Endocrine disruptors and ovine reproductive development

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Summary

Declining fertility and perturbations in reproductive development in a variety of species have been linked to exposure to endocrine disrupting chemicals (EDCs), which are ubiquitous in the environment. Ruminants are largely exposed to such chemicals in sewage sludge fertiliser widely used in animal production systems. This has been investigated experimentally through the deliberate exposure of pregnant ewes to sewage sludge fertilised pastures or control pastures treated with inorganic fertiliser containing no detectable EDCs. Perturbations in the developing fetal hypothalamic-pituitary-gonadal axis have been observed in both male and female fetuses. A sub-population of rams exposed both pre- and post-natally exhibited adversely altered testis development. Periods of developmental sensitivity to EDCs have also been identified. An *in vitro* culture system for ovine fetal ovaries has shown that mixtures have a greater effect than individual chemicals. The dog provides a sentinel model of human dietary exposure to chemicals in ruminant-derived food products. Evidence of altered fertility and testicular cancer, along with EDC detection in dog testes and pet foods, support this concept. Established rodent models of EDC exposure provide a means to investigate mechanisms and transgenerational effects. Overall, monitoring and *in vitro* studies carried out in sentinel species and on human tissues, combined with *in vivo* mechanistic studies carried out in ruminants and rodents, provides a clear picture of the global impact of EDCs on reproductive development. In particular, the ovine model of sewage sludge exposure provides the only currently available "real life" model of exposure to a cocktail of EDCs.

Introduction

Since the publication of Silent Spring in 1962, perturbations in reproductive development in a variety of species have been increasingly associated with exposure to anthropogenic environmental chemicals (Carson 1962). The periods of fetal and early post-natal development are particularly sensitive to such exposures and this reflects the exquisite control of fetal

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organogenesis mediated by endocrine, metabolic and other key biological signalling processes. The term "endocrine disrupting chemicals (EDCs)" is frequently used to describe those chemicals which interfere with the endocrine system, however it is now clear that many of these chemicals perturb a wide range of other biological processes and physiological systems including gametogenesis, steroidogenesis, the neuroendocrine and immune systems as well as nutrient partitioning and metabolism. Since all of these processes are crucial for reproductive development, EDCs have the potential to impact on fertility at multiple levels.

Exposure to environmental chemicals

Chemicals described as EDCs are extremely heterogeneous and originate from a diverse range of sources including plastics, industrial lubricants and solvents, detergents, adhesives, pesticides, cosmetics, fertilisers and even analgesic pharmaceuticals. The main route of exposure to EDCs is through the diet (including by drinking), however less characterised modes of exposure also occur through the inhalation of industrial volatile organic chemicals, the production of which has markedly increased in recent years, and by absorption through the skin (Rhind *et al.* 2010). Ruminants are exposed to EDCs largely through modern day domestic animal production systems, reflecting the replacement of costly inorganic fertilisers with processed human sewage sludge (reviewed in Rhind *et al.* 2010). Sewage sludge contains a cocktail of thousands of environmental chemicals, thus constituting a major source of exposure that may be higher (i.e. accumulating over time) than found in the environment (Rhind *et al.* 2013). Exposure largely occurs through the ingestion of soil and to a lesser extent herbage, and this level of exposure has been described as relevant to "real life". Indeed a controlled sewage sludge experimental model has been characterised as a model for human exposure as described in detail below. Ruminants will also be exposed to pollutants through air inhalation although the level of such contamination has not been characterised.

This review is designed (1) to update current understanding on the impact of EDCs on ruminant reproductive development and production and (2) to discuss the relevance of data obtained from ruminant studies to other species, including humans. The relative advantages and disadvantages of alternative animal models will be reviewed and the ruminant work placed into perspective in relation to the global problem of environmental contamination with EDCs.

Ruminants and endocrine disruptors

An experimental ovine model of exposure to a mixture of EDCs was developed by exposing ewes to pastures to which sewage sludge had been applied. Although a variety of pollutants have been detected in sewage sludge, its application had minimal short-term effects on soil chemical concentrations when compared to control pastures fertilised with inorganic fertiliser (Rhind *et al.* 2002, Stevens *et al.* 2003). Similarly, liver concentrations in ewes grazing the pastures showed only slight changes compared to controls (Rhind *et al.* 2005, 2009). Despite these minor effects of sewage sludge exposure on maternal and fetal liver concentrations, the exposure of pregnant ewes from conception to euthanasia at mid or late gestation, or the continued exposure of the offspring to weaning, markedly perturbs the development of all organs of both the male and female reproductive axes (see below).

Similarly, EDCs have been detected in bovine tissues at low concentrations and are derived from contaminated pastures (Petro *et al.* 2010). Given the low levels of EDCs in sheep purposely exposed to sewage sludge and showing clear effects on reproduction, chemical measurements

alone (frequently at only one end-point) do not preclude an environmental impact on ruminant fertility and reproduction. It is of note that polychlorinated biphenyls (PCBs) in milk are elevated in high-producing dairy cows compared to lower yield dairy cows (Petro *et al*. 2010). This reflects the negative energy balance and associated lipolysis that characterises high milk yield cows and is thus a potential source of exposure for the human consumer. Organochlorine pesticides and PCBs have been detected in bovine and ovine semen and human studies indicate that poor sperm quality may be associated with direct exposure to environmental contaminants (Rozati *et al*. 2002, Kamarianos *et al*. 2003b, Zhang *et al*. 2006). In examining bull fertility in a limited geographic region, a strong statistically significant correlation between the levels of pesticide used and the number of spermatozoa in semen was found (Snoj *et al*. 2013). Overall these data indicate that the multifactorial problem of declining fertility in the dairy cow is likely to include one or more environmental components.

