The 6th International Scientific Conference "Animal Biotechnology 2018"
NATIONAL AGRICULTURAL AND FOOD CENTRE
RESEARCH INSTITUTE FOR ANIMAL PRODUCTION NITRA
Slovak J. Anim. Sci., 51, 2018 (4): 161–170

ISSN 1337-9984

THE INTERPLAY OF MOUSE AND PORCINE BIOMODELS FOR ENDOCRINE DISRUPTOR REPRODUCTIVE IMPAIRMENT

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ABSTRACT

Individual mammalian organisms, used as a biomedical model, represent partial benefits and drawbacks for transfer of knowledge into human medicine. Whereas some models, such as rhesus monkey, are more similar to human, its usage is difficult and controversial. In contrast, well-designed combination of several mammalian models shows effective way how to verify a hypothesis and, based on conservativeness of observed phenomenon, implicate it into the human medicine. The aim of this overview is to compare individual mammalian biomodels, with respect to reproductive biology, elucidation of reproductive toxicology and, in particular, to an effect of endocrine disruptors. The literature search is supplemented with own experimental designs and observations obtained using mouse and porcine models. Our findings point out advantages of *in vivo* exposure of oocyte, sperm or embryos of outbred mice to endocrine disruptors followed by verification using porcine *in vitro* treatment of cumulus-oocyte complexes with identical endocrine disrupting compound. In summary, the association between *in vivo* and *in vitro* exposure suggests about highly-relevant and available model for testing of endocrine disruptors and risk assessment for human reproductive health.

Key words: oocyte; embryonic development; endocrine disruptor; bisphenol; reproductive health

INTRODUCTION

Plastic compounds (bisphenols) (Žalmanová *et al.*, 2016), pesticides (DDT, vinclozoline, pyrethroids) (Petr *et al.*, 2013; AL-Hussaini *et al.*, 2018), flame retardants (organophosphates) (Carignan *et al.*, 2018) and others are continuously introduced into the environment during industrial production and plastic usage. These compounds are considered to be environmental pollutants and, in many cases, represent dangerous agents with endocrine disrupting effect (Gingrich *et al.*, 2018). Endocrine disruptors, firstly defined by Colborn *et al.*, (1993) are generally described as being: i) permanent exposure to humankind due to their ubiquitousness, ii) very low exposure doses that do not achieve toxic effects in a dose-dependent manner, iii) non-monotonic curve of endocrine disruptor effect, often results in more deleterious effects of lower doses than higher ones (Daston *et al.*, 2003; Vandenberg *et al.*, 2012) and iv) temporal window of sensitivity to effects of endocrine disruptors is often observed during specific stages of ontogenetic development, when targets for ED are temporarily present in an organism.

Without doubt, human health is under intensive spatiotemporal pressure of sub-toxic doses of several endocrine disruptors. These agents

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Received: October 1, 2018
Accepted: October 11, 2018

are carried to human organism through various paths of exposure, such as dermal contact, inhalation and, so often, via consumption of contaminated beverages and/or foodstuffs. Although endocrine disruptors are circulating in the bloodstream immediately after the exposure, it remains unexceptionally without clinical manifestations. These circumstances should be taken into account for the appropriate selection of an adequate biomodel for testing individual endocrine disruptors, particularly with concern to which signal pathways are expectedly disrupted.

In contrast to apparent harmlessness, human and animal reproductive health is significantly affected through three different mechanisms for endocrine disruptor action: the first, genetic or genotoxic effect (Smith-Oliver and Butterworth, 1987; Tiwari *et al.*, 2012), is mostly unapplied because of sub-toxic doses. Others, non-genetic (Viñas and Watson, 2013) and epigenetic (Skinner, 2014) molecular action frequently occur. There are major manifestations of these modes of action: hormone imbalance with many physiological consequences and inadequate epigenome changes, respectively. In addition to systemic organism-wide response to hormone imbalance (i. e. non-genetic effect), transgenerational inheritance of epigenetic endocrine disruptor-driven effect personates the risk for further generations (Nilsson *et al.*, 2012; Rodgers *et al.*, 2015).

