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INVITED REVIEW

Reproductive Health

Integrative rodent models for assessing male reproductive toxicity of environmental endocrine active substances

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In the present review, we first summarize the main benefits, limitations and pitfalls of conventional *in vivo* approaches to assessing male reproductive structures and functions in rodents in cases of endocrine active substance (EAS) exposure from the postulate that they may provide data that can be extrapolated to humans. Then, we briefly present some integrated approaches in rodents we have recently developed at the organism level. We particularly focus on the possible effects and modes of action (MOA) of these substances at low doses and in mixtures, real-life conditions and at the organ level, deciphering the precise effects and MOA on the fetal testis. It can be considered that the in vivo experimental EAS exposure of rodents remains the first choice for studies and is a necessary tool (together with the epidemiological approach) for understanding the reproductive effects and MOA of EASs, provided the pitfalls and limitations of the rodent models are known and considered. We also provide some evidence that classical rodent models may be refined for studying the multiple consequences of EAS exposure, not only on the reproductive axis but also on various hormonally regulated organs and tissues, among which several are implicated in the complex process of mammalian reproduction. Such models constitute an interesting way of approaching human exposure conditions. Finally, we show that organotypic culture models are powerful complementary tools, especially when focusing on the MOA. All these approaches have contributed in a combinatorial manner to a better understanding of the impact of EAS exposure on human reproduction.
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INTRODUCTION

During the past half century, temporal trends and geographically marked differences in human reproductive health together with the observation of various reproductive disorders in wildlife have raised concerns about the role of environmentally mediated risk factors.[1] Due to the short period over which those reproductive anomalies were observed, they cannot be explained by genetic changes. Furthermore, over roughly the same period, the production and use of both natural and synthetic chemicals has markedly increased.[2] Among the myriad of chemicals present in air, water, food and in a variety of consumer products, many are capable of interfering with the endocrine system of animals and humans. These endocrine active substances (EASs), as recently qualified,[3] directly or indirectly interfere with the production, release, transport, metabolism, binding action or elimination of natural hormones. They may simply produce biological changes that lie within an organism's homeostatic capacity or be detoxified and would therefore not be expected to cause adverse effects. However, a core of epidemiological and experimental *in vivo* studies shows that some of these EASs, following certain exposure conditions, may exert adverse effects on intact organisms (either humans and nonhumans) with a plausible or demonstrated causal relationship between the endocrine activity and the adverse effect; in that case, the terms 'endocrine disruption' or, 'endocrine-disrupting compounds' (EDCs) should be used[3] (when citing literature references, we shall use both acronyms jointly, EASs/EDCs, because the proper use of each term depend on the experimental context, doses and so on). Most EASs/EDCs are released in the environment through human activity (pesticides, pharmaceuticals, compounds used in industry and in consumer products, industrial by-products and pollutants), but EASs/EDCs of natural origin occurring in plants are part of human regimens (for example, estrogenic compounds in soy such as genistein and daidzein). Among the numerous environmental EASs/EDCs, those that are likely to affect signaling pathways/modes of action (MOA) related to estrogen, androgen, thyroid hormone or steroidogenesis (EATS modalities) are particularly scrutinized because rare epidemiological studies and a contrasting number of *in vivo* and *in vitro* studies point to their deleterious impact on the male reproductive system.[4] Several EASs/EDCs such as bisphenol A or phthalates are ubiquitously detected in the biological fluids of the general population.[5,6] However, the EASs/EDCs multi-exposure condition in humans and the fact that reproductive organs and tissues cannot be directly studied are the main difficulties for

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studying this question in depth (except for associations with semen quality or developmental markers such as the anogenital distance (AGD)).[7–9] In contrast, animal models, mostly rodent models and *in vitro* systems have been widely used to assess the deleterious effects and to decipher the mechanisms of action of a number of EASs in the academic world and for regulatory purposes. This approach has been the object of several in-depth reviews in recent years.[10,11] Recently, the WHO (World Health Organization) report on EDC exposure risks provided an up-to-date state of the science of the laboratory-based research into the reproductive implications of EDC exposure in men,[1] much of it being carried out using the rat (less often, the mouse) as a model.[12] Whilst male rats differ from men to some extent with regards to some specific aspects of their physiology (in particular, with regard to some characteristics of their endocrine and reproductive systems), in general, the processes underlying their development and physiology are thought to be similar. Thanks to conventional *in vivo* experiments and complementary *in vitro* data, a body of knowledge on the effects and MOA of EASs has been accumulated in recent years. Among other studies, those focusing on *in utero* exposure, a crucial developmental period for the differentiation, development and future maintenance of the testis, have helped to identify chemicals that interfere with male reproductive development and emphasize the importance of this developmental window.[13] However, crucial gaps remain, notably those related to the possible effects of low EAS (environmental doses) or EAS mixtures and the confounding role of many other environmental or lifestyle factors in humans.

In the present review, we will summarize the main benefits, limitations and pitfalls of conventional *in vivo* approaches assessing male reproductive structures and functions in rodents in cases of EAS/EDC exposure from the postulate they may provide data that can be extrapolated to humans. Then, we will briefly present some integrated approaches in rodents we have recently developed to particularly focus on the possible effects and MOA of EAS/EDC low doses and mixtures in real-life conditions.

CHARACTERISTICS, BENEFITS, LIMITATIONS AND PITFALLS OF CONVENTIONAL *IN VIVO* APPROACHES ASSESSING MALE REPRODUCTIVE STRUCTURES AND FUNCTIONS IN RODENTS

Only experiments in intact organisms (*in vivo* experiments) can address the question of chemically- or physically-induced reproductive anomalies by accounting for all the complexity that may link harmful reproductive effects to exposure (including metabolism, toxicokinetics and so on). For example, experiments with EASs/EDCs on intact organisms, thus maintaining the hypothalamic-pituitary-gonadal axis integrity, are capable of accounting for both the central and peripheral possible effects of these substances. However, whilst fundamental for understanding human pathologies, data obtained from animal experiments cannot be directly applied to the human situation. In particular, the design, discussion and conclusions of experiments with EASs/EDCs should account for a number of characteristics, such as the choice of species and strains within species, the mode of administration, the exposure window and so on.