Effects on the male

In the human male, exposure to EDCs has been associated with increasing incidences of testicular cancer, congenital abnormalities at birth (cryptorchidism, hypospadias) and a decline in sperm counts in adulthood. Since these problems cluster in specific geographical areas, they probably have a similar aetiology and are collectively referred to as testicular dysgenesis syndrome (TDS) (Skakkebaek *et al*. 2001). The rapid period of time over which this has occurred is indicative of an environmental, rather than principally genetic, cause and there is a vast literature based upon a variety of animal models. Although the decline in sperm counts has received the most publicity, it is also the most controversial. Historically, interest in declining human sperm quality stems from a meta-analysis of studies looking at sperm counts published in 1992 (Carlsen *et al*. 1992). Carlsen reported a decline in human sperm counts between 1938 and 1990 and this was further corroborated by the addition of 47 more studies and re-analysis in 2000 (Swan *et al*. 2000). A number of additional studies have been published, the most notable being that of Rolland *et al*. showing a decline in semen quality across France between 1989 and 2005 (Rolland *et al*. 2013). Nevertheless, these findings have been criticised on the basis of inconsistencies in andrology methods and changes in laboratory quality assurance during the period of analysis (Pacey 2013). More recent studies have cast further doubt on the precise nature of the changes in sperm counts, suggesting a modest reversal (Jorgensen *et al*. 2012). However, they nevertheless show that a worryingly large proportion of young men have below optimal sperm quality. In contrast, studies showing an increased incidence of human male congenital abnormalities at birth (especially in small for gestational age neonates) and testicular cancer are more robust and widely accepted (Jackson *et al*. 1986, Bergstrom *et al*. 1996). A large proportion of genital abnormalities have been reported in rams examined in UK abattoirs (Smith *et al*. 2012), including hypospadias, and these may reflect exposure to EDCs. There is also evidence of a decline in bull semen quality between 1970 and 1985, although post 1985, a number of sperm parameters improved while sperm morphology declined (Wahl & Reif 2009). The variability may reflect potential methodological inconsistencies as suggested for the human. An additional confounder in such studies is the culling of sub-fertile animals which may account for the lack of such a trend in an earlier study (Setchell 1997, Karoui *et al*. 2011).

The sewage sludge model of EDC exposure has been used to investigate the impact of a cocktail of environmental chemicals on testis development. The exposure of pregnant ewes to sewage sludge fertilised pastures from conception to day 110 of gestation reduced both the number of Sertoli cells and Leydig cells in the fetal testis and the hormones produced by

these cell types: inhibin A and testosterone respectively (Paul *et al.* 2005). The perturbation of Sertoli cell function has serious implications for adult male fertility. Sertoli cells are critical for germ cell development and, subsequently, sperm production. Consequently, perturbed Sertoli cell proliferation and/or differentiation will impact on spermatogenesis in adult life. Recently, the period of sewage sludge exposure was extended to include exposure via the mother from conception and the post-natal period until weaning (Bellingham *et al.* 2012), thus ensuring that all periods of Sertoli cell proliferation and development were included (Fig. 1). Five of 12 exposed rams were shown to exhibit significant testicular abnormalities manifest by fewer germ cells, calculated per testis or Sertoli cell, and increased numbers of tubules that only contained Sertoli cells. While these data support the concept that the germ cell is a key target for EDCs (Lagos-Cabre & Moreno 2012), it is uncertain why only a subset of animals appears to be susceptible to EDC exposure. Nevertheless, this finding is more similar to human than rodent studies and factors including differences in grazing or ingestion patterns or differences in maternal and/or fetal metabolism may be responsible. With respect to the latter, polymorphisms in metabolic genes have been reported in humans, e.g. *GSTP1*, *GSTM1* genes and these may modulate polycyclic aromatic hydrocarbon-DNA adducts (PAH-DNA) in human mononuclear white blood cells (Butkiewicz *et al.* 2000). Although sperm counts were unaffected in the 12 rams, repeated ejaculations were not tested and the finding probably reflects the capacity of the ruminant to store sperm.

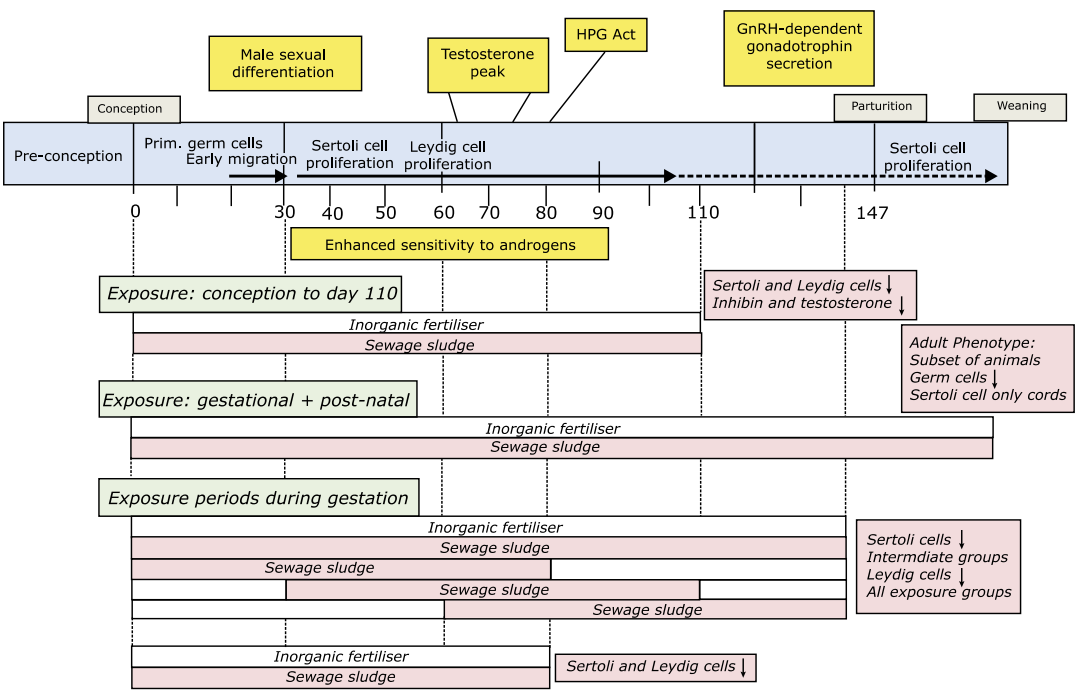

Fig. 1. The effects of EDCs contained in sewage sludge on the developing ovine fetal and post-natal testis. A developmental timeline is presented along with the experimental design and key observations. Pregnant ewes were exposed to sewage sludge fertilised pastures or to control pastures with inorganic fertiliser. Results are presented relating to (a) exposure of the ewes from conception to day 110 followed by euthanasia, (b) exposure throughout pregnancy followed by offspring exposure to weaning and (c) exposed for 80 day periods during early, mid or late pregnancy after which all ewes were euthanized at day 140. A separate subgroup was euthanized at day 80.