Poor health from endocrine disruption-induced hazards, abundant in human and animal health, is obvious. Accordingly, the risk assessment is inevitable. Biomonitoring data have been systematically collected through many countries, however, models serve experimental simulation of endocrine disruptor exposure followed by comprehensive analyses. In general: there are several different models, such as *in vivo*, *in vitro* and *in silico*. Although the *in silico* model means detailed computational simulation with many advantages, *in vivo* and *in vitro* models are based on real live elements (i.e. the animal and the cell, respectively). Both biomodels have individual benefits and drawbacks and, therefore, the selection of the model represents an essential step in the risk assessment of individual endocrine disruptors.

Biomodels

Many well established simple invertebrate models, such as nematodes (roundworm *Caenorhabditis elegans*), insects (fruit fly *Drosophila melanogaster*) and echinoderms (sea urchins), have been historically used for a description of biological processes (Kuo *et al.*, 2000; Wang *et al.*, 2004; Al Rawi *et al.*, 2011), and more recently for pollutant assessment (Bošnjak *et al.*, 2014; Quesada-Calderón *et al.*, 2017; Zhou, 2018). Due to the sensitivity, more often water living organisms (i. e. snails, molluscs), invertebrate species represent easy available models for pollutant screening for vertebrates (deFur, 2004). Similarly, non-mammalian vertebrate species (zebrafish *Danio rerio*, african clawed frog *Xenopus laevis*) are frequently used in toxicological and similar studies (Iwamuro *et al.*, 2003; Barros *et al.*, 2018). There are several specific possibilities, such as neurohormonal study (molluscs) (Shomrat *et al.*, 2010), juvenile or pheromone investigation (insects) (Bomtorin *et al.*, 2014), availability of gametes (*Xenopus* spp.) (Gelaude *et al.*, 2015), that favour the utilization of these lower-class animal species as a bioindicator of environmental pollution (Bouchard *et al.*, 2009; Marquis *et al.*, 2009; Blahova *et al.*, 2018). The presence of the endocrine system and the conservativeness of some hormonal signalling offer the possibility to test endocrine disrupting effect (Keay *et al.*, 2006). However, the complex signal pathways in mammals possibly affected by endocrine disruptors remains largely unknown and the studying molecular action of such compounds requires mammalian biomodels. Presumable targeted pathways in these mammalian models are considered to be more similar to human than non-mammalian species.

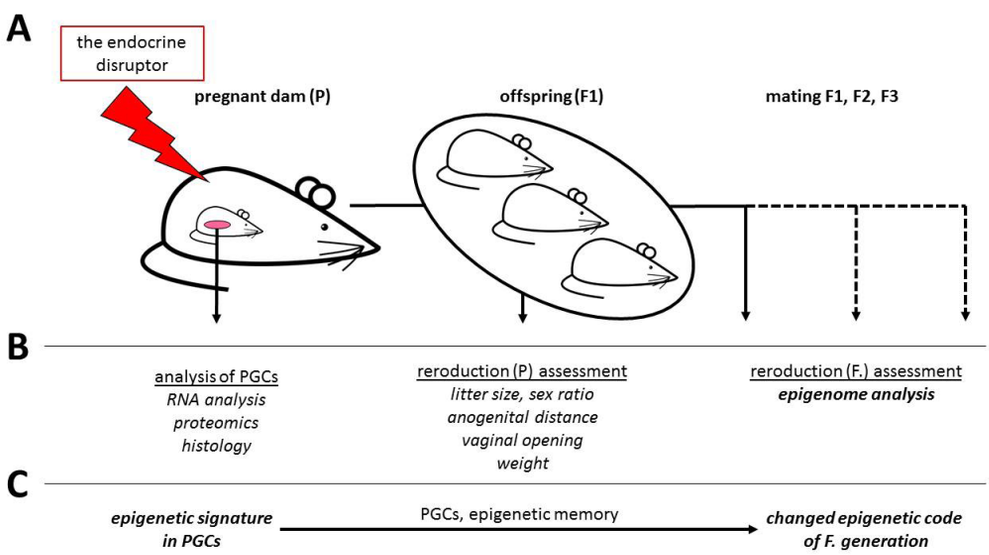
Rodent models for *in vivo* exposure

Laboratory rats and mice have many advantages and are commonly used for *in vivo* testing the effects of endocrine disruptors. The possibility of *in vivo* treatment of primordial germ cells, oocytes, spermatozoa and/or embryos allows the major benefit for evaluating endocrine disruptor effects on such reproductive functions. There are several ways of administrating compounds of interest,

