Molecular and Cellular Endocrinology 376 (2013) 156–172

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Exposure to chemical cocktails before or after conception – The effect of timing on ovarian development ☆

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ARTICLE INFO

Article history:
Received 20 November 2012
Received in revised form 11 June 2013
Accepted 11 June 2013
Available online 20 June 2013

Keywords:
Ovary
Development
In utero exposure
Environmental chemicals
Mixtures
EDCs

ABSTRACT

Exposure of female fetuses to environmental chemicals (ECs) during pregnancy results in a disturbed ovarian adult phenotype. We investigated the influence of pre- and/or post-conception exposure to low-level mixtures of ECs on the structure and function of the fetal ovine ovary. We examined ovarian morphology, expression of oocyte and granulosa cell-specific genes and proteome. Female fetuses were collected at day 110 of gestation, from dams exposed continuously until, and after mating, by grazing in pastures treated with sewage sludge as a fertiliser (TT) or in control fields treated with inorganic fertiliser (CC). In addition, in a cross-over design, fetal ovaries were collected from dams maintained on sludge pastures up to the time of mating but then transferred to control pastures (TC) and, reciprocally, those transferred from control to treated pastures at mating (CT). On examination, the proportion of type 1a follicles (activating primordial follicles) was significantly lower in animals from the CT groups compared with CC and TT groups ($P < 0.05$). Of the 23 ovarian gene transcripts studied, 14 were altered in the ovaries of exposed fetuses (CT, TC, and TT) relative to controls, with the largest number of changes observed in cross-exposure pattern groups (CT or TC). Continuous EC exposure (TT) produced fewer transcript alterations and only two genes (*INHBA* and *GSN*) presented differential profiles between CC and TT. Fetal ovarian proteome analysis (2-DE gels) showed, across all exposure groups, 86 differentially expressed protein spots compared to controls. Animals in the CT group exhibited the highest number (53) while TC and TT presented the same number of affected protein spots (42). Fetal ovarian proteins with altered expression included MVP (major vault protein) and several members of the heat-shock family (HSPA4L, HSP90AA1 and HSF1). The present findings indicate that continuous maternal EC exposure before and during gestation, are less deleterious for fetal ovarian development than a change in maternal EC exposure between pre and post-conception. The pathways by which the ovary responds to this chemical stress were common in TT, CT, TC exposed foetuses. In addition to the period of pregnancy, the pre-conception period appears also as crucial for conditioning long-term effects of EC exposure on ovarian development and primordial follicle reserve and hence future fertility.

1. Introduction

Environmental chemicals (ECs), including endocrine disrupting compounds (EDCs), adversely affect multiple physiological systems in a wide range of animal species (Colborn et al., 1993; Fowler et al., 2012; Magnusson, 2012; Rhind, 2005). Effects on reproductive function of controlled exposures, to large amounts of single chemicals, have been studied using laboratory rodents or cell cultures (Craig et al., 2011; Gray et al., 2000; Meerts et al., 2001), while in other studies, abnormalities have been retrospectively linked to environmental exposure to abnormally high levels of individual

Abbreviations: Anti-ACTB, anti-β actin; DEHP, diethylhexylphthalate; ECs, environmental chemicals; EDCs, endocrine disrupting chemicals; FSH, follicle stimulating hormone; LH, luteinising hormone; WB, Western blot.

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http://dx.doi.org/10.1016/j.mce.2013.06.016

pollutants (Guillette et al., 1994; Wolfe et al., 1995). Using sheep as an experimental model and sewage sludge applications to pasture as a means of exposing them to environmental levels of a mixture of ECs, the relationships between "everyday", background EC exposure, associated tissue chemical burdens and physiological status have begun to be addressed. These studies have shown that small increases in tissue concentrations of selected ECs (Rhind, 2002, Rhind et al., 2005), were associated with perturbations of hypothalamo-pituitary (Bellingham et al., 2009, 2010; Rhind et al., 2010a) testicular (Bellingham et al., 2012; Paul et al., 2005) and ovarian physiology (Fowler et al., 2008) in the offspring of sludge-exposed mothers. Whilst these animals had been continually exposed to sludge treated pastures, and thus theoretically ECs, grazing at different sites and changes in environmental conditions probably means that such EC exposure is not at a constant level over an animal's lifetime. It has been previously shown that among ewes reared in fields showing accumulation of phthalates, the percentage of animals with presence of DEHP increases with age. This is due in part to a longer exposure but it likely also due to the mobilization of body reserves which occurs during pregnancy (Herreros et al., 2010). Therefore, fetuses may only be exposed to biologically significant concentrations of ECs during specific periods of development and the physiological response associated with exposure may be influenced by the timing and extent of placental and fetal hepatic biotransformation of ECs (e.g. O'Shaughnessy et al., 2011). The sensitivity of a fetus to ECs may also differ depending on the time/stage of development.