Selection of species/strains

Most *in vivo* studies in the field of EASs and EDCs have been carried out in rodents, which, from the point of view of the physiological regulation of reproductive functions, are grossly similar to humans (overall, there is a high degree of conservation of the reproductive function among most mammalian species). However, EAS/EDC studies in rodents show a significant diversity in the strain, dose, exposure route and window selected. Additionally, the rodent model presents some limitations that must be considered when designing EAS/EDC studies. Several differences in the developmental timing or the structure of the reproductive organs exist when comparing rodents and humans; there are also differences in metabolism.[14] For example, polyovulation and the short duration of gestation in rodents represent differences from other mammals, including humans. Unlike humans, rats and mice are multiparous species; thus, the effect of a gestational exposure to EASs/EDCs in the progeny is confounded by the fact that exposure to sex steroids during sexual differentiation varies, depending on a hormone transfer between male and female fetuses in rodent uterine horns.[15,16] Other differences in reproductive function exist when comparing humans with other mammals. For example, in humans, the determinants of spermiogenesis are possibly more complex than in most other mammalian species, including rodents.[17] Rodent species/strains also present some important limitations for studying human reproductive disorders. For example, except for rare strains with certain specific genetic backgrounds, there is no germ cell tumor in the testes of mice or rats; whereas, it is the most common human cancer in young adults.[18] Within the same rodent species, it is also worth noting the existence of between-strain differences in certain endpoints sensitive to the effect of reprotoxicants. For example, in response to *in utero* exposure to a certain phthalate (dibutyl phthalate (DBP)), Wistar rats have higher rates of cryptorchidism and lower rates of epididymal agenesis than Sprague-Dawley rats.[19]

Exposure route

In the context of EAS/EDC human exposure, the main exposure route, but not the unique route, is frequently diet. Thus, it seems legitimate to use the same exposure route in rodent studies. However, it is sometimes difficult to know with certainty the amount of food ingested and the administration of exogenous substances can alter food intake, which is why many authors choose gavage, which allows better control of the amount of substance administered (whereas this approach shunts the salivary glands). However, gavage may represent an additional stress that can alter the response to EASs/EDCs. For example, glucocorticoids appear to enhance the effects of phthalates on the fetal testis,[20] and it therefore seems reasonable to consider that maternal stress may contribute to the alterations induced by these compounds. A number of EAS/EDC studies generally designed for understanding the underlying mechanisms of action are based on exposures via unrealistic routes (subcutaneous or intraperitoneal route, osmotic pumps grafted under the skin and so on), thus raising questions about the relevance of these studies to the human situation. In order for the phenotype changes or reproductive disorders that are supposed to be induced by EAS/EDC exposure to be minimally confounded by other 'environmental' factors, it is important to design studies with an environment devoid of contaminants. For example, it should be mandatory that the animals be placed in cages devoid of estrogenic or antiandrogenic contaminants and that they be fed a diet containing no phytoestrogens (such as soy-free diet). However, one must admit that such a controlled design does not at all reflect the conditions of human multiple exposure. For example, bisphenol A exposure data in humans indicate that besides the oral route, which is the main exposure route, cutaneous exposure also occurs.

Exposure window

Hormones are important for the normal development of organs and tissues. Thus, endocrine disruption at critical points such as during the gestational period can result in irreversible changes of the organ/tissue. In mammals, critical periods of development have been identified at

conception and during gestation, infancy, childhood and puberty (for a comprehensive review of endocrine-mediated effects and the timing of exposure in mammals, including humans, see the recent WHO report on the possible developmental early effects of endocrine disruptors on child health).[13] It is widely accepted that in relation to the potential effects from exposure during critical periods of susceptibility, testing *in vivo* is required. In rodents, the length of gestation is very short, 19–21 days, resulting in the birth of very immature pups. This is not the case in large mammals such as humans. It is therefore understandable that selected exposure windows do not follow a strict parallelism between species: perinatal exposure (fetal and early postnatal life) in rodents covers events related only to fetal exposure in humans. Some species, such as ruminants, appear to be closer to humans for these parameters, but handling these animal models is time consuming and expensive, which greatly restricts their use in basic or applied reproductive toxicology. Current internationally validated rodent tests do not cover certain endpoints that might be induced by exposure during fetal or pubertal development, but emerge later in life, such as certain cancers (prostate and testis) and effects on reproductive senescence.[4] Finally, although fetal life is undoubtedly a critical period for the establishment of the reproductive function, it is not obvious that a very short exposure during the rodent fetal life accurately reflects the chronic (and possibly lifelong) human exposure (s).

Positive control

The inclusion of a positive control (for example, with a known potent estrogenic or antiandrogenic substance) may be an interesting point of comparison in EAS/EDC and male reproduction studies. However, a positive control may represent an experimental confounding factor when the mode of action of the EASs *in vivo* is unknown. For example, many authors use flutamide, a nonsteroidal antiandrogen, as a positive control in studies on phthalates in the fetal testis.[21] However, the effects of phthalates appear at least partly independent of the androgen receptor.[22] Thus, in this case, flutamide is more a point of comparison than a 'strictly speaking' positive control. The absence of a valid positive control in some studies cannot be systematically considered as a severe drawback.

Reproductive endpoints studied

Rodent studies can consider a large number of phenotype endpoints related to the male reproductive function, most of which cannot be assessed in humans and are therefore informative points for deciphering EAS/EDC effects. Obviously, some endpoints are more informative than others. For example, significant changes in testis weight (preferably, testis weight relative to the animal body weight) reflects only major alterations of spermatogenesis and is clearly less informative than assessing the daily sperm production (DSP) or the relative ratio of proliferating to apoptotic germ cells. Similarly, a decreased epididymis, prostate or seminal vesicle (relative) weight may grossly reflect an antiandrogenic action, which should be proven by other approaches. A number of sperm characteristics, such as testicular or epididymal sperm count, % motility or % morphological abnormalities are usually assessed. However, considerable variability in sperm parameters is found among studies even when comparing only control groups[23] and this may depend on differences in the experimental design, which may occur at the pre-analytical and/or analytical and/or post-analytical steps (for example, the method used to release sperm from the cauda epididymis or the incubation medium used in the case of motility assessment). As a result, comparison of data across laboratories is difficult. Some guidelines for assessing sperm quantitatively and qualitatively have been proposed, but are not always followed. As for human semen assessment, standardized protocols and quality control (QC) schemes should be used. From this point of view, using reproducible semiautomatic approaches such as those based on computer vision (computer-aided sperm analysis (CASA)) is recommended.[24] Other endpoints are related to the gestation developmental period: measurement of the AGD, frequency of hypospadias, cryptorchidism and nipple presence/retention. Another set of endpoints concerns the fertilizing ability of exposed animals (mating index, fertility index, rates of pre- and/or postimplantation loss and so on), puberty timing (age at preputial separation, a parameter that may be influenced by 'environmental conditions' such as a change of feeding behavior) and hormone levels (testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH), estradiol; measurements of intratesticular testosterone are less variable than serum levels, but this requires the destruction of tissue). Developmental endpoints such as AGD or reproductive anomalies are interesting endpoints because they can be quite easily assessed in both rodents and humans relative to controlled or measured exposures.[9] Rodent studies also often include testis histology and/or immunohistochemistry, a fundamental approach for assessing an adverse effect (for example, an increased apoptosis process as detected by DNA fragmentation using an *in situ* TUNEL (terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling) assay). Note that this type of assessment is prone to a certain amount of subjectivity, and standardized quantitative methods (for example, based on stereology or semiautomatic image analysis) should be preferred over subjective visual approaches.

