Use of ovary culture techniques in reproductive toxicology

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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 post-pubertal 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 estrogen 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 toxicity 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.
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 theca 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 estrus cycle                         | Mouse   | [68]       |
|                                 | Cessation of estrus 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 lutea, 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                           | Mouse   |           |
| 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 estrus cycles          | Rat     | [83]       |
|                                 | Decreased sex steroid hormone levels                     | Mouse   | [84]       |
| Phthalates (DEHP/MEHP/BBP)      | Prolonged estrus 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]       |
| Trichloron (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 synopsis, 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 [59–104]. 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 POI 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, alklyphenols], polychlorinated biphenyls (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 oocyte and 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
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 oestradiol but with stronger bioactivity, and high affinity to ERs (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 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

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**Table 3**

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

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

**Table 4**

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

| Effect of DES                  | In vivo Study | Species | Paper |
|--------------------------------|---------------|---------|-------|
| Accelerated follicular development | Pregnant mice at E15.5 injected (s.c.), F1 females collected at PND7 s.c. injections between PND1 and 14 | Mouse | [142] |
| Slow follicular development    | Daily injections (s.c.) on PND1–4. Neotal mice injected (s.c.) on PND0–1 | Mouse (CD1) Mouse (C57BL/6J) | [60] [55] |
| Meiotic defects                |                |         |       |
| Altered cyst breakdown         | Daily injections (s.c.) on PND1–4. Ovaries collected on PND5 | Mouse (CD1) Mouse (C57BL/6J) | [60] [55] |
| ↓ 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] |
| 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 |
| ↑ apoptosis of follicles                          | Four week old mice treated with DXR                                    | Mouse (CD1)                                                             | [146]  |
|                                                   | Single i.v. injection of DXR. Ovaries collected 24 h later.             | Mouse (B6D2 and FBV)                                                   | [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]   |
|                                                   | Mice xenografted with human ovarian tissue and dosed                    | Human                                                                  | [145]  |
| Stromal damage                                    | Mice xenografted with human ovarian tissue and dosed                    | 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 |
| ↑ 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]   |
|                                                   | s.c. injections between PND1 and 14. Ovaries collected on PND30         | Sheep                                                                  | [59]   |
|                                                   | Females treated with oral doses of BPA for 6–8 days preceding oocyte collection on PND28 | Mouse                                                                  | [51]   |
|                                                   | 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]   |
|                                                   | 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]   |
|                                                   | Females exposed daily from gestation day 6 until labour (gavage), followed by dosage of pups from PND1 until PND90 | Rat (SD)                                                              | [163]  |
| ↑ apoptosis and oocyte loss                       | s.c. injections between PND1 and 4. Ovaries collected on PND5          | Mouse (CD1)                                                            | [60]   |

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.                         | 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]          |
| Reduced number of antral follicles and/or CL       |                                                                        |                         |                | Antral follicles cultured with MEHP for 96 h                         | Mouse (CD1)              | [174]          |
|                                                    |                                                                        | Rat (SD)                | [85]           | Pre-antral follicles from PND14 rats cultured for 48 h with MEHP     | Mouse (CD1)              | [175]          |
|                                                    |                                                                        |                          |                | Antral follicles cultured with MEHP for 24–96 h                     |                          |                |
| † number of mature follicles                      | Pregnant females exposed via oral gavage to MEHP. Females in litter collected on first day of estrous cycle | Mouse (C57Bl/6)        | [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 (C57Bl/6)        | [172]          |                                                                        |                          |                |
| Reduced aromatase expression                       | Pregnant females exposed via oral gavage to MEHP. Females in litter collected on first day of estrous cycle | Mouse (C57Bl/6)        | [172]          | Granulosa cells from adult ovaries cultured with MEHP for 48 h       | Rat (Fisher 344)         | [177]          |
|                                                    |                                                                        |                          |                | Granulosa cells cultured with MEHP                                  |                          |                |
|                                                    |                                                                        |                          |                |                                                                        |                          |                |

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
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 stable 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,
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 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 is a substantial number of in vitro studies showing near-identical effects to those found in vivo (Table 13). There are however, instances where different effects 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 9
Comparison of in vivo and in vitro effects of MXC on the ovary.

| Effect of MXC | In vivo | In vitro |
|---------------|---------|---------|
|               | Study   | Species | Paper |
| ↓ follicle growth/↓ antral follicles | Females received daily s.c. injections of MXC | Rat (SD) | [81] |
|               |         |         |       |
| ↑ 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] |
|               | Females received daily i.p. MXC injections for 20 days | Mouse (CD1) | [195] |
|               | Neontal 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.

