.

### 3.3. Follicle or cumulus–oocyte-complex cultures

For culture of individual follicles, the required follicles are isolated from their surrounding stroma and cultured to allow examination of growth, development and metabolism away from systemic influences. Whole pre-antral follicles can be isolated from the ovaries of immature rodents and are able to survive in culture for up to 12 days, growing from early pre-antral stages to large Graafian follicles which can then be ovulated *in vitro* [91,104,110,121]. This culture system has yielded live pups from cultured pre-antral follicles [91] and is an important culture system for toxicology, as not only does it span the second meiotic division of the oocyte, but it also covers the entire antral stage of follicle development, including ovulation. It also allows for more detailed investigation into the paracrine interactions between the oocyte and the surrounding somatic cells and can also be used to examine interactions between follicles [66,122,123].

## 4. Comparison of *in vivo* and *in vitro* effects of pharmaceuticals and EDCs

As outlined above, chemicals that are suspected to have endocrine disrupting properties include plasticisers (BPA, phthalates, alkylphenols), polychlorinated biphenols (PCBs), organochlorine pesticides (DDT and MXC), flame retardants, parabens, perfluorinated compounds, synthetic hormones used as pharmaceutical drugs as well as the naturally occurring endocrine active substances, such as isoflavones. Despite several cases where environmental toxicants are thought to have influenced reproductive function in various wildlife species [124], as well as a number of *in vivo* exposure studies showing a potential link between environmental compounds and reproductive abnormalities [14,22,27,35,125,126], it remains difficult to prove a causal relationship. Regardless, environmental toxicants are still viewed as contributing factors to diseases that are oestrogen-dependant, including breast cancer and endometriosis [26,127]. Recent *in vitro* research on laboratory animals also strongly point towards the ability of different chemicals to affect the reproductive systems [27,128–133]. This section focuses on a selected number of compounds for which there are data on their effects on the female reproductive system from both *in vitro* and *in vivo* studies. It concentrates more on environmental compounds because they constitute the largest component of chemicals with reported reproductive effects *in vivo* and *in vitro*, with less data available on pharmaceutical compounds, aside from the few chemotherapy agents that have reported effects *in vivo* and *in vitro*.

### 4.1. Pharmaceutical compounds

#### 4.1.1. Cyclophosphamide (Cy)

Cy is a commonly used chemotherapeutic agent with well-recognised ovarian toxicity including follicle destruction and lowered ovarian oestrogen production [72]. Cy causes intra- and inter-strand DNA cross-linking, thereby interfering with cell division. With increasing success of cancer treatments, there are growing concerns about the long-term side effects of alkylating agents such as cyclophosphamide on female reproductive function. There are not many studies using animal models on the reproductive effects of cyclophosphamide, but the ones that have been carried out both *in vivo* and *in vitro* report very similar effects (Table 3), namely, Cy results in a reduced total follicle reserve. To

**Table 3**

Comparison of *in vivo* and *in vitro* effects of cyclophosphamide on the ovary.

| Effect of Cy | In vivo | | | In vitro | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| ↓ total follicle reserve | i.p. treatment for 20 days<br>Single i.p. treatment of Cy<br>Pregnant females received a single i.p. injection on E10.5 and E11.5 | Rat (SD)<br>Mouse (Balb/c)<br>Mouse (L1 or 129) | [134]<br>[136]<br>[41] | 8 day culture of PND4 ovaries with Cy metabolites | Mouse (CD1) | [135] |
| ↑ apoptosis<br>Altered steroidogenesis<br>Failed fertilisation and implantation | i.p. treatment for 20 days<br>i.p. treatment for 20 days<br>6–7 week old females injected (i.p.) with Cy | Rat (SD)<br>Rat (SD)<br>Mouse (Balb/c) | [134]<br>[134]<br>[137] | 8 day culture of PND4 ovaries with Cy metabolites | Mouse (CD1) | [135] |

i.p., intraperitoneal; SD, Sprague Dawley; PND, post-natal day.

the best of our knowledge, no *in vitro* studies have investigated the effect of Cy on steroidogenesis or on fertilisation rates.

### 4.1.2. Diethylstilbestrol (DES)

DES is a non-steroidal synthetic compound functionally similar to natural estradiol but with stronger bioactivity, and high affinity to ERα (ESR1) [138]. It was first synthesised in 1938 and was then prescribed to women between the 1940s and early 1970s as it was believed to reduce the risk of spontaneous abortions and other pregnancy-related complications. It was also thought to improve pregnancy outcome by increasing production of placental steroid hormones [139]. DES was in later years shown to cause reproductive abnormalities in the daughters born to these women, including the otherwise rare clear cell adenocarcinoma of the vagina and cervix [140]: DES was finally banned in 1971 [141]. There are many gaps in the literature where no equivalent *in vitro* studies have been carried out alongside *in vivo* studies, and *vice versa.* However, in the few instances where there has been overlap, the *in vivo* and *in vitro* studies show consistent results, namely demonstrating an increasing incidence of polyovular follicles in mice exposed to DES (Table 4). *In vivo* and *in vitro* studies have not always given consistent results, though, with one study by Karavan [60] finding that DES decelerated follicular development *in vivo*, whereas

other *in vivo* studies by Wordinger [142] and Rivera [59] found the opposite effect.