Recently, 'omics' approaches have increasingly been used in reproductive toxicology with the aims of detecting infraclinical phenotype changes in conjunction with the assessment of more classical endpoints (see above) and of providing information on the possible MOA of the EAS/EDC exposure (s) that may affect molecular structures and the functions of the testis, epididymis, prostate and so on.[25,26] However, whereas transcriptomics provides an abundance of gene expression data, there are no validated and established standards for the analysis and interpretation of these data. One must also keep in mind that a change in gene expression does not necessarily reflect a change in protein expression or indicate an adverse effect. Thus, transcriptome approaches must be used jointly with other endpoints to understand the toxicological meaning behind the changes in gene expression. Specific hypothesis-driven studies investigate candidate genes (usually via RT-qPCR (real-time quantitative reverse transcription polymerase chain reaction)) that may be involved in the EASs/EDCs MOA or have a crucial role in testis differentiation and/or development and/or maintenance. Alternative approaches make no such assumption and use rat DNA-arrays (the full transcriptome) to identify and characterize relevant transcriptional changes related to the exposure. Resulting gene expression patterns may point to a possible MOA (functional change), which in turn can generate additional hypotheses that could be tested further. Incorporating additional 'omics' technologies such as proteomics and metabolomics in conjunction with transcriptomics could help further our understanding of the effects and MOA of EAS/EDC exposure.[27] However, to use these data proficiently requires both validation steps and an expertise in bioinformatics.

Some EASs/EDCs have been shown to disrupt epigenetic programming. For example, the antiandrogenic fungicide vinclozolin was shown to affect spermatogenesis up to three (unexposed) generations following an initial gestational exposure in the rat.[28] Recently, vinclozolin was reported to disrupt the methylation patterns

of several imprinted genes in mouse sperm,[29] a possible link between both phenomena. Currently, there is no validated framework or guidance for determining the best way to incorporate epigenetic endpoints into toxicological risk assessment.

Finally, a currently serious pitfall in the interpretation of the vast majority of studies based on omics endpoints and those investigating the effects of EASs/EDCs on the epigenome are the high dose exposure level of the substances studied, which may not be relevant for human exposure.

Limits of the endocrine active substances/endocrine-disrupting compounds literature based on rodent models for human risk assessment and current controversy on endocrine active substances/endocrine-disrupting compounds low-dose effects

Most studies on the effects and MOA of EASs/EDCs pertaining to the male reproductive function have been carried out with high unrealistic doses in terms of human exposure.[1,4] In recent years, an intense debate has emerged owing to the low (environmental) (multi) exposure to EASs/EDCs in humans, and the contrasted *in vivo* results showing or not low-dose effects. Several studies reporting adverse effects (reproductive or affecting other organs and tissues) used doses (well) below the lowest dose levels expected not to result in adverse effects (no-observed-adverse-effect-level (NOAEL))[30] and EAS low-dose effects have been recently considered in the WHO/UNEP (United Nations Environment Programme) State of the Science of Endocrine-Disrupting Chemicals 2012 report.[1] In addition, some of these studies have revealed a non-monotonic dose-response curve (NMDRC; following, for example, an inverted U-shape), thus challenging the paradigm in toxicology of a linear dose-effect. Most reported low-dose and NMDRC findings have been observed with rodents and are therefore connected to human health, the dose in some studies being very similar to the contamination doses found in human biological fluids. Unfortunately, a number of published low-dose studies are based on noticeably different protocols; for example, those that are internationally validated, such as the OECD (Organization for Economic Co-operation and Development) tests[31] or various in-house academic experimental designs. Many results have not been repeated or papers reporting the results lack a certain amount of crucial information to evaluate the relevance of the author's conclusions. Thus, it should be considered that assessment of the reproducibility of findings that might indicate low-dose effects and/or NMDRC is important to rule out spurious results. Currently, the 'low-dose hypothesis' implies that EASs/EDCs interacting with hormone action can do so in a manner that is quite specific, following various pathways that differ from the MOA previously described in the case of *in vitro* studies or high-dose exposure (such as classical agonist or antagonist action on ER or AR). The current controversy about EAS/EDC low-dose effects is, among other reasons, why national and international agencies in charge of risk assessment still have difficulties rendering recommendations for specific EASs, even when using systematic approaches for evaluating the scientific evidence about the relationship between environmental exposure and health effects. From this point of view, it is crucial in the case of rodent reproductive studies and especially with low-dose EAS exposure (s) to evaluate whether reported data are likely to support, or not, some relevant associations, which can include causality. Because many factors may interfere (see above), to draw firm conclusions and to improve our knowledge of the low-dose effects as the mode of action of various EASs, especially for exposures relevant to human exposure conditions, very stringent criteria incorporating a number of items should be applied in the experimental designs and for the interpretation of studies, as illustrated in **Table 1**.

Other important limits in the conclusions of most EAS/EDC experimental rodent studies and in the current risk assessment of environmental chemicals (which include EASs/EDCs) are related to the fact that each chemical substance is evaluated individually and that the possible modulation by the genetic background and the various environmental factors jointly occurring are not accounted for.[32]

EXAMPLES OF ALTERNATIVE AND INTEGRATIVE APPROACHES TO ASSESS THE ADVERSE EFFECTS AND MODES OF ACTION OF REALISTIC ENDOCRINE ACTIVE SUBSTANCES EXPOSURES

As briefly summarized above, most of the literature on the reproductive effects of EASs/EDCs or EAS/EDC MOA in rodents is based on unrealistic experimental designs (high doses, a very short exposure window, an inadequate exposure route and so on). For example, a majority of studies are based on short exposure periods, at specific crucial developmental steps and/or using a single compound administered at high non-environmental dose ranges sometimes via unrealistic exposure routes. In contrast, the characteristics of human EAS/EDC exposure are prolonged windows of exposure (lifelong for some), multiple exposures at low levels according to human exposure data, with, in addition, a number of other lifestyle and environmental factors that may modulate the EAS/EDC effects. These crucial points and serious gaps in our current understanding of the EASs/EDCs effects were recently acknowledged in the conclusions of the WHO/UNEP report[1] and by EFSA (European Food Safety Authority).[3]

Ideally, EAS/EDC rodent models should make it possible: (i) to jointly scrutinize the various reproductive effects of EASs/EDCs and their origin, either central and/or peripheral, (ii) to precisely study the EAS/EDC MOA and (iii) to provide relevant information for risk assessment (thus implying the study of low-dose and mixture effects and MOA). Unfortunately, there is obviously no experimental model that can simultaneously cover all these desirable goals because it is not possible to mimic the human exposure situation for a given objective due to the complexity and variable nature of human experiences. Thus, both conventional *in vivo* experimental models and epidemiology remain the main cornerstones of the assessment of the effects and mode of actions of EASs/EDCs. However, and notably in rodents, as briefly reported below, new integrated approaches based on intact animals or testis in culture have recently proven useful for producing new pieces of the puzzle pertaining to both effects and MOA in humans.