### 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 |
| ↓ number of primordial, primary and secondary follicles | Daily i.p. injections for 30 days to PND28 females | Rat (F344) | [199] |
|               | PND28 females received single i.p. injection | 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 with VCD for 1, 2 or 8 days | Rat (F344) | [128] |
|               | PND4 ovaries cultured with VCD for 1 or 15 days | Rat (F344) | [128] |
|               | 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 |
| ↓ 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] |
| ↓ 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 Medicinal 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 |
| Lowered or inhibited E2 production | Neonates received s.c. injections of genistein between PND1 and 5 | Mouse (CD1) | [30] |
| Inhibited P4 production | Females received s.c. day of genistein from PND18 to 20 | Rat (Wistar) | [217] |
| ↓ testosterone and cAMP levels (no effect on E2) | Females received s.c. injections of genistein from PND18 to 20 | Rat (Wistar) | [217] |
| Inhibited nest breakdown | Neonates dosed orally with genistein from PND1 to 5 | Mouse (C57BL/6) | [58] |
| ↑ 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 | 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 |
|-------------------------------------------------|----------|------------|
| † 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 | MEHP | [85] | [130,133,173] |
| ↓ number of antral follicles/CL | | | |
| 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] |
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 fecundity [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 toxicity 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 action 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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References