### 4.1.3. Doxorubicin

Doxorubicin (DXR) is an anthracycline chemotherapy agent used to treat a variety of cancers including lymphomas, sarcomas, as well as breast and ovarian cancer. It is thought to intercalate with DNA to prevent replication and transcription, partly by inhibiting topoisomerase II, although its precise mechanism remains unknown [24,144]. Studies into the effect of DXR on the ovary have reported a large dose-dependent rise in the number of double-strand DNA breaks in both oocytes and granulosa cells of the ovary. Stromal and vascular damage have also been reported in exposed ovaries, indicating induction of premature ovarian ageing not only through damage to the germ cells but also *via* the somatic components of the ovary [47,145]. Several papers have reported the effects of DXR on the ovary *in vivo*, and many of their results have been supported by *in vitro* studies, such as increased apoptosis of follicles and increased stromal damage of exposed ovaries (Table 5).

### 4.2. Plasticisers

#### 4.2.1. Bisphenol A (BPA)

BPA is a weak environmental oestrogen whose effects have generated considerable controversy over the last few years. It

**Table 4**

Comparison of *in vivo* and *in vitro* effects of diethylstilbestrol on the ovary.

| Effect of DES | In vivo | | | In vitro | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| Accelerated follicular development | Pregnant mice at E15.5 injected (s.c.). F1 females collected at PND7<br>s.c. injections between PND1 and 14 | Mouse<br><br>Sheep | [142]<br><br>[59] | | | |
| Slowed follicular development | Daily injections (s.c.) on PND1–4. Ovaries collected on PND5<br>Neonatal mice injected (s.c.) on PND0–1 | Mouse (CD1)<br><br>Mouse (C57BL/6J) | [60]<br><br>[55] | | | |
| Meiotic defects | | | | Exposure of COCs during meiosis-I and meiosis-II spindle formation | Mouse (Balb-C) | [50] |
| Altered cyst breakdown | Daily injections (s.c.) on PND1–4. Ovaries collected on PND5 | Mouse (CD1) | [60] | | | |
| ↓ cell death | Neonatal mice injected (s.c.) on PND0–1 | Mouse (C57BL/6J) | [55] | | | |
| ↑ polyovular follicles | Neonatal mice injected (s.c.) on PND0–1 | Mouse (C57BL/6J) | [55] | PND0 ovaries cultured with DES for 5 days and transplanted under the renal capsule for 30 days | Mouse (C57BL/Tw) | [143] |
| | s.c. injections for 5 days from PND0 | Mouse (ICR/Jcl) | [57] | | | |
| ↓ Estradiol and testosterone levels | | | | PND14 ovaries collected, follicles isolated and cultured with DES | Rat (SD) | [138] |

COCs, cumulus–oocyte complexes; PND, post-natal day; s.c., subcutaneous; SD, Sprague Dawley.

**Table 5**
Comparison of *in vivo* and *in vitro* effects of doxorubicin on the ovary.

| Effect of DXR | In vivo | | | In vitro | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| ↑ apoptosis of follicles | Four week old mice treated with DXR | Mouse (CD1) | [146] | GV oocytes cultured with DXR for 20 min | Mouse (ICR) | [47,147] |
| | Single i.v. injection of DXR. Ovaries collected 24 h later. | Mouse (B6D2 and FBV) | [145] | Ovarian cortical pieces cultured for 24–72 h with DXR | Human | [145] |
| ↓ ovarian size/weight | 7–8 week old females injected (i.p.) and ovaries collected 1 day or 1 month later | Mouse (ICR) | [23] | | | |
| ↓ population of secondary and primordial follicles | 7–8 week old females injected (i.p.) and ovaries collected 1 day or 1 month later | Mouse (ICR) | [23] | Ovarian cortical pieces cultured for 24–72 h with DXR | Human | [145] |
| | Mice xenografted with human ovarian tissue and dosed | Human | [145] | | | |
| Stromal damage | Mice xenografted with human ovarian tissue and dosed | Human | [145] | Ovarian cortical pieces cultured for 24–72 h with DXR | Human | [145] |

GV, germinal vesicle; i.p., intraperitoneal; i.v., intravenous; s.c., subcutaneous; COCs, cumulus–oocyte complexes; SCID, severe combined immunodeficiency; SD, Sprague Dawley; PND, post-natal day.