These findings raise the following questions: (a) which period(s) of gestation are more sensitive to exposure to EDCs and (b) do EDCs have different mechanistic effects at different stages of gestation. The key stages of fetal testis development are summarised in Fig. 1. To investigate this, we set up an experiment to investigate the impact of exposure of pregnant ewes to sewage sludge during 80-day windows in early, mid or late gestation periods (0-80, 30-110 and 60-140 days) and additional groups comprised ewes exposed continuously (0-140 days) and control non-exposed animals (0-140 days). Our preliminary findings indicate that numbers of Sertoli cells at day 140 of gestation were reduced in the 0-80, 30-110 and 60-140 exposed groups but not in animals continuously exposed from 0-140 days (Lea *et al.* 2010, R G Lea, B Loup, L Purdie, B Mandon-Pepin, M R Amezaga, S M Rhind, C Cotinot, P A Fowler & K D Sinclair 2014, unpublished observations). In contrast, a sub-group of animals exposed to sewage sludge for 80 days and immediately euthanised, did show reduced numbers of Sertoli cells relative to controls. It is possible therefore that between day 80 and day 140, the Sertoli cells underwent increased proliferation so that no difference was observed at the later time point. Leydig cell numbers showed a different pattern to Sertoli cells and were reduced in animals exposed throughout gestation and in the intermediate groups (Lea *et al.* 2010, R G Lea, B Loup, L Purdie, B Mandon-Pepin, M R Amezaga, S M Rhind, C Cotinot, P A Fowler & K D Sinclair 2014, unpublished observations).

In the sewage sludge model, two chemicals were present at greater concentrations in fetal compared to maternal livers: two polychlorinated biphenyl (PCB) congeners (PCB101, 118) and di(2-ethylhexyl)phthalate (DEHP) (Rhind *et al.* 2009). This observation provided the basis for a second ovine model whereby pregnant ewes were exposed from conception to late gestation (day 140) to environmental concentrations of DEHP, PCB (101,118) or a combination of the two. Our preliminary findings indicate that fetuses from ewes exposed specifically to each chemical independently or to a combination of the two, from conception to day 140, exhibit decreased numbers of Sertoli cells (Lea *et al.* 2011). This contrasts with the Sewage sludge exposure model where no differences in Sertoli cell numbers were observed at the same stage of gestation. Although the reason for this intriguing difference between the two ovine models is uncertain, it clearly reflects the increased complexity of exposure to a cocktail of EDCs in model 1 as compared to the more controlled exposure to two selected chemicals as described in model 2.

Effects on the fetal hypothalamus and pituitary gland

Exposure to sewage sludge is associated with perturbations of the fetal hypothalamus and pituitary gland. The regulation of reproduction, by both internal and external environmental factors, occurs at the level of the hypothalamus via gonadotrophin releasing hormone (GnRH) which in turn regulates gonadotrophin (LH and FSH) release from the pituitary gland. Sewage sludge exposure is associated with reduced expression of hypothalamic *GNRH1* mRNA and pituitary gland *GNRHR* mRNA (Bellingham *et al.* 2010), both of which might result in decreased drive within the hypothalamic-pituitary-gonadal axis. The GnRH neurosecretory system is not directly regulated by steroids and therefore not at direct risk of steroidogenic EDC effects. However, the above studies also documented reduced mRNA expression of the neuropeptide kisspeptin (*KISS1*) (Bellingham *et al.* 2009); a steroid-sensitive GnRH afferent and key regulator of the changes in GnRH neuronal activity required for pubertal transition and the generation of the preovulatory LH surge (d'Anglemont de Tassigny *et al.* 2007, Roa *et al.* 2011). The kisspeptin system is an important potential target for endocrine disruption, which could result in altered reproductive function (Tena-Sempere 2010, Losa *et al.* 2011). The effects of sewage sludge at the level of the hypothalamus is further supported by downstream effects in the pituitary

gland, manifest by decreased numbers of LHB immunopositive cells, a reduced number of cells co-expressing LHB and kisspeptin and reduced LHB/oestrogen receptor alpha (ESR1) co-expression in sewage sludge exposed fetuses relative to control animals (Bellingham *et al.* 2009). These changes could have negative effects on normal regulation of reproductive function. Further effects at the level of the hypothalamic-pituitary gland were seen relative to galanin, a neurotransmitter system involved in the relay of information about the internal environment to the GnRH neurosecretory system. Maternal sewage sludge exposure was associated with reduced fetal mRNA expression of galanin receptor isoforms in both the hypothalamus and pituitary gland (Bellingham *et al.* 2010). These studies demonstrate that continuous maternal grazing on sludge treated pastures before and during pregnancy is associated with significant alterations of fetal hypothalamic and pituitary regulators of GnRH function. Due to the sensitivity of the fetus to exogenous factors and developmental plasticity, such effects could have long term consequences for the reproductive neuroendocrine axis.

Effects on the female

A number of reports claim a link between exposure to endocrine disruptors and breast cancer, precocious puberty, premature menopause, polycystic ovary syndrome (PCOS), infertility and endometriosis in women (Crain *et al.* 2008, Bourguignon *et al.* 2013, Braun *et al.* 2013, Caserta *et al.* 2013). Rodent studies have shown that perinatal exposure to environmental chemicals induces precocious puberty, hastens reproductive senescence and induces other abnormalities of female reproductive development (Dickerson & Gore 2007, Gore *et al.* 2011). However, the data are contradictory and the evidence for a consistent negative effect of EDCs at sub-toxicological doses on fertility in women is controversial (Mascarenhas *et al.* 2012, Rostad *et al.* 2013). Part of the problem in humans is the variability and effects of a large number of confounding factors, including occupational vs environmental exposures, many of which cannot be accurately determined. In addition, in human fertility studies the contribution of the partner has to be included in a way that does not directly translate to animal studies (Buck Louis 2013). In ruminants, some EDCs have been reported in bovine follicular fluid however in other papers, EDCs were below the limit of detection (Kamarianos *et al.* 2003a, Petro *et al.* 2012).

