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Review article

Adverse effects of endocrine disruptors on the foetal testis development: focus on the phthalates

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Abstract: There are great concerns about the increasing incidence of abnormalities in male reproductive function. Human sperm counts have markedly dropped and the rate of testicular cancer has clearly augmented over the past four decades. Moreover, the prevalence rates of cryptorchidism and hypospadias are also probably increasing. It has been hypothesized that all these adverse trends in male reproduction result from abnormalities in the development of the testis during foetal and neonatal life. Furthermore, many recent epidemiological, clinical and experimental data suggest that these male reproductive disorders could be due to the effects of xenobiotics termed endocrine disruptors, which are becoming more and more concentrated and prevalent in our environment. Among these endocrine disruptors, we chose to focus this review on the phthalates for different reasons: 1) they are widespread in the environment; 2) their concentrations in many human biological fluids have been measured; 3) the experimental data using rodent models suggesting a reprotoxicity are numerous and are the most convincing; 4) their deleterious effects on the *in vivo* and *in vitro* development and function of the rat foetal testis have been largely studied; 5) some epidemiological data in humans suggest a reprotoxic effect at environmental concentrations at least during neonatal life. However, the direct effects of phthalates on human foetal testis have never been explored. Thus, as we did for the rat in the 1990s, we recently developed and validated an organ culture system which allows maintenance of the development of the different cell types of human foetal testis. In this system, addition of 10^{-4} M MEHP (mono-2-ethylhexyl phthalate), the most produced phthalate, had no effect on basal or LH-stimulated production of testosterone, but it reduced the number of germ cells by increasing their apoptosis, without modification of their proliferation. This is the first experimental demonstration that phthalates alter the development of the foetal testis in humans. Using our organotypic culture system, we and others are currently investigating the effect of MEHP in the mouse and the rat, and it will be interesting to compare the results between these species to analyse the relevance of toxicological tests based on rodent models.

Key words: endocrine disruptors, phthalates, environment, development, reproduction, health, foetus, testis, germ cells, testosterone

Alterations in male reproductive function

Changes in the environment and their consequences for male reproductive function have been of major concern for the past 20 years [1-3].

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Alterations in male reproduction were first observed in wild animals, in studies reporting the effects of accidental exposure of estrogenic chemicals on wildlife in the natural environment. These changes in male reproductive function vary from very subtle to permanent alterations, such as feminization or changes in reproductive behaviour [2,4]. Guillette *et al* studied the male reproductive function of alligators in two lakes in Florida. These two lakes are located very close

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to each other geographically, excluding the possibility of climate-based bias in these studies. They found that adult male alligators in Apopka Lake, which was polluted with agricultural waste and experienced a major chemical spill in 1980, had lower testosterone levels and presented micropenis and disorganized testes [5,6]. A key part of this story is that no chemicals could be detected in the water of the apparently contaminated lake and thus the alligators were being exposed simply by being at the top of the food chain. Other documented disruptions or alterations of reproductive activity and physiology have been correlated with exposure of contaminants in fish, amphibians, reptiles, birds, and mammals [4,7,8]. Most of the reported effects on wildlife have been observed on aquatic organisms and this is linked to the concentration of pollutants along the food chain. In humans, there is increasing evidence that the birth sex ratio is altered in areas close to industry and exposed to environmental and industrial chemicals. The findings of the recent report on the Aamjiwnaang First Nation community in Canada are striking [9], i.e. the proportion of male live births in this community has been decreasing continually from 1990 to 2003, the sex ratio (number of male births/total number of births) being only 0.3.

The epidemiologic data have also shown an increase in human male reproductive function disorders over the past 50 years, with the suggestion of a relation with the increase in the amounts of endocrine disruptors in the environment. Testicular cancer, which is the most prevalent cancer in young men, has steadily increased in all countries studied, rising, for example, from 3.4 per 10,000 in 1973 to 5.5 per 10,000 in 1997 in North America [10]. Hypospadias and cryptorchidism also dramatically increased from 0.2 and 2%, respectively, in 1970 to 0.38 and 3.5%, respectively, in 1991 [10]. Finally, sperm count decline has been controversial, but large-scale prospective studies using standardized methodologies have shown a decline from 170 to 70 million spermatozoa per millilitre between 1940 and 1990 in Europe [1,11].

There are grounds for linking these four types of disorders. For example, a comparative study in European countries showed that the incidence of each of these four pre-cited abnormalities (sperm count decline, testicular cancer, hypospadias, and cryptorchidism) was maximal in Denmark and minimal in Finland [12]. Moreover, a history of cryptorchidism increases the risk of other three disorders [13], by a factor of 3-17 in the case of testicular cancer [14]. Similarly, hypospadias increases the chances of developing testicular cancer [1], and oligospermia is frequently observed in men who go on to develop testicular cancer [15,16]. Therefore, it has been suggested that these four alterations are symptoms of a single syndrome named the testicular dysgenesis syndrome (TDS) [16,17].