**Table 6**
Comparison of *in vivo* and *in vitro* effects of BPA on the ovary.

| Effect of BPA | In vivo | | | In vitro | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| ↑ recruitment of PMFs | s.c. injections on PND 1, 3, 5 and 7. Ovaries collected on PND8 | Rat (Wistar) | [158] | | | |
| Meiotic defects | Females exposed *in utero* (E11.5–E18.5). Ovaries collected at E18.3, PND28 | Mouse (C57BL/6) | [52] | Chronic exposure to growing pre-antral follicles in culture | Mouse (C57BL/6j × CBA/Ca) | [131] |
| | s.c. injections between PND1 and 14. Ovaries collected on PND30 | Sheep | [59] | BPA exposure of COCs during meiosis-I and the formation of meiosis-II spindle | Mouse (Balb/c) | [159] |
| | Females treated with oral doses of BPA for 6–8 days preceding oocyte collection on PND28 | Mouse | [51] | Oocytes isolated from antral follicles exposed during *in vitro* maturation | Mouse (MF1) | [53] |
| | Pregnant females exposed BPA during GD 50–100. Ovaries collected at GD 100 | Rhesus monkey | [160] | | | |
| Delayed cyst breakdown/MOFs | Injections (s.c.) between PND1 and 4. Ovaries collected on PND5 | Mouse (CD1) | [60] | Newborn ovaries cultured 3 days with BPA | Mouse (CD1) | [161] |
| | Injections (s.c.) between PND1 and 4. Ovaries collected on PND5 | Sheep | [59] | | | |
| | Newborns received 5 daily s.c. injections and ovaries collected at PND40 and 90 | Mouse (ICR/Jcl) | [57] | | | |
| | Pregnant females treated with BPA. F1 pups collected at PND 3, 5, 7 | Mouse (CD1) | [118] | | | |
| | Pregnant females exposed during third trimester (GD100-term) | Rhesus monkey | [160] | | | |
| Altered steroidogenesis | *In utero* exposure through drinking water of pregnant females from day 6 of pregnancy | Rat (SD) | [69] | Granulosa cells cultured with BPA for 48 h | Porcine | [162] |
| | Females exposed daily from gestation day 6 until labour (gavage), followed by dosage of pups from PND1 until PND90 | Rat (SD) | [163] | Theca-interstitial cells and granulosa cells cultured with BPA | Rat (SD) | [164] |
| | | | | Granulosa cells cultured for 72 h with BPA | Porcine | [165] |
| ↑ apoptosis and oocyte loss | s.c. injections between PND1 and 4. Ovaries collected on PND5 | Mouse (CD1) | [60] | Granulosa cells cultured with BPA | Mouse (B6C3F1) | [166] |

GV, germinal vesicle; i.p., intraperitoneal; s.c., subcutaneous; COCs, cumulus–oocyte complexes; SD, Sprague Dawley; PND, post-natal day.

**Table 7**
Comparison of *in vivo* and *in vitro* effects of MEHP on the ovary.

| Effect of MEHP | In vivo | | | In vitro | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| Inhibited follicle growth. Reduced number of antral follicles and/or CL | Adult females were dosed daily by gavage for 1–12 days | Rat (SD) | [85] | Pre-antral follicles cultured individually for 10 days in MEHP | Rat | [173]. |
| | | | | Antral follicles cultured with MEHP for 96 h | Mouse (CD1) | [174] |
| | | | | Pre-antral follicles from PND14 rats cultured for 48 h with MEHP | Rat (SD) | [133] |
| | | | | Antral follicles cultured with MEHP for 24–96 h | Mouse (CD1) | [175] |
| ↑ number of mature follicles | Pregnant females exposed *via* oral gavage to MEHP. Females in litter collected on first day of estrous cycle | Mouse (C57/Bl6) | [172] | | | |
| ↑ atretic antral follicles | Pregnant females received daily doses of DEHP by gavage from day 6 p.c. to lactation day 21 | Rat (Wistar) | [176] | | | |
| Suppressed estradiol production in granulosa cells of pre-ovulatory follicles | Adult females were dosed daily by gavage for 1–12 days | Rat (SD) | [85] | Granulosa cells from adult rat ovaries cultured with MEHP for 48 h | Rat (Fisher 344) | [177] |
| | | | | Antral follicles cultured with MEHP for 96 h | Mouse (CD1) | [174] |
| | | | | Granulosa cells cultured with MEHP | Human | [178] |
| | | | | Pre-antral follicles from PND14 rats cultured for 48 h with MEHP | Rat (SD) | [133] |
| Increased estradiol levels in serum | Pregnant females exposed *via* oral gavage to MEHP. Females in litter collected on first day of estrous cycle | Mouse (C57/Bl6) | [172] | | | |
| Reduced aromatase expression | Pregnant females exposed *via* oral gavage to MEHP. Females in litter collected on first day of estrous cycle | Mouse (C57/Bl6) | [172] | Granulosa cells from adult ovaries cultured with MEHP for 48 h | Rat (Fisher 344) | [177] |
| | | | | Granulosa cells cultured with MEHP | Human | [178] |

SD, Sprague Dawley; PND, post-natal day; p.c., post-coitum.

was formulated around the same time as DES but was considered less potent. Exposure to BPA is ubiquitous, because it is used in combination with other chemicals in the manufacturing of polycarbonate plastic and resins and is one of the highest-volume chemicals produced worldwide [148,149]. BPA is used in the manufacturing of polycarbonate, epoxy and corrosion resistant polyester–styrene resins used to make food containers, tableware, dental sealants and the lining of food cans [150,151]. Due to the ester bonds in BPA-based polymers, they are subject to leaching which increases environmental exposure [152,153]. Analysis of human urine samples in the United States has shown that BPA is present in around 95% of urine samples (>0.1 ng/ml) [154] and since BPA is rapidly metabolised [155], this implies that humans are continuously exposed to BPA probably through more than one source. Relationships between the degree of BPA exposure and ovarian dysfunction, such as polycystic ovary syndrome [156] and recurrent miscarriage [157] have been suggested. BPA is also a selective ER modulator (SERM) and several studies have demonstrated its endocrine disrupting actions in both *in vivo* and *in vitro* (Table 6). Studies that have investigated the effects of BPA both *in vitro* and *in vivo*, have overall reported consistent effects: an increase in meiotic defects, especially at higher doses, altered steroidogenesis, delayed cyst breakdown and increased apoptosis in BPA exposed females/cultured ovaries [52,131,158]. Within that, there is some discrepancy to the exact details of the meiotic defects where some studies report aneuploidy following BPA exposure [51,52] whereas another study reports no aneuploidy, but an induction of meiotic arrest [53].