with respect to considered path of endocrine disruptor exposure. Oral administration through drinking water, feed, dosage in corn oil, or more invasive subcutaneous and intraperitoneal administration of tested compound dissolved in a vehicle (saline, corn oil, etc.) are available and commonly used. Although injections can provide exact dosage of the compound [expressed as ng (µg, mg) g (kg) $bw^{-1}.day^{-1}$], the invasiveness makes these approaches more laborious and stressful for the animal facility and animals, respectively. Moreover, oral intake of endocrine disruptors via water or feed more precisely simulate the real exposure of human population, where systemic organism response, as well as pharmacokinetics of tested compound, are expected.

Rat and mouse model are desirable for a few different experimental schemes, combining each other. Individually, the acute (days) or chronic (weeks) exposure can be used as simple assessment of tested compounds on reproductive functions. More sophisticated approaches are available, such as *in utero* exposure or breastfeeding exposure, treating the pregnant and nursing dam, respectively.

In utero exposure length differs with respect to subsequent physiological features: embryo transport through fallopian tube occurring at embryonic day E0.5 – E3.5, blastocyst hatching at E3.5-4.5, embryo implantation and placental development at E6.5 – 9.5 (Slevin *et al.*, 2006), primordial germ cell (PGC) migration and epigenetic reprogramming at E7.5 – 14.5 (Doyle *et al.*, 2013), including progressive mitosis of PGCs, followed by organogenesis until delivery (Chen *et al.*, 2013). The epigenetic reprogramming window can cause transgenerational inheritance of endocrine disruptor-affected epigenome (Figure 1) and the exposure between E7 – E14 allows the study of this effect separately (Rahman *et al.*, 2017). On the contrast, whole-gestation exposure mimics the real endocrine disruptor impact. However, the beginning of exposure at E7.5, when the strict placental exposure of the foetus is considered, seems



(A) Environmental influence of the endocrine disruptor is capable of modifying the epigenome of foetuses (F1 generation) *in utero* of directly exposed dams (P generation). When the disruptor affects primordial germ cells (PGcs), the 3rd generation of offspring (F2) is affected. Although the rewriting of genetic information (mutation) does not occur, the impact of environment to parental (P) generation is inherited.
(B) Thorough molecular analyses of PGCs, gametes, and embryos (RNA analysis, proteomics and histology), as well as non-invasively achieved data of clinical reproduction (litter size, sex ratio, weight) are required. Oestrogenic or androgenic effects of endocrine disruptors are trackable through selected features (anogenital distance and puberty onset via vaginal opening).
(C) The further transmission of environmental factor-modulated epigenome to F3 and other generations through the epigenetic memory is assumed.

Figure 1. Presumed transgenerational effect of endocrine disruptors

to be more proper and the treatment is equivalent to the transplacental exposure.

In addition to *in utero* exposure, the dosage of milking dams offers the exposure through breastfeeding. This approach serves as a unique model for bisphenol evaluation, for reasons as follows: i) general sign of breastfeeding is exclusive milk intake by pups, ii) liposolubility in high-fatty diet, iii) extreme sensitivity of juvenile organisms to endocrine disruption, iv) breastfeeding as susceptible exposure window for gametogenesis at early stage of development (Sunyer *et al.*, 2006), and v) model the potential risk for babies exposed to polycarbonate milk bottles and plastic toys (Quitmeyer and Roberts, 2007; Andaluri *et al.*, 2018).

Both *in utero* and lactation exposure does not allow the exact dosage for treatment of the F1 generation through pregnant or milking dams. There are two possibilities for indirect estimation of genuine exposure by the compound, with different drawbacks of routine usage – firstly, analytical methods for tracking of endocrine disruptor level in blood plasma (Argmann and Auwerx, 2006) or breast milk (DePeters and Hovey, 2009; Muranishi *et al.*, 2016). In the second way, the ability to calculate the exposure using a physiologically based pharmacokinetic (PBPK) model (Karrer *et al.*, 2018), or even improved pregnancy PBPK model (Sharma *et al.*, 2018). Although this modelling requires comprehensive biomonitoring data, cohort studies and a highly sophisticated mathematical approach, PBPK modelling seems to be potent for experimental designing and, in particular, for implications of experimental data in human or veterinary medicine.