The multiorgan/multi-tissue in vivo approach to low-dose and mixture exposures

In recent years, xenoestrogens and antiandrogens, initially identified for their effects on the reproductive organs and/or fertility parameters, were found to act on other organs, tissues or behaviors. Usually, EAS/EDC studies consider the different organs and tissue separately (generally, on the basis of the discipline/domain of expertise of the investigators). This conventional approach does not make it possible to link adverse effects occurring jointly in different hormonally dependent organs and tissues, and therefore, it cannot firmly demonstrate that a real endocrine disruption process is occurring. From this point of view, jointly studying females may add value by possibly revealing sexual dimorphisms or differences in behavior that may be explained by the substance under study (according to its invoked estrogenic, antiandrogenic and other properties). As briefly summarized above, classical rodent models used either for regulatory purposes or in the academic world do not account for a number of characteristics of EAS/EDC human exposure (most often chronicity, often lifelong rather than a short duration of exposure during a specific window, low doses,

Table 1: prototype of a validation grid from a fictitious example for designing EASs/EDCs studies in rodents and for assessing the results of EASs/EDCs reproduction studies in rodents

Criteria	*Brief description/results*	*Comments*
Complete reference	XXX, *et al*., (2013) Effects of ZKV …. *Endocrine Disrupt Toxicol* 12: 5–13	NA
Type of study (1G, 2G, prenatal)	Perinatal (gestational/lactational) exposure window	NA
Aim (s) of study	Reprotoxic effects of ZKV in postpubertal male rats	NA
Monograph, scientific paper, other	Regular paper, scientific journal	NA
GLP or not GLP study	Not GLP study	NA
Origin of grants (institutional/academic/ industry…)	Academic only	NA
Chemical (s) compound (s) studied, ref. number, purity, composition, vehicle	Purity: unknown	Should be indicated
Species/strain/age/weight	Sprague-Dawley dam and pups	NA
Randomization method for animal allocation	Yes	OK
Blinding	Yes	OK
Accounting for all animals	No	Should indicate how the animals studied were selected
Sex and number of animals/exposure group	8 males per group reproductive study	How the number of animals has been determined?
Control group, 'exposure' conditions	Yes, ?	'Exposure' route of the control should be indicated
Positive control	No	NA
Husbandry conditions (temperature, humidity, light, regimen, number animals/cage) Controlled or uncontrolled environment/ exposure for EASs/EDCs	Mating outside the animal facility (Charles Rivers Lab), dams acquired thereafter Temperature: 18–26 °C, humidity: 30–70%, 1 animal per cage, phytoestrogen residues in regimen, cages containing polycarbonate, water composition unknown, bedding composition unknown	Temperature variation +++ Problem+++ of uncontrolled 'environmental' conditions, which may confound results
Exposure route	Gavage	Not fully adequate for ZKV
Exposure frequency and duration	Daily, from conception to weaning	NA
Doses/exposure concentrations (indicate whether nominal or measured concentrations)	1 ml kg^{-1} ZKV at 50, 250 or 750 mg ml^{-1} (measured concentrations: 47, 242 and 760 mg ZKV per ml)	Irrelevant doses for humans
Observation/endpoints studied	Sacrifice at 25 dpc Malformations, liver and reproductive organ weighing, testis histology	NA
Statistical analysis	Animals from different litters in the various exposure groups	Litter effect accounted for?
Effects observed	Liver weight increased for 250–750 mg kg^{-1} day^{-1} Reduced testis weights at all concentrations Increased Leydig cells aggregates for 750 mg kg^{-1} day^{-1} No observed effect at 250 mg kg^{-1} day^{-1} Unaffected anogenital distance, whatever the dose	Gross weight (what about the relative weight?) Unstandardized/subjective method for assessing the number of Leydig cells NA
Authors' conclusions	Perinatal ZKV exposure alters testis structures and functions	NA
Comments and conclusions from experts/ reviewers	Some crucial points are not indicated and there are some methodological flaws	It is not possible to draw meaningful conclusions
Quality of the report/study (Klimisch score[a])	3	NA

dpc: day post conception; EASs: endocrine active substances; EDCs: endocrine-disrupting compounds; GLP: good laboratory practice; NA: not applicable; ZKV: zalkevedin. [a]Reliability criteria of studies in the field of regulatory toxicology and/or ecotoxicology[66] that may be applied to *in vivo* studies in the field of EASs/EDCs studies. The following categories/codes of reliability seem to be adequate: 1: reliable study without restriction, 2: reliable study with restrictions, 3: not reliable study and 4: not assignable study

mixtures and so on). Thus, in recent years, we have developed a rat model of exposure to EASs/EDCs approaching (but not mimicking) the conditions of human exposure. In a first study, we tested the hypothesis that low (environmental)-dose exposure to estrogenic 'feminizing' and/ or antiandrogenic 'demasculinizing' EASs/EDCs, which may occur simultaneously in the human diet, may affect the male reproductive tract and fertility. We selected the phytoestrogen genistein with a low dose of 1 mg kg^{-1} day^{-1}, representing the amount of this compound in Asian soy-based diets and the antiandrogenic food contaminant vinclozolin, at a low dose of 1 mg kg^{-1} day^{-1}, a dose below the US Environmental Protection Agency NOAEL (1.2 mg kg^{-1} day^{-1}). The rats were exposed from conception to adulthood and then mated with unexposed females to assess fertility endpoints. We found that, among the different exposure conditions, the low-dose mixture produced the most significant alterations in adults: decreased sperm counts, reduced sperm motion parameters, decreased litter sizes, increased post implantation loss and more pronounced differences in testicular mRNA expression profiles.[33] Further, to understand the role of the exposure window, gestational/lactational (GL) *vs* puberty to adulthood (PA), in the effects found in adults; we jointly carried out two studies using the same compounds and doses and we also studied the possible consequences of these low-dose/mixture exposures in F2 unexposed male generations sired by exposed fathers (F1) with control unexposed mothers. In addition, to test the hypothesis that low-dose/mixture EAS/EDC exposures may result in adverse effects via a disruption of the endocrine system, we extended the model to females and other tissues and organs depending on steroids, the mammary gland, bone and cartilage and the salivary glands. Feeding behavior was also jointly studied. This approach clearly raised the question of the effects of mixtures of compounds that coexist in the

food bowl or the environment. It aimed at giving an integrative view of the low-dose/mixture effects of EASs; thanks to a multidisciplinary approach in the context of known or emerging diseases, including possible multigenerational effects. GL exposure to each substance or their low-dose mixture had antagonistic effects on rat fetal germ cell development,[34] and it decreased the AGD, increased the frequency of urogenital abnormalities, delayed puberty and increased the rate of postimplantation loss; thus, confirming previous results with the same substances without other endpoints being affected. The relative adult weight of the testes and epididymides were increased and decreased, respectively; with the puberty-to-adult exposure window and this exposure reduced the sperm production. In the unexposed F2, an increased frequency of urogenital abnormalities was observed when fathers were subjected either to GL or PA exposure modalities, and sperm production was significantly reduced by 50% in F2 animals sired from PA-exposed fathers in comparison to the F2 controls. In addition, testicular gene expression was found to be modified differently in the adult according to the exposure window, and an intriguingly high number of genes pertaining to relevant pathways were found deregulated in the adult testis of the unexposed progeny, with different pathways depending on whether the fathers were subjected to GL or PA exposure (Eustache, unpublished data).