### 4.2.2. Mono(2-ethylhexyl)phthalate (MEHP)

The plasticiser MEHP is the major active metabolite of DEHP, the most abundantly produced phthalate ester and the most potent reproductive toxicant of the phthalates [167]. Phthalates are used in the manufacturing of products such as packaging materials, food wraps, medical devices including tubing, blood bags and disposable medical examination gloves, and children's toys to increase their flexibility [167]: over time, phthalates can leach from the plastic. Due to their lipid-soluble and volatile nature, they are ubiquitous environmental contaminants and humans are daily exposed to phthalates from a wide variety of sources [168]. MEHP can cross the placenta, and it is therefore possible that effects might occur from exposure *in utero*. A group of factory-working women who were chronically exposed to phthalates were shown to have increased numbers of miscarriages and decreased pregnancy rates [169]. A later study carried out on pregnant women living near a plastic manufacturer also found a correlation between the phthalate levels in their urine and pregnancy complications [170]. Mother and foetus may also be exposed to DEHP through every-day beauty and consumer products [167]. In 2005, the European Union banned phthalates with potential reproductive toxicity in all toys and childcare objects [171]. Studies carried out on the effects of MEHP on the pre- and post-natal ovary suggest that phthalates can induce

**Table 8**
Comparison of *in vivo* and *in vitro* effects of DDT on the ovary.

| Effect of DDT | In vivo | | | In vitro | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| ↑ expression of VEGF, Flk-1 and IGF-1 | Females treated with DDE at PND28 and 31. Ovaries collected at PND50 | Rat | [186] | Granulosa cells treated after 72 h in culture for 24 h with DDE | Human | [186] |
| ↑ progesterone synthesis | | | | Granulosa cells cultured with DDE | Porcine | [187] |
| ↓ progesterone synthesis | | | | Granulosa cells from pre-pubertal gilts cultured with DDE for 48 h | Porcine | [188] |
| | | | | Granulosa cultured with DDE for 24 h | Porcine | [189] |
| ↑ proliferation of granulosa cells | | | | Granulosa cells from pre-pubertal gilts cultured with DDE for 48 h | Porcine | [188] |

PND, post-natal day.

adverse responses in females at all stages of development, with *in vivo* and *in vitro* studies reporting consistent results, namely that the main effect in adult females is suppressed estradiol production by the ovary, most likely through suppression of aromatase activity (Table 7). A few studies reported opposite findings, for example, Moyer and Hixon [172] reported that PND56 females exposed *in utero* had elevated serum estradiol levels (as opposed to the majority of findings that reported reduced estradiol levels). However, estradiol levels in the same females were significantly reduced compared with controls by PND365.

### 4.3. Organochlorine pesticides

#### 4.3.1. Dichlorodiphenyltrichloroethane (DDT)/dichlorodiphenyldichloroethylene (DDE)

DDT is used in a variety of agricultural fertilizers, household insecticide sprays and parasitic medications. It has the ability to persist in the environment and accumulate in the food chain [179]. It has been reported to result in reproductive abnormalities in wildlife, with a strong association found between dichlorodiphenyldichloroethylene (DDE), the most persistent metabolite of DDT, and egg shell thinning in predatory birds [180]. Exposure of female rabbits to DDT has been reported to lead to decreased fertilisation rate as well as pre- and post-implantation embryonic loss [87]. DDE is ubiquitous and can be found in various human tissues, as well as serum and follicular fluid [181–183], suggesting that DDE has direct access to the oocyte. Higher DDE levels in follicular fluid of women undergoing IVF were associated with lower fertilisation rates [183], although the precise mechanism through which DDE affects the ovary is unknown. Another study suggested an association between DDE and polycystic ovary syndrome (PCOS) [184]. Even though the use of DDT was discontinued in North America in 1972, it is still used, *e.g.*, in Mexico as a way of controlling malaria, and therefore is still able to enter the environment [185]. The only study into both *in vitro* and *in vivo* effects of DDT on the ovary reported increased VEGF, Flk-1 and IGF-1 expression following treatment. Another study reported an increased proliferation of granulosa cells, but several *in vitro* studies looking at progesterone synthesis do not report consistent effects (Table 8).

#### 4.3.2. Methoxychlor (MXC)

MXC is an insecticide that was developed to replace DDT as it is considered less toxic [130,190], none-the-less, it has been found to affect the reproductive system at all stages of development [191]. It is metabolised to mono-hydroxy MXC in the body [192], and ovaries of adult mice exposed to MXC have been reported to contain higher

numbers of large atretic follicles, and have lower weight than control ovaries [191]. It has been debated whether these pesticides (MXC/DDT) have the ability to impair fertility by directly acting on the ovaries and affecting the meiotic progression of oocytes, or whether they affect fertility indirectly due to action on the genital tract [193]. *In vivo* and *in vitro* studies on the effects on MXC have produced consistent results (Table 9), reporting reduced follicle growth and increased follicle atresia. This consistency between *in vivo* and *in vitro* studies strongly indicates that the effects are direct.

### 4.4. Occupational chemicals

#### 4.4.1. 4-Vinylcyclohexene diepoxide (VCD)

VCD is an occupational chemical, an epoxide metabolite of 4-vinylcyclohexene (VCH). The VCH family of compounds are occupational-hazard chemicals released into the environment at low concentrations as by-products of the synthesis of plasticisers, flame retardants and rubber. VCD has been shown to selectively deplete the rodent ovary of primordial and primary follicles [197,198]. Daily treatments of VCD in mice and rats results in primordial and primary follicle depletion, greatly reducing the number of follicles that can be recruited for the formation of antral follicles, thus subsequently affecting E2 secretion [199]. However, only rodents are able to metabolise VCH to its active form VCD, and since human exposure to VCH and its metabolites is minimal, VCH has been considered as a model toxicant for reproductive toxicology studies and for studying their effects on primordial follicle loss [200]. All studies on the effects of VCD on the ovaries, *in vitro* and *in vivo*, report consistent results: reduced numbers of pre-antral follicles and an increase in apoptosis (Table 10).