In female mammals, including the human, the two key events of ovarian differentiation i.e. oogonia meiosis and follicle formation starts during *in utero* development and only concludes when the oocyte is fertilized (Sarraj & Drummond 2012) (Fig. 2, 3). Meiotic cell division is unique to germ cells, allowing them to produce haploid cells for sexual reproduction. During fetal life, the first meiotic prophase occurs, and then oocytes may remain arrested at dictyonema for many years in long-lived species (Spiller *et al.* 2012). In contrast to rodents, early folliculogenesis occurs *in utero* in ruminants and humans (Fig. 3) (Sathananthan *et al.* 2000, Sawyer *et al.* 2002, Baillet *et al.* 2008), beginning with recruitment of pre-granulosa cells to the oocyte to form the primordial follicle (Pepling 2012). The pool of primordial follicles constitute the ovarian reserve throughout the reproductive period in females (Forabosco & Sforza 2007) and some are stimulated to grow and develop into primary, secondary or antral follicles. Environmental compounds could, potentially, perturb the function of each of the cell types involved (oocytes and follicular cells). Moreover, EDC exposure during the fetal period can reduce the female's lifetime reserve of oocytes, which cannot be renewed, unlike males in which continuous spermatogenesis may quench transient EDC effects.

In the sewage sludge model, exposure of the female fetus *in utero* from conception to day 110 of gestation disrupts fetal ovarian development (Fowler *et al.* 2008). In a follow-up study, the timing of exposure was investigated: ewes were exposed pre-mating, post-conception

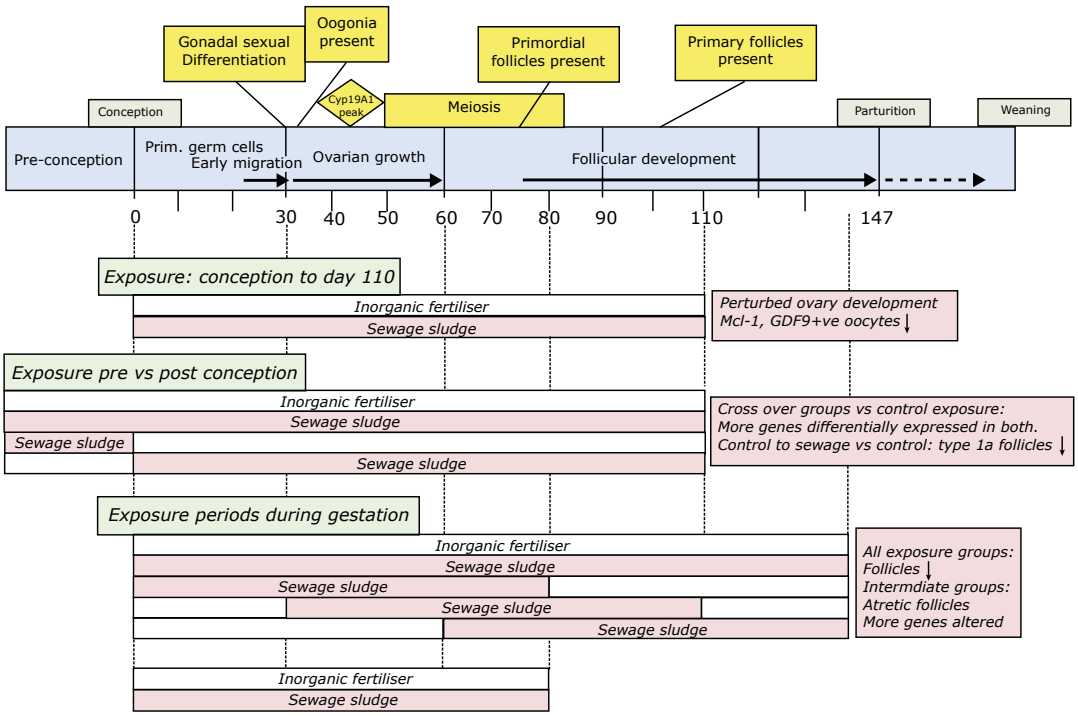

Fig. 2. The effects of EDCs contained in sewage sludge on the developing ovine fetal and post-natal ovary. A developmental timeline is presented along with the experimental design and key observations. Pregnant ewes were exposed to sewage sludge fertilised pastures or to control pastures with inorganic fertiliser. Results are presented relating to (a) exposure of the ewes from conception to day 110 followed by euthanasia, (b) exposure prior to conception or post-conception, with a cross over to control or sewage sludge exposure through to day 110, (c) exposed for 80 day periods during early, mid or late pregnancy after which all ewes were euthanized at day 140. A separate subgroup was euthanized at day 80.

or throughout both periods (Fig. 2). Ewes switched from control to sewage sludge at mating exhibited fewer follicles than those exposed throughout both periods (Bellingham *et al*. 2013). In addition, both cross exposure groups exhibited a greater number of gene expression changes compared to ewes continuously exposed to sewage sludge or control pastures. Thyroid glands from these animals were also most affected in these groups (Hombach-Klonisch *et al*. 2013). Consequently, a change in fetal ovarian environment, even to a reduced exposure to EDCs, appears to be more detrimental than a consistent period of exposure to normal or elevated levels of EDCs. Chronic exposure probably induces an increased detoxification in both mother and fetus highlighting the resilience capacity of the reproductive system.