In addition to the effects on the male reproductive system, this multidisciplinary program has demonstrated that dietary exposure to relatively low doses of genistein and vinclozolin disrupt the organization and function of many tissues and organs subjected to regulation by steroid hormones. For example, the GL exposure affected the development and growth factor mRNA expression of the submandibular salivary gland in immature female rats, and it affected sweet preference and submandibular development in male rats.[35,36] It resulted in immaturity of the mammary gland during the peripubertal period,[37] and it showed that minute amounts of these EASs targeted the rat chondrogenesis (Auxietre, unpublished data). All these results question the conventional scheme of a linear dose-effect, which should have led to an absence of effects given the doses used. The results also raise questions about the MOA of substances/mixtures usually only considered from the point of view of their estrogenic/antiandrogenic properties. MOA studies of substances/mixtures for the different organs or tissues studied are still ongoing. Finally, in a recent program based on the same rat model, we chronically exposed animals, from conception to adulthood, to a low dose of bisphenol A approaching environmental levels (5 $\mu g kg^{-1} day^{-1}$) alone or in combination with low doses of genistein and vinclozolin with the aim of studying the effects of these EAS mixtures on various organs, tissues and behaviors in the exposed males of the F1 generation and the unexposed F2 generation sired by these rats. We studied the impact of exposures on the male reproductive tract in adults and for a subset of animals during the prenatal period and in pre- and postpuberty to study the dynamics of effects according to the exposure modalities, developmental windows and the fact that there was a continuous exposure reminiscent of human exposures. In addition, a selection of targets potentially associated with reproduction, development and feeding behavior in rats and humans, and for which we have previously characterized low-dose effects (see above), were jointly studied. Another target, closely associated with feeding behavior targets and possibly reflective of the effects of exposure, was studied: the tooth. Among numerous results, we provided evidence of delayed puberty onset in both F1 and F2 males and reduced adult testis and epididymis weights in the F1. Sperm production was significantly decreased in all exposure groups in the second unexposed generation. The growth rate of the testis and prostate during the puberty period were significantly different in exposed rats than in controls. In addition, the testis transcriptome studies in the F1 at each developmental period indicated differential impacts of the exposure conditions on the basis of the networks of genes significantly modified see (**Figure 1**), (which summarizes some of the findings of this multidisciplinary program). Among the results following the same exposure conditions in other organs and tissues, prepubertal rats exposed to bisphenol A from conception until postnatal day 30 had an abnormal accumulation of organic material in erupted enamel, an adverse effect reminiscent of human incisor hypo-mineralization, a pathological condition indicative of some adverse events occurring during early childhood.[38] We also provided evidence that a gestational exposure to bisphenol A alone or in a mixture with other EASs/EDCs feminized the digit-length ratio.[39]

In aggregate, the multiorgan/multi-tissue rat model revealed a number of phenotype changes, some showing real adverse effects on targets associated with reproduction, development and feeding behavior. Thus, we have provided evidence that such an integrative rodent model has the advantage of jointly addressing an important number of endpoints related to the crosstalk through all organs participating in the reproductive function. Due to the low (realistic) doses used with effects in various organs and tissues, we believe our results have implications for human studies. Furthermore, the effects found in the unexposed F2 offspring suggest an impact on fetal programming and/or the involvement of epigenetic mechanisms with chronic low-dose exposures, which also pertain to human conditions. Mixture effects reflect endocrine disruption that may result from complex interactions between endocrine chemicals, which will necessitate in-depth further studies on the possible MOA. Finally, the various results of the program illustrate the difficulty in predicting the effects of low-dose EASs/EDCs alone or in cocktails on the sole basis of their endocrine properties at high doses or *in vitro*. Taking the example of an exposure to low 'environmental' doses of BPA, **Table 2** summarizes the characteristics and results of our approach compared with those of an internationally validated reproductive study and of an academic male reproductive study to illustrate their similarities and differences, advantages and limits.

The fetal testis in culture

In vitro models are widely used for screening the deleterious effects of EASs/EDCs. Among these, cell lines or primary cultures provide fast data, sometimes including precious mechanistic elements, but they are limited as most only poorly mimic the physiological situation. The relevance of the information obtained in such models thus requires confirmation *in vivo*. Intermediary between dissociated cells and *in vivo* conditions, the organ culture makes it possible to study the response to EASs/EDCs in a model preserving the intercellular relationships within a tissue. This approach has been developed and used for fetal testes, an organ proven to be especially sensitive to EASs/EDCs during development. Organ cultures of developing gonads were first set up with tissues from rodents. The main model relies on a developing gonad set on a support to allow its development at the interface between the air (5% CO_2) and medium. Filters and agar discs are the two main supports widely used. For early stages, the whole gonad can be set in culture, and for late gestational stages (over 16.5 gestation day in mouse embryos), the testes require sectioning in small parts to permit the correct diffusion of nutrients and gases. Such models were proven to faithfully mimic the kinetics of development of the main testicular cell types (i.e. Sertoli, Leydig and germ cells) over a few days.[40,41] Of interest, the culture of fetal or post-natal testes can facilitate the development of germ cells over a longer time period. Haploid cells able to fertilize an