#### 4.4.2. Polychlorinated biphenyls (PCBs)

PCBs are pollutants that were generated on a large scale commercially until their production was banned in the 1970s [203]. PCB contamination was first detected when environmental samples were being screened for DDT and other environmental compounds [204]; they have since been detected in the environment worldwide. They are also used in the industrial community due to their stabile and lipophilic properties [205]. This adds to their persistent contamination in the environment, since they can accumulate in food chains [35]. Furthermore, due to their lipophilic properties, they can accumulate in adipose tissues, breast milk and are able to cross the placenta. This means that the offspring can be exposed to high concentrations of PCBs during pre- and post-natal development [206]. PCBs and their congeners have been shown to affect the reproductive system of several species [35]. One of the congeners,

**Table 9**
Comparison of *in vivo* and *in vitro* effects of MXC on the ovary.

| Effect of MXC | *In vivo* | | | *In vitro* | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| ↓ follicle growth/↓ antral follicles | Females received daily s.c. injections of MXC | Rat (SD) | [81] | Antral follicles from PND30 to 35 females were cultured for 96 h with MXC | Mouse (C57BL/6) | [129] |
| | | | | Antral follicles were cultured for 96 h with mono-OH | Mouse (CD1) | [192] |
| ↑ AMH positive follicles | Female rats received daily s.c. injections of MXC | Rat (SD) | [81] | | | |
| ↑ follicle atresia | Females received MXC by oral gavage for 14 days | Rat | [194] | Antral follicles from PND30 to 35 females were cultured for 96 h with MXC | Mouse (C57BL/6) | [129] |
| | Females received daily i.p. MXC injections for 20 days | Mouse (CD1) | [195] | | | |
| | Neonatal females received 14 daily i.p. injections of MXC. Ovaries collected at 3, 6 and 12 months | Mouse (ND4 Swiss Webster) | [196] | | | |
| ↓ progesterone synthesis | | | | Granulosa cells from pre-pubertal gilts were collected and cultured for 48 h with MXC | Porcine | [188] |
| | | | | Granulosa cells were exposed to MXC for 24 h | Porcine | [189] |

i.p., intraperitoneal; s.c., subcutaneous; SD, Sprague Dawley; PND, post-natal day.

3,3′,4,4′-tetrachlorobiphenyl (TCB), is believed to act as an ovarian toxicant through AhR-binding and consequently altering steroidogenic pathways in the ovary [35].

Few *in vitro* studies have been carried out on the effects of PCBs on the ovary, therefore the only correlation between *in vitro* and *in vivo* studies relates to examining the effects on meiotic division of oocytes, with a decreasing percentage of oocytes reaching metaphase II when ovaries were exposed to PCBs, demonstrated *in vivo* in the mouse model and *in vitro* in the bovine model (Table 11). Other findings, such as decreased ovary weight, increased follicle atresia and decreased follicle numbers show consistency between *in vivo* experimental approaches.

### 4.5. Phyto-estrogens

#### 4.5.1. Genistein

Genistein is a naturally occurring isoflavone found in soy, with the main human route of exposure through the consumption of soy products or soy-based infant formula [35]; it is also taken as a form of hormone replacement therapy by post-menopausal women. It has received much scientific interest since it was discovered that it had a negative effect on fertility in ewes [213]. Genistein has been shown to have weak estrogenic and anti-estrogenic properties and to induce cell differentiation [214]. Its actions are thought to result

mainly from binding to ERs due to structural homology with E2 [215]. There are only two studies that appear to find similar effects *in vitro* and *in vivo*, with both studies finding that germ cell nest nest breakdown was inhibited when neonatal mouse ovaries were exposed to genistein: other effects have all been reported in only one model (Table 12).

### 5. Comparison of results from *in vivo* and *in vitro* studies

Despite a wide range of study designs, which can vary in terms of species, dosage routes and concentrations, timing and duration of exposure, as well as whether single or repeated doses of compounds are used, there are a substantial number of *in vitro* studies showing near-identical effects to those found *in vivo* (Table 13). There are however, instances where different effect/s are observed, either between *in vivo* and *in vitro* studies, or despite the similarity in the model used (Table 14). Possible explanations include the compound having off target effects that could lead to a secondary effect on the ovaries *in vivo* but not *in vitro*. Alternatively, the effects of a compound might not follow a normal dose–response relationship; exposure to a compound at a low dose can have a different, even a more significant effect on the ovaries, than exposure to higher concentrations [162,196]. Thus, low concentration of BPA stimulated granulosa cell E2 production, whereas it

**Table 10**
Comparison of *in vivo* and *in vitro* effects of VCD on the ovary.

| Effect of VCD | *In vivo* | | | *In vitro* | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| ↓ number of primordial, primary and secondary follicles | Daily i.p. injections for 30 days to PND28 females | Rat (F344) | [199] | PND4 ovaries cultured with VCD for 1, 2 or 8 days | Rat (F344) | [132] |
| | PND28 females received single i.p. injection | Rat (F344) | [128] | PND4 ovaries cultured with VCD for 1 or 15 days | Rat (F344) | [128] |
| | PND28 females received daily i.p. injections | Rats and mice | [201] | | | |
| ↑ apoptosis | Daily i.p. injections between PND4 and 19 | Rat (F344) | [202] | PND4 ovaries cultured for 15 days with VCD | Rat (F344) | [202] |

i.p., intraperitoneal; PND, post-natal day.