The choice of appropriate genetic background is the general advantage of the rat and mouse as a biomodel. With respect to the major features of an experiment, i. e. risk assessment of tested compound for human population (Chemek *et al.*, 2016) or the molecular action and endocrine disruptor-affected signalling pathways (Dolinoy *et al.*, 2007), outbred and inbred strains are available for use (Table 1). Additionally, genetically modified inbred strains can be used for precise study of molecular mechanism of endocrine disrupting effects (Liu *et al.*, 2015).

Besides the advantages of the aforementioned *in vivo* exposure, several weaknesses are obvious: inter-species differences in organs (uterus, placenta), tissues (placental barriers) and cells (molecular regulation of oocyte maturation). These reasons support the usage of advanced biomodels like pigs (*Sus scrofa*) and cattle (*Bos taurus*), in particular for the explanation of molecular mechanism and conservativeness of the mode of action of endocrine disruptors.

Mammalian i*n vitro* models for elucidation

Abovementioned disadvantages of lower mammalian species establishes higher mammals as more appropriate for *in vivo* study. Historically, laboratory cats and dogs were sporadically used, however, they have not become widely utilized. Rhesus monkey (*Maccaca mulatta*) or other non-hominin primates are most appropriate, mainly for the similarity of macaque genome to human. Nevertheless, unavailability, expensiveness or ethic problems arise resulting in rare utilization, mostly for basic study (Sutovsky *et al.*, 1999; He *et al.*, 2014; Song *et al.*, 2016), cancer research (Lertpiriyapong *et al.*, 2014; Dray *et al.*, 2018) and regenerative medicine (Higginbotham *et al.*, 2015; Kim *et al.*, 2018) due to the similarity of macaque genome to human. Alternatively, farm animals are exploited for similar studies, however, the *in vivo* treatment-based experiments are poor applicable

Table 1. Overview of frequently used strains of mouse and rat

	Mouse	Rat
Inbred strains	C57BL/6 BALB/c C3H	F334 LEW SHR
Outbred strains	ICR (CD1) NMRI (HsdWin:NMRI) MF1 (HsdoLA:MF1)	Sprague Dawley Wistar Han Lister Hooded

alike. Therefore, the utilization of farm animals destined for breeding (males) or meat industry (females) is widespread. Porcine and bovine models are the most available and allow *in vitro* culture of gametes.

In vitro maturation (IVM) of animal oocytes represents a relevant tool for endocrine disruptor testing as the highly-similar cell to human. Oftentimes, no interpolation is necessary for knowledge transfer to veterinary sciences being an identically used biomodel. Moreover, the presence of surrounding cumulus cells, creating cumulus-oocyte complex, provide the measurement of cumulus expansion (Zámostná *et al.*, 2016), other biological phenomenon accompanying oocyte maturation (Procházka *et al.*, 1998) and a biomarker of oocyte quality (Nevoral *et al.*, 2015; Blaha *et al.*, 2017). Generally, *in vitro* culture does not simulate the systemic response of the organism to endocrine disruption. On the other hand, oocyte IVM combined with biomonitoring obtains the relevant model for human oocyte exposure *in vivo* (unpublished). Specifically, quantified concentration of tracked endocrine disruptors in human follicular fluid is easily usable for *in vitro* treatment via the supplementation of culture media at equal concentration (Žalmanová *et al.*, 2017a). This IVM model simulates the oocyte enclosed in the ovarian follicle and surrounded with a matrix containing defined amounts of the endocrine disruptor.