Further recent studies have investigated which periods of gestation are the more sensitive to chemical exposure, using the experimental design as described above for males. Our preliminary findings indicate that follicle numbers are reduced in all exposure groups, with higher proportions of atretic follicles in the mid and late gestation groups. Moreover, transcriptomics revealed greater numbers of differentially expressed genes in the mid and late gestation windows. Thus similar to the pre- to post-conception switch over study, a change in environment appears to be more detrimental than continuous exposure. However, the preliminary data also suggest that primordial follicle formation, arrest and development may be particularly susceptible to exposure to EDCs

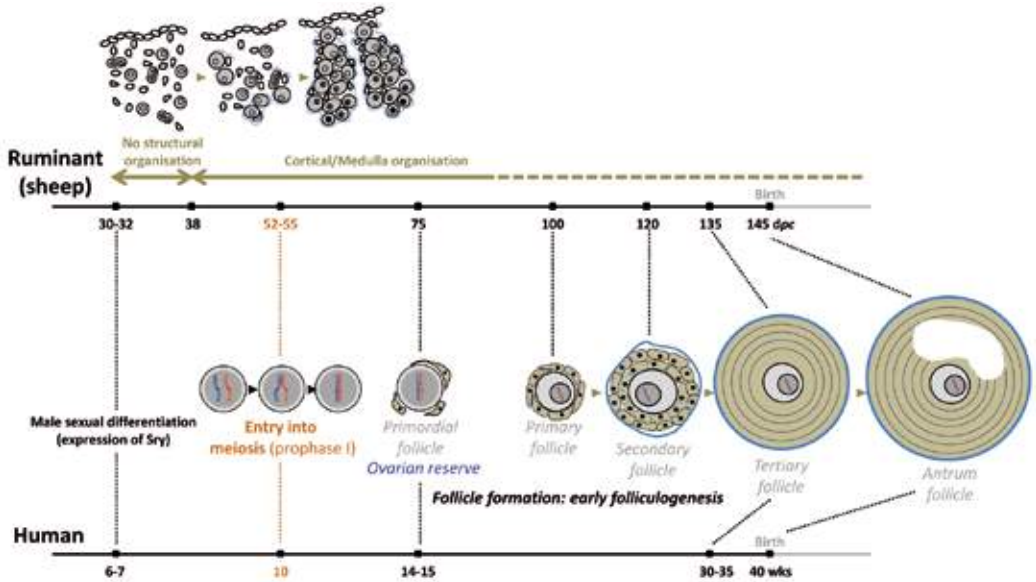

Fig. 3. Timeline of the major cellular events in mammalian ovary morphogenesis. During embryogenesis, the bipotential gonad is formed at the end of the first month of pregnancy in humans and sheep. The switch between male and female occurs a few days later. In the XX gonad, where *SRY* is absent, events in testis morphogenesis do not happen. Specification of the granulosa cell lineage defines the first step of ovary organogenesis (35-38dpc in sheep, 8 weeks in humans). This step is under the control of genes expressed specifically in the female such as *FOXL2* and *RSPO1*. Female germ cells within the germ cell nest begin to enter meiosis at 52-55dpc in sheep (9-10 weeks of gestation in humans) and arrest in the diplotene stage of meiosis I beginning at 75dpc (14-15 weeks in humans). The germ cell nests break down soon after to form primordial follicles. Follicle activation compartmentalizes the somatic cell environment, accompanied by the recruitment of theca cells to the primary follicle (100-110dpc in sheep, 20 weeks in humans).

contained within sewage sludge (Loup *et al.* 2011, R G Lea, M R Amezaga, B Loup, B Mandon-Pépin, M Fraser, C Kyle, C Kerr, M Osprey, Z Zhang, C Allen, L Purdie, K D Sinclair, C Cotinot, S M Rhind & P A Fowler 2014, unpublished observations).

In a separate study, ovine female foetuses, maternally exposed to relatively low concentrations of bisphenol A (BPA), showed altered expression of ovarian steroidogenic enzymes and micro RNAs relevant to gonadal development and function. In support of the sewage sludge model data, gene expression investigated at days 65 and 90 revealed clear gestation day differences (Veiga-Lopez *et al.* 2013). Although we have not yet characterised a female adult phenotype in the sewage sludge model, pre-pubertal exposure to BPA or the organochlorine pesticide, methoxychlor (MXC), has been shown to alter post-pubertal reproductive function. This was manifest by a reduced LH surge amplitude in response to BPA and a delayed onset of the LH surge in response to MXC (Savabieasfahani *et al.* 2006). Interestingly, recent mechanistic studies indicate that this likely reflects altered follicular development and steroidogenesis in the ovary (Abi Salloum *et al.* 2013).

In vitro models parallel *in vivo* studies

Biologically relevant bioassays are needed for dose-response tests of EDC effects on steroidogenesis and gametogenesis and to understand the mechanisms of action by which

EDCs act on their targets to disrupt the normal processes of cellular differentiation. This has been demonstrated in the male where biologically relevant concentrations of the pesticide dieldrin have been shown to reduce testosterone secretion by human fetal testis explants (Fowler *et al*. 2007). However, because disturbances in the endocrine system can affect multiple systems, understanding the effects of EDCs is best accomplished by combining *in vitro* with *in vivo* approaches. Mouse follicle culture models are useful in studying folliculogenesis and oogenesis, (Cortvrindt & Smitz 2002) and in analysing the endocrine disruption of steroid biosynthesis (Myllymaki *et al*. 2005, Lenie & Smitz 2009). One mouse follicle culture model allows the individual and synchronous growth of a large number of follicles from early preantral to preovulatory stages of folliculogenesis (Cortvrindt & Smitz 2002). BPA exposed oocytes present unbalanced chromosome sets, decreased conceptions and are more prone to pregnancy loss than non-exposed oocytes *in vitro* (Can *et al*. 2005, Eichenlaub-Ritter *et al*. 2008, Lenie & Smitz 2009). Furthermore, the effects of several PCB mixtures have been tested on *in vitro* maturation and fertilization of mouse oocytes (Kholkute *et al*. 1994, Kholkute & Dukelow 1997). The addition of PCB mixtures at concentrations ranging from 0.01 to 10 μg/ml, affected the fertilisability of the oocytes. The exposure of bovine oocytes to a mixture of PCBs showed a dose-dependent increase in the percentage of oocytes unable to complete the maturation process to metaphase II. Moreover, the adverse effects of exposure to PCBs extended to fertilization and further embryonic development even after the removal of PCBs from the culture medium (Pocar *et al*. 2001). Exposure of bovine cumulus-oocyte-complexes (COC) to coplanar PCBs affected maturation and a significant increase in apoptosis and *BAX* mRNA expression occurred at doses as low as 100.6 pg/ml (Pocar *et al*. 2005).