**Table 11**
Comparison of *in vivo* and *in vitro* effects of PCB on the ovary.

| Effect of PCB | In vivo | | | In vitro | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| ↓ ovary weight | Pregnant females exposed to PCBs. F1 females sacrificed on PND21 or 84 | Mouse (CD1) | [203] | | | |
| ↑ follicular atresia | Pregnant females exposed to PCBs. F1 females sacrificed on PND21 or 84 | Mouse (CD1) | [203] | | | |
| ↓ % of oocytes reaching metaphase II/impaired fertilisation | Pregnant females exposed to PCBs. F1 females sacrificed on PND21 or 84 | Mouse (CD1) | [203] | COCs were exposed to Arochlor-1254 | Bovine | [207] |
| ↓ follicle numbers | Females received i.p. injections on day 13 p.c. F1 females sacrificed on PND28 | Mouse (C57/B1) | [208] | | | |
| | Pregnant females injected (i.p.) between 7 and 13 d.p.c. Females sacrificed on PND24/25 | Rat (Long–Evans) | [209] | | | |
| Altered steroidogenesis | | | | Granulosa and theca cells cultured with PCB126 or PCB153 | Porcine | [210] |
| | | | | Co-cultures of granulosa and theca cells were supplemented with PCB | Porcine | [211] |
| | | | | Co-cultures of ovarian theca and granulosa cells were exposed for 48 h with PCB | Porcine | [212] |

GV, germinal vesicle; i.p., intraperitoneal; s.c., subcutaneous; COCs, cumulus–oocyte complexes; PND, post-natal day.

was inhibited by higher concentrations [162]. Effects of reproductive toxicants can also be time-dependent, with different effects observed between single-dose studies and those where animals received repeated doses [218].

## 6. Regulatory requirements for reproductive toxicological testing

Most countries have statutory bodies regulating the testing of potentially toxic compounds. Agencies such as EFSA (the European Food Safety Authority), USEPA and FDA (the Food and Drug Administration), were created for the purpose of protecting human health and the environment by providing scientific advice and by communicating on risks associated with the food chain. The pharmaceutical industry also has rigorous regulatory testing requirements, controlled by the EMA (European Medical Agency), FDA (USA) and PMDA (Pharmaceuticals and Medical Devices Agency, Japan). The guidelines are laid out in the core tripartite harmonised guideline issued by the International Conference on Harmonisation of Technical Requirements for Registration

**Table 12**
Comparison of *in vivo* and *in vitro* effects of genistein on the ovary.

| Effect of genistein | In vivo | | | In vitro | | |
|---|---|---|---|---|---|---|
| | Study | Species | Paper | Study | Species | Paper |
| Lowered or inhibited E2 production | | | | Granulosa cells cultured in presence of genistein | Porcine | [215] |
| Inhibited P4 production | | | | Granulosa cells cultured in presence of genistein | Porcine | [215] |
| ↓ testosterone and cAMP levels (no effect on E2) | | | | Follicles isolated from PND14 females and cultured for 5 days with genistein | Rat (SD) | [138] |
| Inhibited nest breakdown | Neonates received s.c. injections of genistein between PND1 and 5 | Mouse (CD1) | [30] | Ovaries from PND1 females cultured for 7 days with genistein | Mouse (CD1) | [216] |
| ↑ atretic follicles | Females received s.c. day of genistein from PND18 to 20 | Rat (Wistar) | [217] | | | |
| ↓ follicle numbers | Females received s.c. injections of genistein from PND18 to 20 | Rat (Wistar) | [217] | | | |
| ↑ MOFs | Neonates dosed orally with genistein from PND1 to 5 | Mouse (C57BL/6) | [58] | | | |
| | Neonates received s.c. injections of genistein | Mouse | [31] | | | |
| | Females were dosed orally with genistein from PND1 to 5 | Rat (SD) | [56] | | | |

i.p., intraperitoneal; s.c., subcutaneous; SD, Sprague Dawley; PND, post-natal day.

**Table 13**
Consistent results from comparisons of *in vivo* and *in vitro* experiments.

| Comparable results between *in vivo* and *in vitro* | Compound | References | |
|---|---|---|---|
| | | *In vivo* | *In vitro* |
| ↑ number of polyovular follicles | DES | [55] | [143] |
| Inhibited germ cell nest breakdown | GEN | [30] | [216] |
| ↓ number of pre-antral follicles | VCD | [128,199] | [128,132] |
| ↑ apoptosis | VCD | [202] | [202] |
| ↓ follicle growth and fewer antral follicles | MXC | [81] | [129,192] |
| ↑ follicle atresia | MXC | [194–196] | [129] |
| Suppressed E2 production in pre-ovulatory follicles | MEHP | [85] | [133,174,177] |
| Inhibited follicle growth ↓ number of antral follicles/CL | MEHP | [85] | [130,133,173] |
| Meiotic defects | BPA | [52,59] | [131,159] |
| Altered steroidogenesis | BPA | [69] | [131,162,164] |
| ↑ apoptosis | BPA | [60] | [166] |
| Stromal damage | DXR | [145] | [145] |
| ↑ apoptosis in oocytes | DXR | [145,146] | [145,147] |
| ↑ apoptosis | Cy | [134] | [135] |

of Pharmaceuticals for Human Use (ICH) (S5) 1993 which provides guidance on tests for reproductive toxicity [221].