[1] Baillet A, Mandon-Pepin B. Mammalian ovary differentiation – a focus on female meiosis. Mol Cell Endocrinol 2012;356:417–33.
[2] Anderson LD, Hirschfield AN. An overview of follicular development in the ovary: from embryo to the fertilized ovum in vitro. Mod Med J 1992;41:614–20.
[3] Adams IR, McLaren A. Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. Development 2002;129:1155–64.
[4] McGee EA, Hsieh AJ. Initial and cyclic recruitment of ovarian follicles. Endocr Rev 2000;21(2):200–14.
[5] Craig J, Oryiska M, Wang H, Oryiska S, Thompson W, Zhu C, et al. Gonadotropin and intra-ovarian signalig during follicle development and atresia: the delicate balance between life and death. Front Biosci 2007;12:3628–39.
[6] Woodruff TK, Walker CL. Fetal and early postnatal environmental exposures and reproductive health effects in the female. Fertil Steril 2008;89:67–51.
[7] Bellingerham M, Sharp RM. Chemical exposures during pregnancy: dealing with potential, but unproven risks to child health. RCOG; 2013 [Scientific Impact Paper 37].
[8] Tiemann U. In vivo and in vitro effects of the organochlorine pesticides DDT, DCP, methoxychlor and lindane on the female reproductive tract of mammals: a review. Reprod Toxicol 2008;25:316–26.
[9] Birklett JW. Scope of the problem. Endocrine disruptors in wastewater and sludge treatment processes. Boca Raton, FL: Lewis Publishers; 2003.
[10] Rahman MF, Yanful EK, Jasim SY. Endocrine disrupting compounds (EDCs) and pharmaceuticals and personal care products (PPCPs) in the aquatic environment: implications for the drinking water industry and the global environmental health. Water Res 2003;37:2243–43.
[11] Sharp RM, Irvine D. How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health. BMJ 2004;328:447–51.
[12] Propper CR. The study of endocrine-disrupting compounds: past approaches and new directions. Integr Comp Biol 2005;45:194–200.
[13] Fowler PA, Bellingham M, Sinclair KD, Evans NP, Pocar F, Fischer B, et al. Impact of endocrine-disrupting compounds (EDCs) on female reproductive health. Mol Cell Endocrinol 2012;355:231–9.
[14] Petro EML, Leroy JMLR, Van Cruchten SM, Covacci A, Jorssen EPA, Bols PEJ. Endocrine disruptors and female fertility: focus on (bovine) ovarian follicular physiology. Theriogenology 2012;78(9):1887–904.
[15] Rhind SM. Are endocrine disrupting compounds a threat to farm animal health, welfare and productivity. Reprod Domest Anim 2005;40:282–90.
[16] Gray J, Evans N, Taylor B, Rizzio J, Walker M. State of the evidence (review): the connection between breast cancer and the environment. Int J Occup Environ Health 2009;15:43–78.
[17] Johnson MD, Kenneu N, Stoica A, Hilaikiva-Clarke L, Singh B, Chegkpo G, et al. Cadmium mimics the in vivo effects of estrogen in the uterus and mammmary gland. Nat Med;2003(9):8201–4.
[18] Martino-Andrade AK, Chahoud I. Reproductive toxicity of phthalate esters. Mol Nutr Food Res 2010;54:148–57.
[19] Ranaldi R, Gambuti G, Eichenauer-Bitter U, Pacchierotti F. Trichlorfon effects on mouse oocytes following in vivo exposure. Mutat Res 2008;651:125–30.
[20] Ishibashi H, Matsunuma N, Hirano M, Matsuoka M, Shirauchi H, Ishibashi Y, et al. Effects of triclosan on the early life stages and reproduction of medaka Orzyias latipes and induction of hepatic vitellogenin. Aquat Toxicol 2004;67:167–79.
[21] Hujf J. Carcinogenic bioassays of bisphenol A, 4-vinylcyclohexene diepoxide and 4-vinylcyclohexene. Toxicol Sci 2001;64:282–3.
[22] Uzumcu M, Zachow R. Developmental exposure to environmental endocrine disruptors: consequences within the ovary and on female reproductive function. Reprod Toxicol 2007;23(3):337–52.
[23] Ben-Aharon I, Bar-Joseph H, Tzafaryy G, Kuchinsky L, Rizel S, Sommer MW, et al. Doxorubicin-induced ovarian toxicity. Reprod Biol Endocrinol 2010;8:20.
[24] Morgan S, Anderson RA, Courley C, Wallace WH, Spears N. How do chemotherapeutic agents damage the ovary? Hum Reprod 2012;18(5):525–35.
[25] Daston GP, Gooch JW, Breslin WJ, Shuey DL, Niforov AF, Tico FA, et al. Environmental estrogens and reproductive health: a discussion of the human and environmental data. Reprod Toxicol 1997;11(4):465–81.
[26] Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho SM, Hunt P, et al. Female reproductive disorders: the roles of endocrine-disrupting compounds and developmental timing. Fertil Steril 2008;90(4):911–40.
[27] Diamanti-Kandaridou E, Bourguignon JG, Guideri LC, Hauser R, Prins GS, Soto AM, et al. Endocrine-disrupting chemicals: an endocrine society scientific statement. Endocr Rev 2009;30:293–342.
[28] Rochester JR. Bisphenol A and human health: a review of the literature. Reprod Toxicol 2013;42:132–55.
[29] Adewale IR, McLaren A. Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. Development 2002;129:1155–64.
[30] Jefferon E, Newbold R, Padilla-Banks E, Pepling M. Neonatal gestation treatment alters ovarian differentiation in the mouse: inhibition of oocyte nest breakdown and increased oocyte survival. Biol Reprod 2006;74:161–8.
[31] Jefferon E, Padilla-Banks E, Newbold R. Disruption of the female reproductive system by the phytoestrogen genistein. Reprod Toxicol 2007;23:308–16.
[32] Newbold R. Lessons learned from perinatal exposure to diethylstilbestrol. Toxicol Appl Pharmacol 2004;199:142–50.
[33] Swan SH. intrauterine exposure to diethylstilbestrol: long-term effects in humans. Annu Rev Publ Health 2000;21(2):1793–804.
[34] Newbold R, Hanson RB, Jefferon WN, Bullock BC, Haseman J, McLachlan JA. Proliferative lesions and reproductive tract tumors in male descendants of mice exposed developmentally to diethylstilbestrol. Carcinogenesis 2000;21(7):1355–63.
[35] Miller KP, Borgerst C, Greenfeld C, Tomix D, Flaws JA. In utero effects of chemicals on reproductive tissues in females. Toxicol Appl Pharmacol 2004;198:111–31.
of oocyte.

of endocrine-disrupting chemicals on female reproduction: an ovarian perspective. Front Neuroendocrinol.