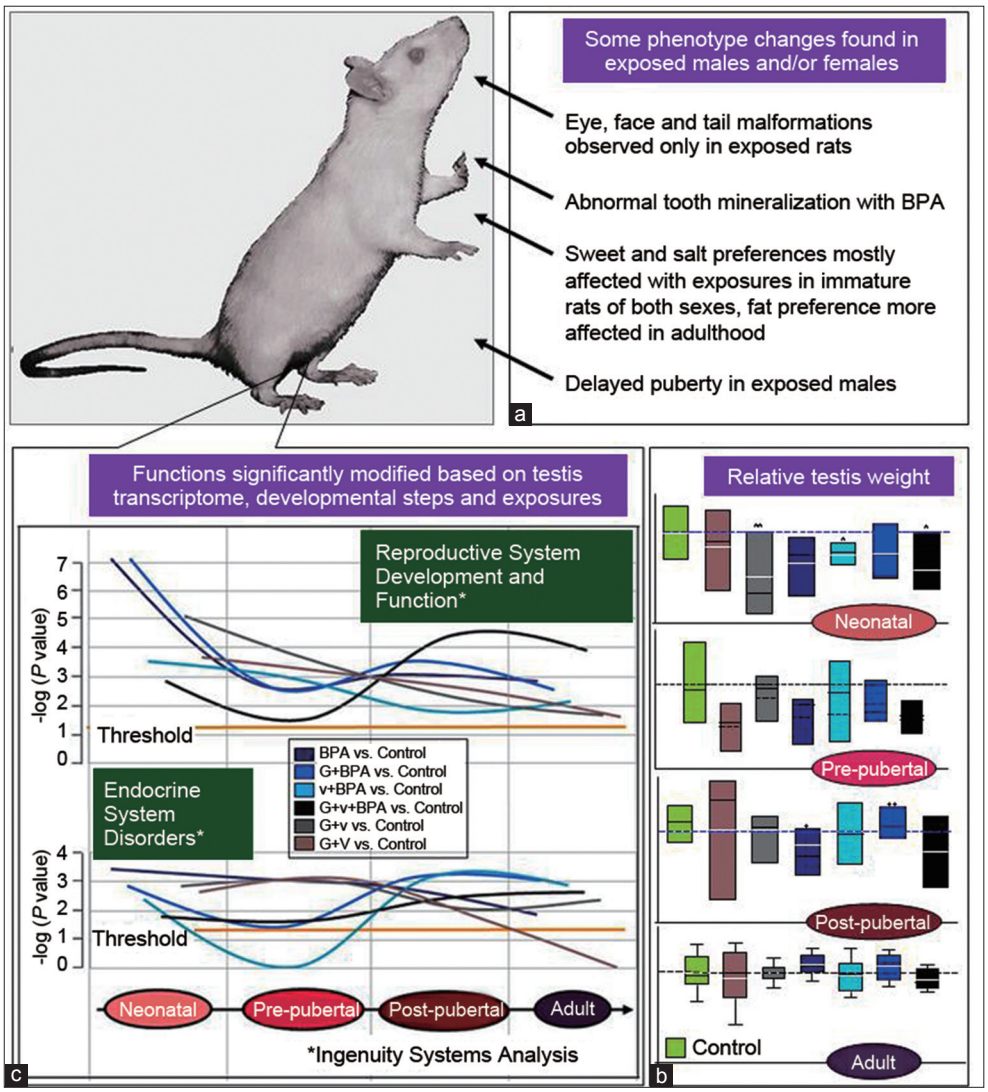

Figure 1: Main features of the integrated rat model for the assessment of the effects and modes of action of endocrine active substances. **(a)** Some phenotype changes using the multiorgan/multi-tissue rat model following a continuous exposure from conception to adulthood to low (environmental) doses of bisphenol A, BPA: 5 μg kg^{-1} day^{-1}; vinclozolin, v or V: 10 μg kg^{-1} day^{-1} ('low') or 1000 μg kg^{-1} day^{-1} ('high'); and genistein, G: 1000 μg kg^{-1} day^{-1} administered alone (BPA) or in double or triple association. **(b)** Box plots showing the temporal changes in relative testis weight at different developmental steps, neonatal, pre- and postpubertal and young adult, following the same exposure conditions. **(c)** Systems and functions (according to Ingenuity analysis software) significantly modified based on data from testicular transcriptome at different developmental periods according to the various exposure conditions (Auger J, unpublished data).

egg have even been obtained *in vitro* with this type of model.[42] More recently, similar organotypic culture protocols were developed to study the human fetal gonads.[43] This approach is particularly useful as it can provide experimental evidence of the potential deleterious effect of a given chemical substance in humans. **Figure 2** summarizes the principles of the culture of fetal or post-natal testes with illustrations in mouse, rat and human.

The advantages of organotypic cultures of fetal testes to study EAS/EDC effects are numerous. These can be performed in a fully defined medium (usually with no phenol red), thus avoiding cross-contamination with other potentially estrogenic compounds. The exposure level is precisely defined. This model also provides a very convenient way to analyze the effect of EASs/EDCs by exposing one gonad to the substance and the contralateral (i.e. the second gonad of the embryo) to the vehicle. Analyzing the effects of EASs/EDCs in the developing gonads *in vivo* sometimes requires numerous animals due to small changes (e.g. regarding the actual developmental stage), resulting in consequent between-litter variability and though less pronounced, inter-embryo variability within the same litter. Having 'control' and 'exposed' testes from the same embryo considerably limits the variability and thus provides a highly sensitive method. In rodents, pregnant females can produce up to 15 embryos. This also makes it possible to assay various doses or different compounds with a single pregnant female; whereas, *in vivo*, this requires using different animals. Thus, the organ culture makes it possible to reduce the number of animals that have to be sacrificed. Lastly, the functions of the testis result from a complex paracrine dialog. For instance, gametogenesis relies both on supporting somatic (Sertoli cells) and germ (gonocytes) cells that form testicular cords. Maintaining, *in vitro*, the three-dimensional structure, cellular contacts and paracrine exchange offers a way to determine the global effect of a given EAS/EDC. In this way, organ culture offers a suitable model to measure several parameters and endpoints simultaneously. The two parameters that are most frequently assayed in the organotypic culture of fetal testes are usually the testosterone secretion (in the culture medium) and germ

Table 2: comparison of three reproductive/general BPA studies using an internationally validated design (i), an in-house academic protocol (ii) or our integrative model (iii). Main advantages and limits