The development of an *in vitro* culture system mimicking cellular interactions and supporting the growth and differentiation of fetal ovaries is a crucial step for testing and understanding EDC effects. Indeed, *in vitro* culture systems allow kinetic and dose-dependent studies of toxicological agents in human fetal organs. Organ culture is an approach requiring a small quantity of biological material and may be an alternative to animal experiments. Several groups have developed human fetal ovarian *in vitro* cultures to test the effects of EDC exposures (Angenard *et al*. 2011, Brieno-Enriquez *et al*. 2011, 2012, Poulain *et al*. 2012). Human fetal BPA-exposed oocytes showed a delay in meiotic progression and oocytes exposed to BPA show an up-regulation of genes involved in double strand break generation, signalling and repair (Brieno-Enriquez *et al*. 2011, 2012).

The development and validation of a ruminant *in vitro* organ culture system allows individual chemical (BPA, DEHP and PCB) and mixture effects on early ovarian development to be tested (C Cotinot, personal communication). For this purpose, we optimised the culture conditions for two fetal sheep developmental stages (50-70 days and 90-120 days post coitum (dpc) (Fig. 4). Day 50-70pc corresponds to prophase I of meiotic division of oocytes (11-22 weeks of development of human fetal ovaries, Fig. 3) while 90-120dpc corresponds to ovarian follicle formation (early folliculogenesis, 18-28 weeks in human, Fig. 3). Ovarian folliculogenesis is complex and is regulated by various endocrine, paracrine, and autocrine factors. For these reasons, we developed a long-term organ culture system in which three ovarian fragments per fetal ovary were placed on an insert floating at the surface of culture medium and maintained for 20 days (Fig. 4). This enabled the study of the onset and completion of prophase I meiosis (50-70 dpc), the transition of primordial to primary follicles (90-110dpc) and the transition of primary to secondary or antral follicles (110-130dpc). Mixtures of chemicals, at all environmental concentrations tested (10^{-6}M and 10^{-8}M), induced more changes in ovarian transcriptomes than chemical molecules tested alone (Mandon-Pépin *et al*. 2013). However, there were no significant differences in morphology or follicle numbers between exposed and non-exposed cultures.

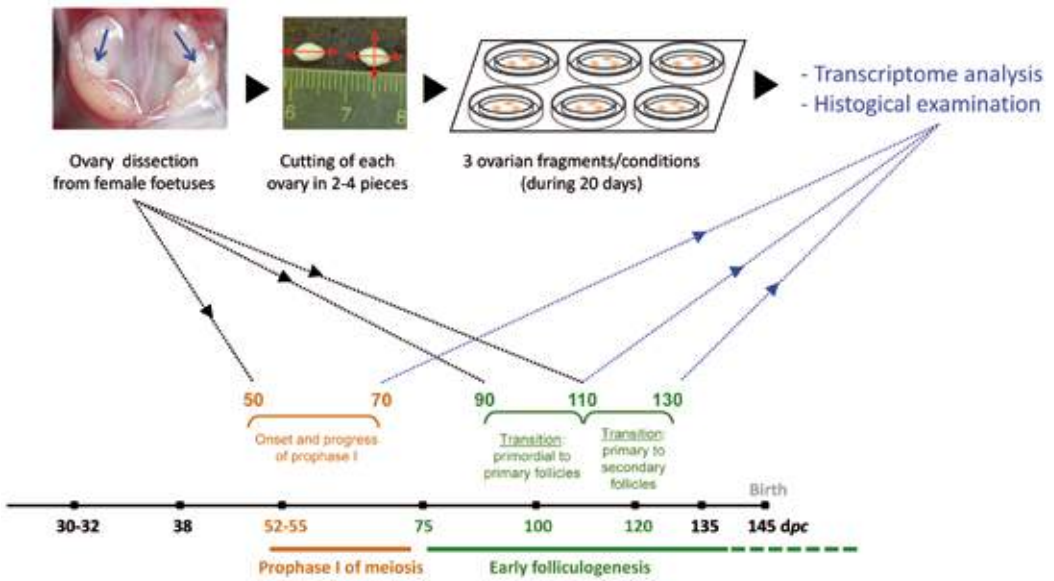

Fig. 4. Ruminant *in vitro* ovary culture system. The ovaries were removed aseptically from the fetuses at different developmental stages. Ovary explants were cut into pieces of a similar size, specifically two (50dpc), or four (90-130dpc) according to the stage. Three ovarian fragments were used for each condition. The filters bearing the explants were floated on 0.5 ml (early stages) or 1.2 ml (later stages) culture medium in tissue culture dishes and incubated at 37°C, in a humidified atmosphere containing 95% air/5% CO_2. The ovaries were cultured for 20 days with the medium being replaced once per day. Cultured ovaries were fixed in Bouin's fluid, embedded in paraffin and cut into 7-μm-thick sections for histological examination. Ovaries were also frozen at -80°C for molecular analyses.

The impact of BPA, DEHP and PCB on the fetal ovarian transcriptome is greater following *in vivo* exposure than following *in vitro* exposure. These differences may reflect i) chemical biotransformation in intact animals that does not occur in organ culture (metabolites were not tested *in vitro*); ii) other chemicals present *in vivo* acting in synergy with tested chemicals or their metabolites; iii) indirect effects *in vivo* through adverse effects on the hypothalamic-pituitary axis or other organs. These findings suggest that *in vitro* studies must be used for the understanding of the action mechanisms of one molecule rather than the characterization of gonad phenotype after exposure to a complex mixture.

Sentinels of EDC exposure: from ruminants to companion animals

In recent years, increased public awareness of toxic environmental chemicals has led to changes in manufacturing practices for food packaging, baby bottles and other products. It follows therefore that heightened awareness of EDCs in agriculture will focus further attention on domestic animal production systems and the potential consequences of dietary exposure to the consumer. A Swedish study examining levels of EDCs in food products identified fish as the primary source of dietary EDCs with dairy and meat coming second and third respectively (Tornkvist *et al.* 2011). While dairy products contain higher concentrations of polybrominated diphenyl ethers (PBDEs), they contribute less than meat overall due to consumption levels (Voorspoels *et al.* 2007). In Belgium, a range of EDCs have been detected in bovine tissues and in Scotland, a similar range has been detected in ovine meat albeit at variable levels depending

on the regional source (Petro *et al*. 2010, Rhind *et al*. 2011). Therefore the presence of EDCs in ruminant derived food products and contamination of agricultural land with toxic substances contained in processed human sewage sludge fertiliser, pesticides and herbicides emphasizes the importance and relevance of a ruminant sentinel model.