In vitro fertilization (IVF), the technique familiar for human reproductive medicine (Balaban *et al.*, 2014) and biotechnology for farm animals (Pavlok *et al.*, 1989; Abeydeera *et al.*, 1998) follows both *in vivo* and *in vitro* maturation of oocytes. These oocytes acquire developmental competence during meiotic maturation and, therefore, the maturation presupposes the success of the embryonic development (Kim *et al.*, 2008; Nevoral *et al.*, 2014). Accordingly, the exposure of *in vitro* mature oocytes offers relevant observations of the endocrine disruptor impact on embryonic development through the oocyte quality (unpublished). A possible effect of endocrine disruptors on embryos in the fallopian tube and uterus is eliminated in this experimental scheme. Thus, observed phenotypes of oocytes exposed to endocrine disruptors after IVF directly represent the effect of an endocrine disruptor on the oocyte developmental competence acquired during IVM.

In vitro maturation and early embryonic development show several inter-species differences. There is physiologically prolonged nuclear envelope breakdown in prophase-arrested porcine oocyte when the maturation is physiologically initiated (Fulka *et al.*, 1986; Motlik *et al.*, 1998). Therefore, this phase provides an endocrine disruptor-sensitive window specific for porcine oocytes. Strongly affected chromatin changes induced by the endocrine disruptor *in vitro*, such as epigenetic modifications (Wang *et al.*, 2016) and/or aneuploidy incidence (Žalmanová *et al.*, 2017a), can be considered in porcine oocytes. This model extends the possibility of molecular study of candidate compounds during oocyte maturation. Chromatin is highly error-prone during oocyte maturation (Hornak *et al.*, 2011) and, therefore, molecular analyses after endocrine disruptor exposure are highly relevant. Aneuploidy disorders (Down syndrome, Patau syndrome), genetic disorders (haemophilia, cystic fibrosis) and epigenetic imprinting failures (Prader-Willi syndrome, Angelman syndrome) are clinical manifestations of oocyte aneuploidy-derived consequences and present serious issues for human health. Endocrine disruptors are capable to increase the incidence of oocyte aneuploidy (Hunt *et al.*, 2003; Žalmanová *et al.*, 2017b; Nevoral *et al.*, 2018) and, thus, the risk of aneuploidy-derived diseases.

Evaluation of early embryonic development represents another way to assess endocrine disruptor impact on the oocyte and embryo quality. The genetic analysis provides the comprehensive approach, including advanced methods of epigenome analysis. For *in vitro* embryo production, the porcine model seems to be less appropriate due to high incidence of polyspermic fertilization and polyploidy of embryos. Comparing to other farm animal models, the bovine model provides highly reliable methods for IVF and *in vitro* embryo production, based on protocols often used intensively for commercial purpose. Overall, the mouse embryo offers the unique model of embryonic development beyond the gastrula stage (Sozen *et al.*, 2018), incompatible with embryos of other species. Taken together, the combination of individual *in vitro* models meets the requirement for a suitable model.

CONCLUSIONS

Many animal models for biological study have been well established through the decades, followed by biomedical applications and bioindicators. The interplay of models offers the observation of exposure-dependent phenotype, followed by the study of the molecular action of tested endocrine disruptor. There is the potent scheme for risk assessment of an endocrine disruptor for human health, combining a few approaches in the following order: i) simultaneous biomonitoring on human body fluids, followed by simultaneous ii) PBPK usage for *in vivo* exposure of the rodent model and iii) the *in vitro* exposure of oocytes and embryos of farm animals. This approach provides studying the effect and molecular mechanisms of endocrine disruptors acting at environmentally exposed doses, based on human biomonitoring. Additionally, the elucidation of attained observations and the test of conservativeness are available using individual *in vivo* and *in vitro* models. The results obtained from the model interplay, represent highly relevant outputs of the endocrine disruptor testing towards the elimination of environmental pollutants and the human health protection.

ACKNOWLEDGEMENTS

We are grateful to Dr. Karl Kerns (University of Missouri-Columbia) for his kind help and fruitful discussion.

FUNDING

The study of endocrine disruptors was supported by the Czech Health Research Council (NV18-01-00544), the National Agency of Agriculture Sciences (NAZV QJ1510138), the Czech Ministry of Agriculture (MZeRO 0714), European Human Biomonitoring Initiative HBM4EU provided by H2020, the Charles University Research Fund (Progres Q39) and the National Sustainability Programme I (NPU I) Nr. LO1503 provided by the Ministry of Education, Youth and Sports of the Czech Republic.

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