Am. J. Reprod. Endocrinol. 2006;14(4):289–96.

Am. J. Reprod. Endocrinol. 2006;14(6):801–10.

Am. J. Reprod. Endocrinol. 2006;14(7):543–9.

Am. J. Reprod. Endocrinol. 2006;14(7):657–80.

Am. J. Reprod. Endocrinol. 2006;14(8):819–28.

Am. J. Reprod. Endocrinol. 2006;14(8):829–38.

Am. J. Reprod. Endocrinol. 2006;14(9):433111.

Am. J. Reprod. Endocrinol. 2006;14(9):453–62.

Am. J. Reprod. Endocrinol. 2006;14(9):463–74.

Am. J. Reprod. Endocrinol. 2006;14(9):483–90.

Am. J. Reprod. Endocrinol. 2006;14(9):490–8.

Am. J. Reprod. Endocrinol. 2006;14(9):508–16.

Am. J. Reprod. Endocrinol. 2006;14(9):517–25.

Am. J. Reprod. Endocrinol. 2006;14(9):525–34.

Am. J. Reprod. Endocrinol. 2006;14(9):534–29.

Am. J. Reprod. Endocrinol. 2006;14(9):543–50.

Am. J. Reprod. Endocrinol. 2006;14(9):549–58.

Am. J. Reprod. Endocrinol. 2006;14(9):558–70.

Am. J. Reprod. Endocrinol. 2006;14(9):567–70.

Am. J. Reprod. Endocrinol. 2006;14(9):570–83.

Am. J. Reprod. Endocrinol. 2006;14(9):583–90.
Health 2009;550:137–57.
[128] Devine JP, Sipes IG, Hoyer PB. Delayed initiation of ovdlet toxicity by in vitro and in vivo exposures of rat ovaries to 4-vinylcyclohexene diepoxy. Toxicop Reprod 2004;19:71–7.

Miller KP, Gupta RK, Greenfield C, Babus JK, Flaws JA. Methoxychlor directly affects ovarian antral follicle growth and atresia through Bel-2 and bad mediators. Environ Health Perspect 2005;113:121–21.

[129] Gupta RK, Miller K, Babus JK, Flaws JA. Methoxychlor inhibits growth and induces atresia of antral follicles through an oxidative stress pathway. Toxicol Sci 2006;93:382–9.

[95] O’Brien MJ, Pendola FL, Epple JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental potential. Bio Reprod 2003;68:2–6.

[130] Lee S, Cortivert R, Eichenlaub-Ritter U, Smitz J. Continuous exposure to bisphenol A during in vitro follicular development induces meiotic abnormalities. Mutat Res 2008;651:71–8.

[96] Picton JL, Knappey RJ, Sen SK, Ryner AL, Mikes E, Sipes G, Kohnlab J, et al. Inhibition of ovarian KIT phosphorylation by the ovoxtoxic 4-vinylcyclohexene diepoxy in rats. Bio Reprod 2011;85:755–62.

[97] Jin, SY, Lei L, Shikanov A, Shea LD, Woodruff K. A novel two-step strategy for in vitro culture of early stage ovarian follicles in the mouse. Fertil Steril 2010;93(8):2633–9.

[98] Roy SK, Treacy BJ. Isolation and long term culture of human pre-antral follicles. Reprod Dev Endocrinol 2011;23(3):403–49.