From the first reference in the academic literature a century ago of the canary being used to detect dangerous levels of carbon monoxide (Burrell & Seibert 1914), through the 'dancing' cats of the Minamata Bay region of Japan showing symptoms of methyl mercury poisoning (Rabinowitz *et al*. 2009), to bees and their honey being used as a biomonitor of pesticide use (Panseri *et al*. 2014) and brominated flame retardants in the environment (Mohr *et al*. 2014), the idea of a sentinel model is not new. In identifying an appropriate sentinel or biomonitor (term often used interchangeably with sentinel) for a target species, interspecies variability due to longevity, trophic level and diet, metabolism and habitat must be considered. Examples of sentinels are summarised in Table 1.

Table 1. Literature matrix showing strength of sentinel models with the human as the target species.[1]

Sentinel Model	Research Focus	Animal Category	Criteria Match	Reference
Feline	Low level chronic exposure to PBDE	Companion	Trophic level, Habitat	(Dye *et al*. 2007)
Canine	Metal concentration in tissue.	Companion	Trophic level, Diet, Metabolism, Habitat	(Lopez-Alonso *et al*. 2007)
Canine	PCBs	Companion	Trophic level, Diet, Metabolism, Habitat	(Schilling *et al*. 1988)
Canine/ Feline	TCDD	Companion	Trophic level, Diet,[2] Metabolism, Habitat[2]	(Schilling & Stehr-Green 1987)
Canine	Lead	Companion	Trophic level, Diet, Metabolism, Habitat	(Enriquez *et al*. 2009)
Ovine	Tissue levels of many EDCs, reproductive development and adult phenotype	Ruminant	Habitat: exposure to EDCs in sewage sludge fertilizer	(Rhind *et al*. 2009)
Bovine	Sperm quality	Ruminant	Habitat: as Ovine	(Wahl & Reif 2009)
Bovine	Tissue and fluid levels of PCBs, OCPs, PBDEs	Ruminant	Habitat: as Ovine	(Petro *et al*. 2010)
Primate	Germ cell differentiation/ testicular germ cell tumour	Wildlife	Longevity, Trophic level, Diet, Metabolism	(Mitchell *et al*. 2008)
Mink	Mercury, PCB	Wildlife	Trophic level, Diet	(Basu *et al*. 2007)
Polar Bear	OHCs	Wildlife	Longevity, Trophic level, Diet, Metabolism	(Sonne *et al*. 2006)
Marine Mammals	Anthropogenic toxins	Wildlife	Longevity, Species dependent	(Bossart 2011)
Domestic Animals	EDCs	General	Species dependent	(Majdič 2010)
Companion Animals	Public health/environmental contaminants	Companion	Species dependent	(Schmidt 2009)
Mammals	Toxic environmental contaminants	General	Species dependent	(O'Brien *et al*. 1993)
Animals	Environmental Chemicals	General	Species dependent	(van der Schalie *et al*. 1999)

[1]Criteria considered in model validity are longevity, trophic level, diet, metabolism and habitat. [2]Canine only. EDC: endocrine disrupting chemical, PBDE: polybrominated diphenyl ether, PCB: polychlorinated biphenyl, TCDD: tetrachlorobenzodioxin, OCP: organochlorine pesticides, OHC: organohalogen pollutants

Companion animals have been used as sentinel models for human exposure to EDCs. Established in Japan, the feline model is a useful sentinel for methyl mercury (Takeuchi *et al.* 1977) and PBDE in humans (Dye *et al.* 2007, Guo *et al.* 2012). Similarly, the dog has been proposed as a sentinel of human exposure to EDCs and we have recently identified a decline in canine semen quality which parallels that in humans (Byers *et al.* 2012) while an increased incidence of testicular tumours in dogs is evidence for canine TDS (Grieco *et al.* 2008). We have also identified specific EDCs in adult dog testes and the same chemicals in a range of commercially available dog foods is indicative of a nutritional link (Byers *et al.* 2012). Since the dog occupies the same environment as the human and is a carnivore, it may be an excellent sentinel of human exposure to EDCs particularly, but not exclusively, via the diet. Since a majority of the meat consumed by humans and companion animals is derived from ruminants, studies of the dog as a sentinel for human EDC exposure provides key information on the potential impact of ruminant meat-derived EDCs on the consumer. Interestingly, the accumulation of EDCs in Greenland people through the consumption of a high fat marine mammal based diet has been modelled by feeding Minke whale blubber fed to sled dogs (Verreault *et al.* 2008, Bonefeld-Jorgensen 2010). EDCs were not only traced through to adipose tissue in these animals but also to the offspring at pre- and post-weaning stages (Kirkegaard *et al.* 2011).

The use of sentinel models, especially for chronic exposure to ambient levels of environmental EDCs, provides insight not gained from experimental models. The canine sentinel model, based on the consideration of longevity, trophic level and diet, metabolism and habitat, is most useful in understanding EDCs and human health. The dog may be a useful biomonitor of dietary exposure for other target species such as polar bears (Verreault *et al.* 2008), which proves the versatility of these sentinel models. In the case of the ruminant, the bull has already contributed as a sentinel model to the weight of the argument suggesting a temporal decrease in semen quality.

Rodent models of EDC exposure: transgenerational and epigenetic effects

Many published studies on the effects of EDCs use rodent models, and often focus on single chemicals applied at relatively high concentrations *in vivo*. However, this by no means applies to all rodent studies. Indeed, a rat model of *in utero* exposure to di(n-butyl) phthalate at human relevant levels exhibits many of the features characterising human TDS (Fisher *et al.* 2003). Moreover a window of sensitivity (masculinization programming window) has been identified during which environmental chemicals are effective in perturbing testis development and inducing TDS like symptoms in male offspring (Welsh *et al.* 2008). This therefore provides a model whereby mechanisms can be established and tested in other sentinel and experimental model species. A key advantage of the rodent model is the investigation of mutigenerational or transgenerational effects of EDC exposure *in utero* and/or during lactation. Pocar *et al.* recently reported that the exposure of mice during pregnancy and lactation to a mix of PCB congeners (101 and 118) at concentrations comparable to human exposures impaired testis development, sperm counts, ovarian development and oocyte quality in F1 offspring (Pocar *et al.* 2012). Furthermore, reduced sperm viability and altered seminiferous tubule development up to the F_3 generation were observed.