Reference	*Tyl, et al., 2002*[i]	*Salian, et al., 2009*[ii]	*Our integrative model*[iii]
Type of study	Multigenerational (male and female reproductive toxicity study according to OECD/ EPA guidelines)	Multigenerational (male reproductive toxicity study, in-house protocol)	Transgenerational (multiorgan/multi-tissue/behavioral study in males and females, in-house protocol)
Exposure window	Chronic exposure in the F0, F1, F2 and F3	Gestational/lactational (from GD12 to PND21) in the F1, unexposed F2 sired from exposed F1 males and unexposed females, F3	Chronic exposure from conception to adulthood in F1, unexposed F2 sired from, exposed F1 males and unexposed females
Species/strain	Rat/Sprague-Dawley	Rat/Holtzman	Rat/Wistar
Number of animals per group	30	24	20
Dose (s) selected, mg per kg body weight per day (current NOAEL: 5 mg per kg body weight per day; current TDI: 0,05 mg per kg body weight per day)	0.001, 0.02, 0.3, 5, 50, 500	0.0012/0.0024	0.005
Exposure route	Oral (in diet/controlled feed consumption)	Oral (gavage)	Oral (via a micropipette)
BPA mixtures tested	No	No	Yes (with low doses genistein and or vinclozolin)
Sacrifice timing	PND105	PND125	PND3, PND25, PND50, PND100
Phenotype changes (exposed vs control)[a]	No reproductive effects for the - low 'environmental' dose (0.001 mg kg^{-1} day^{-1}) in the F0, F1, F2 and F3 (more than 50 endpoints assessed in males and females)	Increased body weight, F1, F2 and F3 -Decreased epididymal sperm count, F1, F2 and F3, id. for % sperm motility -Increased time taken for copulation, F1, F2 and F3 -Decreased litter size, F1, F2 and F3 -Increased postimplantation loss, F2 -Decreased number of AR and ERβ in testis, F1, F2 and F3; and increased ERα and F1 -Decreased levels of FSH, LH, T, E2 (tested only in F1)	Male reproduction -Delayed puberty onset, F1, F2[b] -Decreased relative weights, testis, epididymis, seminal vesicles, prostate, F1[b] -Decreased epididymal sperm count in cauda epididymis, F2[b] ~10% of testis genes with a significantly modified expression in adults, F1 (F2 in progress)[b] Other: -Eye, head and caudal malformations, F1[b] -Altered tooth enamel prisms (more numerous with larger diameters) and decreased calcium/phosphorus ratio and, enamel volume increased in males, F1[38] -Feminization of digit ratio in males, F1 and F2[39] -Increased body weight in both male and female adults, F2[b] -Food behavior: effects on sweet, salty and fatty preferences in F1, increased in F2[b] -Decreased relative liver weight, F1, F2[b]
Relevance of exposure scheme for human risk assessment	+++ yes (only for the lowest doses), limited to traditional reproductive endpoints	++ yes for both doses but exposure window not fully representative of human chronic exposure and limited to male reproduction endpoints	++ yes for the dose but the absence of exposure in the F2 not representative of human exposure
'Environmental' contamination by EAS/ EDC (bottle, cage, diet and so on) accounted for	No for diet (contains genistein and daidzein)	Yes (for diet and water) No information for cage and bottle	Yes
Mechanistic approaches	NA	+	++ (from collected reproductive organs and tissues and other organs and tissues; ongoing studies, from cellular biology to omics approaches) → allows an integrated views of effects and MOA
Weight of evidence of a low-dose effect	?	+	++
Critical developmental steps studied	No	No	Yes Effects and possible modes of action studied at different developmental stages at the time of exposure (neonatal, prepuberty, postpuberty and adulthood) allowing a dynamic study (see figure 1)
Other	Sprague-Dawley rat strain is generally considered to have a lower susceptibility to endocrine disruption compared with other rat strains		

AR: androgen receptor; BPA: bisphenol A; EAS: endocrine active substance; EDC: endocrine-disrupting compound; EPA: environmental protection agency; ER: estrogen receptor; E2: estradiol; FSH: follicle stimulating hormone; LH: luteinizing hormone; MOA: modes of action; NOAEL: no-observed-adverse-effect-level; OECD: organization for economic co-operation and development; PND: post-natal day; T: testosterone. [a]Only for BPA exposed groups. [b]Auger J and Canivenc-Lavier MC, unpublished data

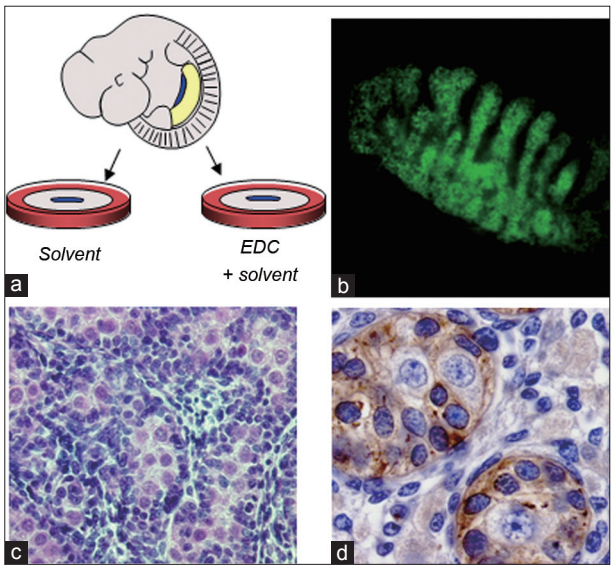

Figure 2: Organotypic cultures of fetal testes. Schematic drawing of the organotypic culture. (**a**) The two testes of the same embryo are set as independent cultures at the gas/medium interface. One is exposed to EDC and the other serves as a control. Mouse fetal testis in culture. (**b**) A testis from a 12.5 dpc (day post conception) OCT4-GFP embryo was cultured for 3 days. Green dots represent germ cells enclosed in testicular cords. Rat fetal testis in culture. (**c**) A 14.5 dpc testis was cultured for 9 days, fixed, sectioned and stained with hematoxylin and eosin. Human fetal testis in culture. (**d**) A piece of 9-week post-fertilization testis was cultured for 4 days and anti-Mullerian hormone, a Sertoli cell marker, was retrieved by immunodetection.

cell death. Those can supply valuable information about the potential impairment of the masculinization potential or about the stock of gamete progenitors in the testis. Histological and RT-qPCR analyses can provide further detailed and integrated information. As with each model, organ culture also has inherent limits, the main one being that the metabolism of EASs/EDCs *in vivo* can generate several bioactive sub-products. One needs thus to carefully identify which chemical substance (s) should be tested *in vitro* and what the expected concentration is in physiological fluids. Another obvious limit is that such a model only makes it possible to assess direct effects on the testis; effects mediated through the hypothalamic-pituitary axis cannot be studied. Altogether, the use of organotypic culture was proven to confirm *in vitro* some of the defects observed with *in vivo* studies (in rodents) for several compounds. Among many examples, DEHP (di (2-ethylhexyl) phthalate) was proven to lower testosterone production and increase the occurrence of abnormal multinucleated germ cells in the developing testis when administered to pregnant rats and its active metabolite, MEHP, was reported to induce the same defects in organ culture. Similar effects of phthalates were also observed *in vivo* and in organ culture in the mouse developing testis.[44]

A further refinement of the organ culture model comes from coupling this approach with the use of transgenic animals. Identifying the mechanism of action of a given EAS/EDC can help in categorizing compounds. Most EASs/EDCs are thought to have proestrogenic or antiandrogenic properties. Mice carrying homozygous gene invalidation of either estrogen (ESR1 and ESR2) or androgen receptors (AR) have thus been exposed to such EDCs. This strategy was proven highly potent when applied to fetal testes. For example, diethylstilbestrol (DES) was proven to decrease testosterone production in mouse fetal testes. The use of ESR1 knockout (KO) mice identified this receptor as mediating the effect of DES on steroidogenesis.[45] In contrast, MEHP alters both gametogenesis and steroidogenesis in the mouse embryonic testis, and these effects were proven independent of ESR or AR.[28] This provides key elements to understand the complexity of EDC effects and should help in proposing compounds that could have additive effects.