[99] Lee S, Cortivert R, Eichenlaub-Ritter U, Smitz J. Maturation of mouse oocytes from a two-step culture system supports development of human oocytes from primordial follicles in the presence of activin. Hum Reprod 2008;23(5):1151–4.

[100] McLaughlin M, Dingle C, Tong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles. Reproduction 2010;139:971–8.

[101] Xue M, Hui SY, Jin Z, Yaping Z, Xili MA, Zhen L, Fei W, et al. A novel method for toxicology: in vitro culture system of a rat preantral follicle. Toxicol Ind Health 2011;27(7):637–45.

[102] Murray A, Spears N. Follicle development in vitro. Semin Reprod Med 2000;18(2):109–22.

[103] Mucha N, Akatani-Hasegawa A, Saka K, Ogino M, Hosoda Y, Wada R, et al. Live births from isolated primary/early preantral follicles following a multi-step culture without ovarian organ in mice. Reprod Med Biol 2013;12(4):362–7.

[104] Hartshorne GM. In vitro culture of ovarian follicles. Rev Reprod 1997;2:94–104.

[105] Devine J, Rajapaksa KS, Hoyer PB. In vitro ovarian tissue and organ culture: a review. Front Biosci 2002;7:1979–89.

[106] Picton HM, Harris SE, Muruvi W, Chambers EL. The in vitro growth and maturation of follicles, Reproduction 2008;136(6):703–15.

[107] Sun F, Betzendahl J, Shen Y, Cortivert R, Smitz J. Eichenlaub-Ritter U. Pre-antral follicle culture as a novel in vitro assay in reproductive toxicology testing in mammalian oocytes. Mutagenesis 2004;19(1):13–25.

[108] Anderson RA, McIlwain L, Coutts S, Kinnell HL, Fowler PA, Childs AJ. Activation of the aryl hydrocarbon receptor by a component of cigarette smoke reduces germ cell proliferation in the human fetal ovary. Mol Hum Reprod 2014;20(1):42–8.

[109] Eichenlaub-Ritter U. Parental age-related anomalies in human germ cells and offspring: a study of past and present. Environ Mol Mutagen 1996;28:211–36.

[110] Eichenlaub-Ritter U. Genetics of oocyte ageing. Maturitas 1998;30:58–69.

[111] Plachot M. Chromosomal abnormalities in mouse. Mol Cell Endocrinol 2001;183(Suppl. 1):59–63.

[112] McLaren A, Southee D. Entry of mouse embryonic germ cells into meiosis. Dev Biol 2001;236:489–94.

[113] Chuma S, Nakatsuji N. Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gpl30-mediated signalling. Dev Biol 2001;242:489–94.

[114] Motohashi H, Sankai T, Nariai K, Sato K, Kada H. Effects of in vitro culture of mouse fetal gonads on subsequent ovarian development in vivo and oocyte maturation in vitro. Hum Cell 2000;22(2):43–8.

[115] Zhang Z, Lian G, Zhang X, Zhang G, Chao H, Li L, et al. Growth of mouse oocytes to maturity from premeiotic germ cells in vitro. PlasOne 2012;7(4):e17171.

[116] Parrott JA, Skinner MK. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. Endocrinology 1999;140(9):339–50.

[117] Devine JP, Hoyer PB, Keating AF. Current methods in investigating the development of the female reproductive system. Methods Mol Biol 2009;507:137–57.

[118] Cortivert R, Smitz J, Van Steirteghem AC. In vitro maturation, fertilization and embryo development of immature oocytes from pre-epubertal follicles from pre-pubertal mice in a simplified culture system. Hum Reprod 1999;11:2656–66.

[119] Spears N, De Bruin JP, Gasden RG. The establishment of follicular dominance in co-cultured mouse ovarian follicles. J Reprod Fertil 1995;106(1):1–6.

[120] Campbell L, Trendell J, Spears N. Identification of cells migrating from the thecal layer of ovarian follicles. Cell Tissue Res 2013;353(1):189–94.

[121] Guilleit LJ. Endocrine disrupting contaminants – beyond the dogma. Environ Health Perspect 2006;114(Suppl. 1):9–12.