These abnormalities first arise during foetal development

The foetal testis is formed by the mixing of a somatic anlage growing at the surface of the mesonephros and primordial germ cells that colonize the gonad and are called gonocytes (reviews in [18], Rouiller-Fabre *et al* in this issue). The foetal testis carries out crucial endocrine and gametogenic functions. Foetal Leydig cells produce the testosterone and insulin-like factor 3 (Insl3), which are absolutely necessary for phenotypic masculinisation of the embryo [19], (review in [20]). After birth, gonocytes give rise to the adult stem spermatogonia and thus correct development of the germ cell lineage during foetal/embryonic life is essential for the establishment of the ability of the individual to produce spermatozoa throughout his life.

It is currently thought that TDS is probably caused by disturbances in the development of the foetal testis [16] because the origins of all four characteristics of TDS can be traced to foetal development.

Hypospadias results from defects in androgen production or action during foetal development, while cryptorchidism results from abnormalities in the production and/or activity of Insl3 and/or the androgens respectively regulating the transabdominal and transinguinal descent of the testes [19].

The aetiology of testicular cancer remains unclear, but there is considerable evidence to suggest that it originates early in development [17] when gonocytes would normally have differentiated into spermatogonia. Carcinoma in situ (CIS) is a local malignant lesion that precedes testicular cancer (seminomas and non-seminomas) [21]. CIS cells closely resemble foetal germ cells in terms of morphology and immunohistological markers (c-kit, alkaline phosphatase, etc) [22]. Moreover, CIS has been reported in boys only a few months old [16].

Finally, sperm counts may have decreased for multiple reasons as the regulation of spermatogenesis remains poorly understood, but is known to involve complex endocrine, intratesticular, and intracellular regulation processes. The stock of gonocytes is determined during foetal development and takes part in determining the number of germ stem cells present in adulthood, since experimentally induced decreases in the number of gonocytes during foetal development lead to decreases in sperm count in adulthood [23,24] Similar results are obtained if the number of Sertoli cells is reduced during perinatal life [25]. Thus, adult sperm production depends partly on foetal seminiferous development.

Numerous clinical, epidemiological and experimental data support the hypothesis that TDS is caused by endocrine disruptors acting during foetal or neonatal life (reviews in [2], [26-28]). Endocrine disruptors have been quantitatively and qualitatively increasing

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in our environment during the last decades. They originate from various sources including plants (phytoestrogens), agriculture (insecticides, herbicides, fumigants and fungicides) and many chemicals (pharmaceutical products, plasticisers, resins, detergents, PCB, flame retardant, bisphenol A, antimicrobial parabens, dioxins). In the present study we focused on phthalates.

Environmental exposure to phthalates

Phthalates (phthalic acid esters) are industrial chemicals that have been used increasingly since 1930. Their worldwide production grew from 1.8 to 4.3 million tons between 1970 and 2006. Phthalates are plasticisers that are added to polymers and essentially to PVC to make them softer and more flexible. They are widely used in a wide range of soft-PVC products including building and construction materials such as cabling, flooring, wall covering, profiles and roofs. They are components of medical equipment, hoses, shower curtains, films and plastic gloves, household furnishings, toys, car interiors, clothing, food and beverage packaging, pharmaceutical products, etc. Phthalates are also used as solvents for oil-soluble dyes, insecticides, peroxides, and other organics. They are added to paints and lacquers, adhesives and sealants, cosmetics, lubricants, putty, perfumes, deodorants, sprays. Di(2-ethylhexyl) phthalate (DEHP) is the most widely used phthalate. One and half million tons of DEHP are produced every year worldwide (40% in Europe). Phthalates are not covalently bound to the product matrix and can leach out over time from these products. As an example, they are recovered in domestic dust [29]. Dermal exposure via clothes and cosmetics may also occur. Small population groups may be exposed via medical equipment. So humans are constantly exposed to phthalates through oral, dermal and inhalation routes [30,31]. In the body, phthalates are rapidly hydrolyzed by esterases in the gut and other tissues into monoesters, which are the active molecules [32]. For example, DEHP is metabolized to its monoester metabolite, mono-(2-ethylhexyl) phthalate (MEHP), and DBP is converted into mono-butyl phthalate (MBP).

Whereas many endocrine disruptors are persistent in the environment and accumulate in fat, the half-life of phthalates does not exceed 36 h in the body. In humans, 75% of the DEHP ingested is metabolized and excreted in urine within 2 days [33]. However, phthalates are so widespread in the environment that humans are largely exposed. As an example, according to a study published in 2003, 12% of the German population has a daily intake of DEHP that exceeds European recommendations [34]. In an epidemiologic study, 75% of the 289 human subjects tested were positive for the presence of four different types of phthalates in their urine samples [35]. The concentration of phthalates in biological fluids in humans show large individual variations [36-41]. Values for MEHP are reported in Table 1. Values for MBP are similar or higher, and its urinary concentration in pregnant mothers can reach 5.10^{-6} M. The most recent reports indicate median levels in urine and amniotic fluid of 4.10^{-7} M for MBP and 8.10^{-8} M for MEHP [41].