In addition, the level and composition of the chemical mixture to which animals are exposed may differ according to its origin, i.e. through environmental/dietary exposure or as a result of maternal tissue mobilisation during gestation (Bigsby et al., 1997). In an extension of the ovine studies indicated above, we examined whether such differences in the profile, timing and/or rate of EC exposure might influence the physiological responses induced in the exposed animals, in particular, how did the timing of maternal, and thus fetal exposure, affect their respective tissue EC concentrations? Tissue EC concentrations were assessed in 3 different groups of ewes and their offspring where the mothers were exposed to sludge-treated pastures during different life stages: (1) throughout life (fetal exposure to ambient and maternally stored ECs), (2) before mating, but not thereafter (fetal EC exposure primarily attributable to release from mobilised tissue), (3) during the first 110 days of gestation (principally ambient exposure), a period of rapid tissue differentiation and development in the fetus which were then compared to unexposed (control) animals. Despite very few differences in EC profiles in either maternal or fetal sludge-exposed tissues, many previous studies using this model (Rhind et al., 2011, 2009, 2010b) have shown that the absence of significant differences in measured tissue burden at slaughter is not necessarily indicative (or predictive) of the previous pattern of exposure or of the occurrence of physiological effects.

The present paper reports the results of extensive investigations into effects of maternal exposure to sludge-treated pastures on fetal ovarian physiology, in three exposure groups and a control group maintained on sludge free pasture throughout life outlined above. Effects on fetal ovarian physiology were investigated by means of changes in fetal ovarian histomorphology, endocrinology, the proteome and the transcription of essential genes for fetal ovarian development.

2. Material and methods

2.1. Ethics statement

All animals used in this study were treated humanely with due consideration to the alleviation of pain, suffering, distress or lasting harm according to the James Hutton Institute's (formerly the Macaulay Land Use Research Institute) Local Ethical Committee and fully licensed by the United Kingdom's Animals (Scientific Procedures) Act 1986 under Project License authority (60/3356). Project license approval automatically includes a prior ethical committee evaluation and approval process, is legally binding, and a legal necessity. All in vivo components of the study were conducted at the James Hutton Institute under this legal framework operating at the highest ethical standards. Therefore, separate ethics approvals from the individual research institutions receiving ex-vivo tissue samples (University of Glasgow, University of Aberdeen, INRA and MRC Centre for Reproductive Biology) are superseded.

2.2. Experimental animals, management and monitoring

The experimental design has been described previously (Hombach-Klonisch et al., 2013; Rhind et al., 2010b) and is summarised in Fig. 1. Briefly, groups of Texel ewes from a single flock were maintained at conventional stocking rates at the James Hutton Institute's research station at Hartwood in Scotland. Pastures were fertilised twice annually (early spring and late summer) with either thermally dried digested sewage sludge (2.25 metric tons of dry matter/ha; Treated; T) or inorganic fertiliser containing equivalent amounts of nitrogen (225 kg /ha/year; Control; C). Ewes of all experimental groups were mated to Texel rams at a synchronised oestrus during the normal breeding season (November). Ewes were randomly assigned to one of 4 experimental groups (n = 12/group). Two groups were exposed to either the sludge-treated (TT) or control (CC) pastures, throughout their breeding lives up until the time of slaughter at 110 days gestation. Two additional groups of ewes (n = 12/group), one of which had been maintained on sludge-treated and one on control pastures, were then swapped onto, and maintained on, the opposite pasture type up until slaughter at 110 days gestation giving rise to CT and TC groups (Fig. 1). Ewes from the CT and TC groups were mated approximately 2 weeks after the CC and TT ewes for enforced husbandry reasons (Additional Information found in Appendix A). Day 110 of gestation was selected for tissue collections since the sheep fetal ovary contains the main classes of follicles by this stage of development: primordial, primary, preantral and few small antral (Fowler et al., 2008; McNatty et al., 1995; Sawyer et al., 2002) at this time.

2.3. Tissue collection

At slaughter, maternal and fetal liver (from one fetus/ewe) was collected for determination of EDC content, as reported previously (Rhind et al., 2010b). Fetal ovaries were either snap-frozen in liquid nitrogen for mRNA extraction or fixed for 5 h in Bouins solution before storage in 70% ethanol prior to processing to wax for histological analysis. Fetal and maternal blood samples were also collected and the plasma stored at −20 °C for hormone measurements.

2.4. Hormone assay

Plasma estradiol concentrations were estimated in duplicate, diethyl ether extracts of 200 μl of plasma (3 assays) using a modification (Evans et al., 1994) of the Serono Estradiol MAIA assay (Serono-Baker Diagnostics, Inc., Allentown, PA). Mean intra- and inter-assay coefficients of variation (CV) were 8.5% and 6.15% respectively and assay sensitivity averaged 0.27 pg/ml. Plasma concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured, in duplicate samples (0.1–0.2 ml), by radioimmunoassay that has been described and validated previously for sheep (McNeilly et al., 1986); the assay standards used were NIDDK-FSH-RP2 and NIH-LH-S12, and assay sensitivities were 0.1 and 0.2 ng/mL for FSH and LH, respectively.