[122] Benigni A, Bergamaschi M. 4- and 4-nonylphenol in a human reference population. Environ Health Perspect 2005;113:391–5.

[123] Krishnan AV, Mathis P, Perrmutt SF, Tokes L, Feldman D. Bisphenol A: an estrogenic substance is released from polycarbonate disks during autolacing. Endocrinology 1993;132:2279–86.

[124] Brotons JA, Ole-Serrano MF, Villalobos M, Pedraza V, Olea N. Xenobiotics released from lacquer coatings in food cans. Environ Health Perspect 1995;103:608–12.

[125] Calafat AM, Kuklenyik Z, Reddy RA, Caudill SP, Ekong J, Needham LL. Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environ Health Perspect 2003;113:391–5.

[126] Volkel W, Colton T, Casgady GA, Fisler JC, Dekant W. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chem Res Toxicol 2002;14(5):633–46.

[127] Takeuchi T, Tsutsui O, Ikuzuki Y, Takay A, Taketani Y. Positive relationship between androgenic and oestrogenic disruption, bisphenol A, in normal women with ovariopathy dysfunction. Endocr J 2004;51(2):165–9.
[157] Sugiuira-Ogasawara M, Ozaki Y, Sonta S, Makino T, Suzuki M. Exposure to bisphenol A is associated with recurrent miscarriage. Hum Reprod 2005;20:2325–9.

[158] Rodriguez HA, Santambrosio NS, Santamaria CG, Munoz-de-Toro M, Luque EH. Neonatal exposure to bisphenol A reduces the pool of primordial follicles in the ovary of the rhesus monkey. Proc Natl Acad Sci U S A 2012;109:17525–30.

[159] Can A, Semiz O, Cinar O. Bisphenol A induces cell cycle delay and alters censored and spindle microtubular organization in oocytes during meiosis. Mol Hum Reprod 2005;11(6):389–96.

[160] Hunt PA, Lawton C, Giske M, Murdoch B, Smith H, Murre A, et al. Bisphenol A alters early oogenesis and follicle formation in the ovary of the rhesus monkey. Proc Natl Acad Sci U S A 2012;109:17525–30.

[161] Zhao T, Li L, Qin X, Zhou Z, Zhang Y, Wang D. Di-(2-ethylhexyl) phthalate and bisphenol A exposure impairs mouse primordial follicle assembly in vitro. Environ Mol Mutagen 2014;55(4):343–53.

[162] Grassel F, Baratta L, Baimo I, Bussolati S, Ramoni R, Grolli S, et al. Bisphenol A and estradiol induce cell function. Dev Mol Endocrinol 2010;30:34–59.

[163] Delcos KB, Camacho L, Lewis SM, Vanlenningham MM, Latendresse JR, Olson GR, et al. Toxicity evaluation of bisphenol A administered by gavage to Spargue Dawley rats from gestation day 6 through postnatal day 90. Toxicol Sci 2014;139(1):174–97.

[164] Zhou W, Liu J, Liao L, Han S. Effects of bisphenol A on steroid hormone production in rat ovarian theca-interstitial and granulosa cells. Mol Cell Endocrinol 2008;283:12–8.

[165] Mlynarcikova A, Kolenka A, Kolenkova F, Mckova S, Sulkova S. Alterations in steroid hormone production by porcine ovarian granulosa cells caused by bisphenol A and bisphenol A dimethacrylate. Mol Cell Endocrinol 2009;284:57–62.

[166] Xu J, Osuga Y, Yano T, Morita Y, Tang X, Fujitara W, et al. Bisphenol A induces apoptosis and C2-M arrest of ovarian granulosa cells. Biochem Biophys Res Commun 2002;292(2):456–62.

[167] Lupton-Jamp-Sawant I, Davis BJ. Mechanisms of phthalate estrogenicity in the female reproductive system. Environ Health Perspect 2003;111:139–45.

[168] Thomas JA, Thomas MJ. Biological effects of (2-ethylhexyl) phthalate and other phthalic acid esters. Crit Rev Toxicol 1984;13:283–317.