There is compelling evidence from rodent studies that some of the effects of environmental chemicals, such as diethylstilbestrol (DES) (Alworth *et al.* 2002) and the isoflavonoid

phytoestrogen genistein (Tang *et al*. 2008), are mediated via epigenetic changes to DNA methylation. Anway and colleagues (Anway *et al*. 2005) went further to demonstrate male-germline mediated epigenetic transgenerational effects of the anti-androgenic compound vinclozolin (an agricultural fungicide) and the oestrogenic compound methoxchlor on gonadal sex determination, which persisted to the F_3 generation in the rat. However, because the F_0 gestating female is exposed to environmental stimuli, both the F_1 embryo and F_2 generation germ line are also directly exposed (Jirtle & Skinner 2007). Consequently, for epigenetic modifications to chromatin to be considered a plausible mechanism for transgenerational inheritance of phenotypic change, effects need to persist to at least the F_3 generation. However to date there is no direct link between exposure to environmental chemicals and epigenetic dysregulation of gene expression that relate to aberrant gonadal development and adverse fertility phenotypes in farm animal species. This is primarily because these mechanisms have not been investigated in this context in these species.

Conclusions

The evolutionary void between human and ruminant is even wider than between human and rodent. However, in many ways the patterns of reproductive and developmental events are much more similar between ruminant and human while key developmental events, such as primordial follicle formation, do not occur *in utero* but, instead, occur post-natally in the rodent. While in mechanistic terms there is much in common between ruminants, humans and rodents in terms of the processes, the events occurring post-natally in the rodent will, inevitably, be exposed to different chemicals (e.g. via lactation) and environmental influences than the human or ruminant e.g. direct exposure to the environment instead of *in utero* via the mother. In terms of digestion and metabolism there are, of course, some marked differences and similarities between ruminants, humans and rodents, such as hepatic recirculation (Mazur *et al*. 2010), which can be associated with considerable differences in internal chemical exposures despite a similar external dose. Notwithstanding the role of the rumen in exacerbating human-ruminant differences in digestion, internal dosage is still likely to be more similar between humans and ruminants. In one respect almost all species conduct their pregnancies at relatively low oestrogen levels compared with humans (human gestational steroid reviewed in Kuijper *et al*. 2013). In addition, the activity of the human fetal liver as a detoxification and steroidogenic organ (O'Shaughnessy *et al*. 2013) is markedly different from the rodent. The take-home message is that endocrine disruption is best studied in a number of laboratory and sentinel species in order to properly account for inter-species differences in exposure, internal dosage, metabolism and phenotypic response (Fig. 5). The sheep, especially housed outdoors with biosolids exposure is therefore an excellent model to better understand the reproductive consequences of endocrine disruption.

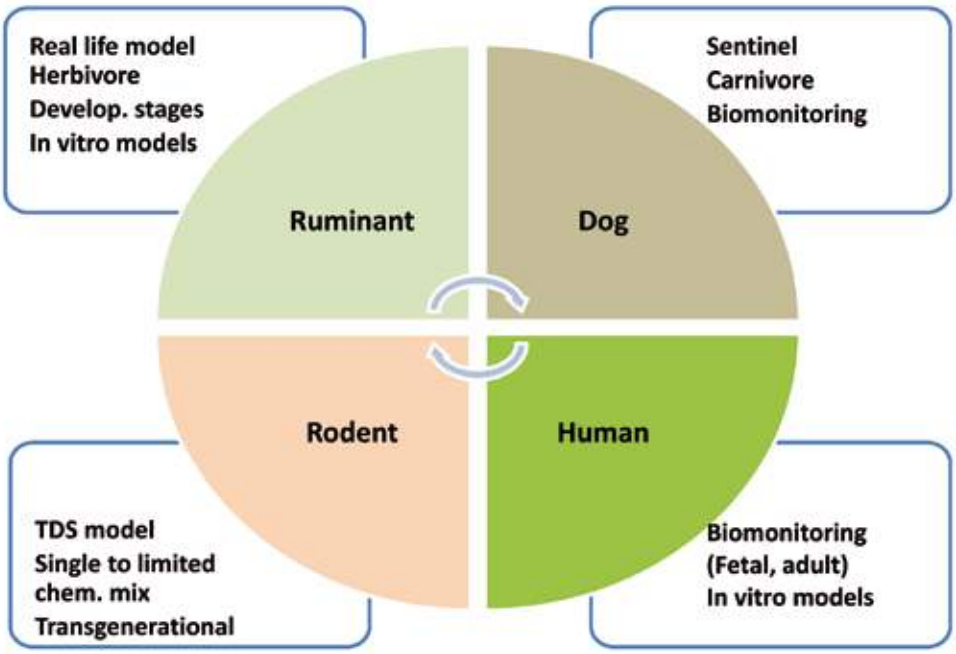

Fig. 5. Roles of experimental animal models and sentinel species in determining the reproductive impact of endocrine disrupting chemicals. **Ruminant:** Sheep exposed to a cocktail of chemicals contained in sewage sludge constitute a real life ruminant (herbivore) model of human exposure. The long period of gestation and well characterised stages of fetal development facilitate the elucidation of EDC effects at specific developmental stages. An established *in vitro* ovary culture system enables individual chemical and mixture effects to be determined. **Rodent:** An established rodent model of testicular dysgenesis syndrome (TDS) provides information on mechanism. The short gestation period has been exploited to investigate transgenerational effects. **Dog:** The dog is a recognised (carnivore) sentinel for human exposure to EDCs and gonad chemical analysis provides a means of biomonitoring. **Human:** Analysis of chemical profiles in adult and fetal samples/tissues provides a means of direct biomonitoring and gonad explant culture systems enable the direct effects of EDCs on human fetal tissues to be determined.

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