### 6.1. Regulation of pharmaceuticals and agrochemicals

In the pharmaceutical industry the majority of reproductive toxicity testing is carried out *in vivo*. Potential toxic effects on both male and female fertility, foetal and post-natal development must be taken into consideration. Reproductive toxicology testing follows guidelines issued by the ICH as well as by The Organisation for Economic Co-operation and Development (OECD), with the aim of creating standardised reproductive toxicology tests [44,222]. The ICH M3 (S5) guideline contains a description of the testing concept and recommendations, especially those addressing pre-mating treatment duration and suggested observations to assess for reproductive toxicity [221]. It defines the periods of treatment to be used in animals to assess for reproductive risk: fertility, implantation through organogenesis to closure of the hard palate and the pre- and post-natal period through to the end of lactation. This allows specific identification of stages of the reproductive cycle that are at risk following human exposure to medicinal compounds.

Preclinical designs to assess effects of potential toxicants on the pre-natal ovary are fairly similar between pharmaceutical compounds and agrochemicals, but they differ in that pharmaceutical companies analyse effects on long-term outcome, such as pregnancy after exposure *in utero*, whereas agrochemical testing also

includes a more detailed qualitative and quantitative histological assessment of the primordial follicle pool following pre-natal exposure.

### 6.2. Regulation of environmental compounds

In 1996 the U.S. congress, through the Food Quality Protection Act (FQPA) and the Safe Drinking Water Act Amendments (SWDA), directed USEPA to develop a screening system that uses scientifically relevant information to examine whether certain compounds can have hormonal effects in humans. The fertility and reproduction study protocol includes exposure of male and female rodents prior to mating and throughout the mating period. The testing process involves a two-tiered screening system, where the first aims to identify chemicals with the potential to interact with the endocrine system, and the second aims to determine endocrine-related effects caused by each individual chemical, gathering information about their effects at different doses.

In 2013, the Scientific Committee (SC) of EFSA delivered a considered opinion on the existing information relating to the testing and assessment on EDCs. The SC concluded that although a complete range of standardised assays are available to test for endocrine modalities in mammals and fish, although no single assay will provide all the information required to decide whether a substance falls into the ED category. The SC therefore expressed a need to further develop the screens and test methods in order to generate

**Table 14**
Conflicting results from *in vivo* and/or *in vitro* experiments.

| Compound | Effect | Study type | References |
|---|---|---|---|
| Genistein | Lowered PMF numbers | *In vivo* | [217] |
| | Increased PMF numbers | *In vivo* | [219] |
| DDT | Suppressed progesterone synthesis | *In vitro* | [188] |
| | Increased progesterone synthesis | *In vitro* | [187] |
| MEHP | Suppressed estradiol production | *In vivo* and *In vitro* | [85,133,174,177,178] |
| | Increased estradiol production | *In vivo* | [172] |
| DES | Decelerated follicular development | *In vivo* | [59,142] |
| | Accelerated follicular development | *In vivo* | [55,60] |
| DES | Increased testosterone levels | *In vivo* | [220] |
| | Decreased testosterone levels | *In vitro* | [138] |

**Table 15**
Pros and cons of *in vivo* compared to *in vitro* studies.

| In vitro | | In vivo | |
|---|---|---|---|
| Pro | Con | Pro | Con |
| Highly controlled, *i.e.*, exposure window, concentration of compound and stage of follicle growth | Will never tell the 'whole story' | Best way of predicting the 'real life' situation | Difficult to assess exactly how much an animal is exposed to through the placenta |
| Can study a specific follicle type/stage | Do not pick up on effects on the reproductive tract | Can pick up effects on the reproductive tract | Compounds can still be passed through breast milk |
| Good for assessing direct effects of a compound on the ovary | Do not pick up any effects on the HPG axis | Can pick up detect effects on the HPG axis | Compounds can persist in the animal long after exposure |
| The only procedure required for the animal is culling for tissue collection | Does not take into account metabolism of chemical | Metabolism can be considered – are compounds being detoxified? | Requires more animal procedures than the *in vitro* method |
| Pre-natal testing does not require exposure to an animal that is not of interest to the study | An effect *in vitro* does not necessarily mean that the same effect will be observed *in vivo* | Extensive use over decades demonstrates its effectiveness | Pre-natal testing requires exposure to the mother–an animal that may not be of interest for the study |
| Easier to assess whether it targets germ cells or somatic cells | | Can be used with ease to study transgenerational effects | |
| Allows for higher throughput, including testing of complex chemical mixtures | | | |

sufficient data for identifying and assessing endocrine disrupting properties [223].

### 6.3. Limitations of regulatory testing

Since EDC and pharmaceutical exposure can occur in the form of individual chemicals or as chemical mixtures, it has proven difficult to establish which situation causes more harm to reproductive function, in particular because the effects might not become evident until years later [11]. Furthermore, different periods of vulnerability mean that a foetus might not be affected by a chemical in the same way as an adult, making the testing of such chemicals on reproductive function difficult, yet that much more crucial.