[169] Aldyrevu MV, Klimova TS, Izuimova AS, Timofeevskaya LA. Effect of phthalate plasticizers on the generative function. Gig Tr Prof Zabol 1975;19:25–9.

[170] Tabacova S, Little R, Babalava L. Maternal exposure to phthalates and complications of pregnancy. Epidemiol Rev 1999;21(1 Suppl):127 (abstract 368 0; 1999 Annual conference of the ISEF and ISEA).

[171] European Commission. Restrictions on marketing and use of certain dangerous substances and preparations (phthalates in toys and childcare articles) amendment of 2005/48/EC. Off J Eur Union 2005;147:13–23.

[172] Moyer B, Hixon M. Reproductive effects in F1 adult females exposed in utero to moderate doses of mono-2-ethylhexylphthalate (MEHP). Reprod Toxicol 2012;34:43–50.

[173] Wan X, Zhu Y, Ma X, Zhu J, Zheng Y, Hou J, et al. Effect of DEHP and its metabolite MEHP on in vitro rat follicular development. Wei Shen Jiao Yian 2010;39(3):268–70, 724.

[174] Gupta RK, Singh JM, Leslie TC, Meacham S, Flaws JA, Yao HH. Di-(2-ethylhexyl) phthalate and mono-2-ethylhexyl phthalate inhibit growth and retard levels of antral follicles in vitro. Toxicol Appl Pharmacol 2010;242(2):224–30.

[175] Wang W, Chakrabarti R, Basavarajappa MS, Hafner KS, Flaws JA. Mono-(2-ethylhexyl) phthalate induces oxidative stress and inhibits growth of mouse ovarian antral follicles. Biol Reprod 2012;87(6):152.

[176] Grande SW, Andrade AJM, Talens CE, Grote K, Golombiewski A, Sternen-Kock A, et al. A dose–response study of follicular maturation exposure to di-(2-ethylhexyl) phthalate (DEHP): reproductive effects on adult female offspring rats. Toxicol Sci 2007;99:114–22.

[177] Lovekamp TN, Davis BJ, Mono-(2-ethylhexyl) phthalate suppresses aromatase transcript levels and estradiol production in cultured rat granulosa cells. Toxicol Appl Pharmacol 2001;172(3):217–24.

[178] Reinberg J, Wegen-Toper P, van der Ven K, van der Ven H, Klingmuelder D. Effect of mono-(2-ethylhexyl) phthalate on steroid production of human granulosa cells. Toxicol Appl Pharmacol 2009;239:116–23.

[179] Snedeker SM. Pesticides and breast cancer risk: a review of DDT, DDE and dieldrin. Environ Health Perspect 2001;109:35–47.

[180] Voogt DJ, Dybing E, Greim HA, Ladedoglu O, Lambrecht C, Tarazona JV. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Crit Rev Toxicol 2000;30:71–133.

[181] Rauhlof V, Bohnet HC, Trapp M, Heesch H, Feichtinger W, Kemetter P. Bio-cides in human follicular fluid. Ann NY Acad Sci 1985;442:240–50.

[182] Jarrell J, Villeneuve D, Franklin C, Bartlett S, Wrixon W, Kohut J. Contamination of human ovarian follicular fluid and serum by chlorinated organic compounds in three Canadian cities. CMJ 1993;148:1321–7.

[183] Younglai EV, Foster WG, Hughes EG, Trim K, Jarrell J. Levels of environmental contaminants in human follicular fluid, serum and seminal plasma of infertile men undergoing in vitro fertilization. Arch Environ Contamin Toxicol 2002;43:121–6.

[184] Gotz F, Thieme S, Donner G. Female infertility – effect of perinatal exposure on reproductive functions in animals and humans. Folia Histochem Cytobiol 2009;47:40–4.

[185] Ayotte P, Giroux S, Devalia I, Avila MH, Farias P, Danis R. DDT spraying for malaria control and reproductive function in Mexican men. Epidemiology 2001;12:366–7.
ICH-harmonised Postnatal follicle genistein on swine granulosa cell function. J Anim Physiol Anim Nutr (Berl) 2010;94:e374–82.