The use of organotypic culture of the human fetal testis has identified several compounds that may alter the proper functioning of this tissue: cadmium, MEHP, bisphenol A, metformin and so on.[43,46] Organ cultures also facilitate a comparison of the sensitivity of various species (including rodents used for safety assessment and humans) in a strictly identical system. Such a comparison was recently performed on the bisphenol A effect on fetal testicular steroidogenesis.[46] Interestingly, this study identified a much greater sensitivity of the human testis in comparison to the rat and mouse testis. In contrast, in this model, some EDCs did not induce the expected alterations based on previously characterized effects in rodents. For instance, MEHP and DES were reported to have no effect on the steroidogenesis,[43,46] though several studies reported that these clearly impair testosterone production in rat fetal testes (both *in vivo* and *in vitro*). One may consider that the slow development of the human gonad that spans several months compared with that of rodent gonads (i.e. a few days) would require exposure times that far exceed the capacity of *in vitro* models that are limited to a few days (approximately 4–15 days).

QUESTIONS FROM THE PANEL

Q1: *To which extent can species and strain differences in sensitivity to EDCs be explained, and does this have a predictive value for the sensitivity of humans to these EDCs?*

A1: There are noticeable interspecies differences in endocrine biology, for example, in the timing of critical windows of vulnerability to EASs/EDCs during development or in the hormones required to maintain pregnancy and similar interspecies differences in endocrine-mediated pathogenesis. It was reported that the same EAS/EDC exposure may result in different phenotype modifications according to the rodent species or strains.[47,48] Numerous differences in toxicokinetics are described from one species to another.[49] This may depend, for example, on differences in the liver metabolism of xenobiotics in different species or strains,[50] which can either differently detoxify EASs or produce active metabolites. Also, it has been reported that different reproductive malformation profiles produced by *in utero* phthalate exposure in Wistar and Sprague-Dawley rats could result, at least in part, from strain differences in fetal Leydig cell function and the manner in which these cells respond to DEHP treatment.[51] There is a lack of scientific consensus on the appropriate selection of the species and strain of laboratory animal for the study of EASs/EDCs.[52] Thus, owing to the multiple and complex differences in toxicokinetics and physiology between rodent species and even between strains within the same species, a desirable goal is to select the species/strains that are the most relevant for humans by comparing, when possible, human data and experimental data in rodents: this could lead to selecting different species or strains depending on the endpoints studied.

Q2: *could you comment on the recent studies on human fetal testis xenografts showing different response from rodent models.*

A2: xenograft models are likely to provide relevant information that would be out of reach with organotypic culture, specifically gametogenesis. To overcome this apparent limitation and the absence of EAS/EDC metabolism, some xenograft models were recently developed. These provide an elegant way to analyze the '*in vivo*' effect of EASs/EDCs in human fetal testes exposed over long periods of time (several weeks) and within a framework involving other tissues able to transform parent compounds into bioactive metabolites. This

approach permits exposure via the circulation of the host animals. This model relies on grafting small pieces of embryonic human testis under the skin of castrated nude mice. Although such a model does not perfectly mimic the human situation in the absence of the placental barrier or due to differences in rodent metabolism compared with human metabolism, it comes as close as possible from a physiological standpoint. In such a model, phthalates (DBP) and DES were equally devoid of effects on steroidogenesis. [53–55] Data obtained with organ culture and xenograft models are thus in agreement. This information is critical for our understanding of the deleterious effect of EASs/EDCs on human reproductive parameters.

Indeed, the germ cell development in the human fetal testis displays an additional complexity, especially during the second and third trimesters, with stages of development that span over a long period of time and that have no exact equivalent in rodents. Ultimately, xenografts also have limitations, especially when one tries to assess numerous parameters with a rare and precious tissue. It was reported that testosterone secretion in the plasma of a grafted host mouse was somewhat variable. Such a limit requires the destruction of the tissue to analyze the expression of mRNA coding for steroidogenic enzymes as a surrogate. Another questionable feature in the xenograft protocol is the dose of EASs/EDCs that efficiently reaches the graft. Such models are still recent, and their improvement should be encouraged.

Altogether, organ culture and xenograft now allow the comparison of the effects of EASs/EDCs in the testis of rodents to that in the human fetal testis. These approaches have already identified some conserved response, such as the induction of multinucleated germ cells following exposure to MEHP. Such an effect was retrieved in the mouse, rat and human. It is likely that all models are 'good enough' for predicting potential deleterious effects, even though such alterations were never documented to occur in human fetuses in response to phthalates *in vivo*. Most importantly, such comparisons also point towards striking differences; the absence of an effect of DES on testosterone production in the human fetal testis is a major argument against the proposal of a deleterious effect of 'proestrogenic' EASs/EDCs directly in the human testis. Such data remain scarce, but are obviously important for identifying the targets and mechanisms impaired by EASs/EDCs. Because the DES effect is mediated via estrogen receptor 1 (ESR1) in rodents, the absence of ESR1 in the human fetal Leydig cells[54] furnishes a plausible explanation for the lack of effect of DES on human steroidogenesis in this tissue. The identification of the mechanism of action of EASs/EDCs, notably through the use of transgenic mice, appears thus to be a key criterion to predict whether the deleterious effect observed in rodents will be observed in humans. Unfortunately, the knowledge of EAS/EDC mechanisms of action remain poorly documented in many cases, and the data related to basic signaling pathways present in human gonads currently suffer serious limitations.

CONCLUSIONS

In summary, the *in vivo* experimental EAS/EDC exposure of rodents remains the first choice for studies and is a necessary tool for understanding the reproductive effects and MOA of EASs, provided the pitfalls and limitations of applying the rodent models to humans are known and considered. We have provided evidence that classical rodent models may be refined for studying the multiple consequences of EAS/EDC exposure not only on the reproductive axis but also on various hormonally regulated organs and tissues, among which, several are implicated in the complex process of mammalian reproduction. Such models constitute an interesting way to approach human exposure conditions. There is now a body of evidence that additional models such as organotypic culture models are powerful complementary tools, especially when focusing on the EAS/EDC MOA (which are often far from being strict estrogenic and/or antiandrogenic compounds, but rather possess a number of other MOA at the organ, tissue, cellular or molecular level). All these approaches have recently contributed in a combinatorial manner to a better understanding of the impact and mechanisms of EAS/EDC exposure on male reproduction.

COMPETING INTERESTS

All authors declare that there are no competing interests.

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