The large majority of regulatory testing outlined above requires *in vivo* experiments, using end-points such as pregnancy, implantation and number of offspring, parameters that do not identify any potential effects on the primordial follicle pool. Consequently, current *in vivo* study designs might not pick up long-term effects on the primordial follicle pool since it may not affect immediate ovulation rates and subsequent pregnancies, but might have longer term consequences on reproductive lifespan. On the other hand it is also possible that an effect seen in the neonatal ovary may, in fact, correct itself in later life [224]. Furthermore, testing of chemicals *in vivo* is time consuming and costly.

To date, *in vitro* models have been used primarily as a preliminary or secondary screening protocol for toxicity testing, but the lack of an alternative test system to available *in vivo* study designs has been commented on Refs. [44,225,226]. Non-mammalian species including zebrafish, frogs, *Caenorhabditis elegans* and yeast have been found useful in regulatory toxicity testing as they offer numerous advantages such as rapid development, ease and cost of maintenance and high fecundidy [183,227,228]. However, to date, these models have primarily been used identify the molecular targets of EDCs, to study toxicogenomics, to screen for oestrogen, androgen and thyroid hormone disruption, or for threshold measurements: as such, they are not ideal models for investigating reproductive effects of toxicants and are not accepted models for regulatory testing in the pharmaceutical industry [227,229]. This has lead to an increased demand for adequate mammalian *in vitro* models that may be used to gain an insight into the mechanisms of chemical exposure and pinpoint potentially hazardous products on reproductive function. As can be seen in the section above, *in vitro* cultures have made a useful and powerful contribution to the field of reproductive toxicology testing. Not all regulatory works have to be carried out *in vivo* and there is a strong case for considering *in vitro* testing to replace some of the currently required *in vivo* tests.

### 7. Conclusions

Animals, including humans, are exposed to a very wide range and number of compounds and chemical mixtures in their lifetime. Consequently, it is becoming increasingly crucial to develop and improve the *in vivo* assessments and *in vitro* culture techniques necessary to elucidate the toxic effects of pharmaceuticals and EDCs on the ovary, to allow for faster screening of potential developmental and/or reproductive toxicants. The type and length of analysis chosen for female reproductive toxicology research requires careful consideration. The main pros and cons of *in vitro* compared with *in vivo* studies are outlined in Table 15.

The key point of *in vivo* studies is to assess the potential toxic risk of a drug on the body, when administered at a therapeutic dose. Although this is more representative of the 'real life' situation, it can also be difficult for *in vivo* studies to assess how much is reaching the gonads, since compounds can be detoxified, activated or eliminated in the body. Calculations to determine the amount of compound reaching the gonads are complex and vary between species and life stage. The method of exposure also needs careful consideration as subcutaneous injections and oral ingestions of the same amount of the same compound will not necessarily result in the same ovarian exposure [230,231]. Other issues reproductive toxicologists face when using *in vivo* studies include attempting to limit the duration of exposure to a single dose of compound, since some compounds can, for example, persist in the animal. Exposure of compounds can change due to mobilisation of maternal body reserves during pregnancy [232] or can be passed through breast-milk a long time after the exposure window, making it difficult to predict the precise time and duration of exposure [35,230]. *In vivo* studies often use end-points such as pregnancy, implantation and number of offspring, which are parameters that do not identify the site of action, the mechanism(s) of toxic damage or the effect on the primordial follicle pool. However, female reproductive function requires effective communication between the ovary, the neuroendocrine system, the hypothalamic–pituitary–gonadal (HPG) axis and the reproductive tract, and *in vivo* studies will be able to detect toxic effects on any of these systems, which could result in a secondary effect on the gonads. For example, effects on the oestrogen-dependent endometrium, could subsequently lead to ovary-independent infertility [37,57]. One drawback of *in vitro* studies is, therefore, that, although they can be useful for assessing

direct effects on the ovary, they cannot account for any indirect action that might modulate hormone-signalling pathways such as the HPG axis. Studies carried out *in vitro* are also unable to take metabolism into consideration and care must be taken when examining effects of a compound that has no effect until it has been metabolised. This is the case with DEHP, which is administered *in vitro* as its active metabolite MEHP [167]. Even if a compound has demonstrated interference with receptor binding/hormone production *in vitro*, the same activity may not be observed *in vivo* [233]. Despite this, *in vitro* models such as the ones described in Section 4 are a promising area in toxicology, allowing pragmatic and mechanistic studies of action of reproductive toxicants and are able to reduce the number of animals required for *in vivo* studies. *In vitro* systems are proving to be an invaluable preliminary method to investigate direct effects of potentially harmful compounds because it is practicable to test a very large dose-range from sub-environmental right up to toxicological levels. This holds very true for the female reproductive system, especially where appropriate care has been taken to administer doses that reflect human exposure levels in at least part of the dose–response curve design. Crucially, they require relatively little time to yield precise answers, and can cover various, yet specific, stages of ovary and follicle development. *In vitro* studies allow scientists to examine the precise mechanisms of action of a reproductive toxicant on the different stages of growth and development, as well as to pinpoint whether a specific chemical targets the stroma, the oocyte, the somatic compartment of the follicle, or the follicle as a whole.

To summarise, *in vitro* studies are proving to be an invaluable part of reproductive toxicology, to enable clear analysis of whether a compound acts directly or indirectly on the ovarian follicle. Although they are less useful for studying indirect toxic effects on the reproductive system, they still do have great potential to provide an important preliminary or secondary screening protocol for toxicology testing alongside *in vivo* studies. The combination of *in vivo* and *in vitro* work is a powerful one to detect and understand mechanisms of damage to the ovary, its follicles and oocytes, and their consequence for adult fertility and subsequent generations.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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