[216] Chen Y, Jefferson WN, Newbold R, Padilla-Banks E. Estradiol, progesterone and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse in vitro and in vivo. Endocrinology 2007;148(8):3580–90.

[217] Medigovic I, Ristic N, Trifunovic S, Manojlovic-Štojanosić M, Milosevic V, Žikic D, et al. Genistein affects ovarian folliculogenesis: a stereological study. Microsc Res Tech 2012;75:1691–5.

[218] Wojtończyk AK. Effects of single and repeated in vitro exposure of ovarian follicles to αp-DDT and p,p' DDT and their metabolites. Pol J Pharmacol 2004;56:465–72.

[219] Zhuang XL, Fu YG, Xu JJ, Kong XX, Chen ZG, Luo LL. Effects of genistein on ovarian follicular development and ovarian life span in rats. Fitoterapia 2010;81:998–1002.

[220] Assies J, Vonk J, Bleker O, Lumey L. Diethylstilbestrol (DES) – related endocrine disturbances in women. A story with no end, yet. Ann N Y Acad Sci 1995;761:369–72.

[221] ICH. Detection of toxicity to reproduction for medicinal products and toxicity to male fertility S5 (R2). ICH harmonised tripartite guideline; 1993.

[222] Muller L, Kikuchi Y, Probst G, Schechtman T, Shimada H, Sofuni T, et al. ICH-harmonised guidelines on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. Mutat Res 1999;436(3):195–225.

[223] EFSA. Scientific opinion on the hazard assessment of endocrine disruptors: scientific criteria for identification of endocrine disruptors and appropriateness of existing test methods for assessing effects mediated by these substances on human health and the environment. EFSA J 2013;11(3):3132.

[224] Bristol-Gould SK, Kreeger PK, Selkirk CC, Kilen SM, Cook RW, Kipp JL, et al. Postnatal regulation of germ cells by activating: the establishment of the initial follicle pool. Dev Biol 2006;298(1):132–48.

[225] Davila JC, Rodriguez RJ, Melchert RB, Acosta D. Predictive value of in vitro model systems in toxicology. Annu Rev Pharmacol Toxicol 1998;38:63–96.

[226] Jackson MR. Priorities in the development of alternative methodolo-gies in the pharmaceutical industry. Arch Toxicol Suppl 1998;20(Suppl.):61–70.

[227] Scholz S, Renner P, Belanger S, Busquet F, Davi R, Demeneix BA, et al. Alternative to in vivo tests to detect endocrine disrupting chemicals (EDCs) in fish and amphibians – screening for estrogen, androgen and thyroid hormone disruption. Crit Rev Toxicol 2013;43(1):45–72.

[228] Segner H. Zebrafish (Danio Rerio) as model organism for investigating endocrine disruption. Comp Biochem Physiol C Toxicol Pharmacol 2009;149:187–95.

[229] Ankley GT, Daston GP, Degitz SJ. Toxicogenomics in regulatory exotoxicology. Environ Sci Technol 2006;40(13):4055–65.

[230] Doerge DR, Twaddle NC, Vanlanningham M, Fisher JW. Pharmacokinetics of bisphenol A in neonatal and adult Sprague–Dawley rats. Toxicol Appl Pharma-col 2010;247(2):158–65.

[231] Fisher JW, Twaddle NC, Vanlanningham M, Doerge DR. Pharmacokinetic modeling: prediction and evaluation of route dependent dosimetry of bisphenol A in monkeys with extrapolation to humans. Toxicol Appl Pharmacol 2011;257(1):122–36.

[232] Herreros MA, Gonzalez-Bulnes A, Inigo-nunez S, Letelier C, Contreras-Solis I, Ros-Rodriguez JM, et al. Pregnancy-associated changes in plasma concentration of the endocrine disruptor di(2-ethylhexyl) phthalate in a sheep model. Theriogenology 2010;73:141–6.

[233] Munn S, Goumenos M. Key scientific issues relevant to the identification and characterisation of endocrine disrupting substances. JRC Scientific and policy reports; 2013.