Effects of phthalates on the development of the rat testis

The first observation of phthalate-induced testicular injuries was reported using adult rats in 1945 [42]. The oral administration of DEHP at dietary concentrations of 0.075, 0.75, 1.5 and 5.0% to rats for 90 days resulted in tubular atrophy and testicular degeneration at the two top dose levels. Subsequently, Harris *et al*. found occasional incidence of tubular atrophy in rats fed 0.5% DEHP in the diet for periods of 3 or 24 months [43]. Many other confirmed and explored the testicular effects of phthalate in experimental animals (review in [44]).

Numerous studies in the rat foetus have shown that *in utero* exposure to di (n-butyl) phthalate (DBP) or DEHP [27,45]). Much attention has been paid to the analysis of Leydig cell development and function [46-52]. *In utero* exposure to phthalates induces an abnormal aggregation of the foetal Leydig cells, an occurrence of intratubular Leydig cells, a reduction of foetal testosterone production and Leydig cell Insl3 gene expression. This leads to epididymal agenesis, reduced ano-genital distance, hypospadias and cryptorchidism. Furthermore, *in utero* exposure to phthalates induces subnormal Sertoli cell proliferation [53], and possibly function [54,55]. Lastly, formation of multinucleated gonocytes during neonatal life and impaired spermatogenesis/infertility in adult have been observed [54,56]. Thus *in utero* exposure to phthalate results in a TDS-like syndrome in the male offspring, except for testicular cancer which is not induced.

The *in vitro* approach has also been used for time and dose analyses of the effects of phthalates on the rat foetal testis. The main limitation on the toxicological use of *in vitro* studies is that the activities and fates of the cells *in vitro* must reproduce those existing *in vivo*. In mammalian cell culture systems, foetal Leydig cells dedifferentiate in the absence of specific gonadotropic stimulation [57-59]. Isolated gonocytes display poor survival in such systems [60-62]. Organ culture systems preserving testicular architecture and intercellular communications appear to us as a relevant method to maintain the development of the foetal, embryonic or neonatal testis. Furthermore, one testis can be cultured in the presence of the tested molecule whereas

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Table 1. Concentrations of MEHP in differents biological fluids in human

Biological liquid	Mininal concentration	Maximal concentration	Reference
Urina from pregnant women	5.10^{-9} M	8.10^{-8} M	Silva et al. 2004 [37]
Urine from pregnant women	5.10^{-9} M	3.10^{-8} M	Swan et al. 2005 [38]
Urine from pregnant women	2.10^{-8} M	5.10^{-7} M	Huang et al. 2009 [41]
Amniotic fluid	$< 3.10^{-9}$ M	4.10^{-7} M	Huang et al. 2009 [41]
Blood from the umbilical cord	not detectable	8.10^{-6} M	Latini et al. 2003 [36]
Human milk	5.10^{-9} M	5.10^{-6} M	Main et al. 2006 [39]
Human milk	2.10^{-9} M	2.10^{-8} M	Högberg et al. 2008 [40]
Human serum from nursing mothers	2.10^{-9} M	2.10^{-8} M	Högberg et al. 2008 [40]
Human urine from nursing mothers	10^{-8}M	2.10^{-7} M	Högberg et al. 2008 [40]

the contralateral testis is cultured in its absence. Thus, an original technique using a filter system was been set up by Habert's team in 1991 [63] (Fig 1). This technique reproduces the development of the Leydig, Sertoli and germ cells observed *in vivo* [58,59,64-67]. Thus, this organotypic culture system is an important tool to study the age-, time- and dose-dependent direct effects of endocrine disruptors on the development of the foetal testis [67]. We named this method the rat foetal testis assay (rFETA) Then, we extended this method to mouse and human foetal testis [66,68-70] and named the methods mFETA and hFETA, respectively. Using this system, the effects of MBP and MEHP were investigated in the rat [71-74]. When explanted at 13.5 or 14.5 days post partum (dpc), one group found an MEHP-induced decrease in the number of gonocytes, and in the production of testosterone and AMH [74] which were not observed by others [72,73]. The reasons for these discrepancies are unclear, but are probably linked to experimental conditions. With foetal testes explanted at 18.5-19.5 dpc, MEHP or MBP induced reductions in the proliferation and AMH expression of Sertoli cells and a reduction in LH-stimulated testosterone production [71,72]. At 3 days post partum, when proliferation of the gonocytes resumes, MEHP induced apoptosis in this cell type [72].

Effects of phthalates on the development of human foetal testis

Despite the growing body of literature data on phthalate reproductive toxicity in animal models, and data demonstrating extensive human exposure, very few studies have examined the effects of these chemicals on human reproductive development.

Epidemiological studies are highly difficult to perform because of the numerous factors that can act on foetal testis development, including hundreds of endocrine disruptors, alcohol, tobacco, individual stress. Furthermore, it is important to take into account the period of exposure. For instance, an association between a low sperm count in adult men with the treatment of their mother with diethylstilbestrol (DES) during pregnancy can be demonstrated only if the data relate to treatment during the first trimester [75]. Recently, anogenital distance was chosen in humans as an index of masculinisation and therefore an index of androgenic activity of the testis. An inverse correlation has been found between anogenital distance measured in male infants 2-36 months of age (mean 12.6 months) and maternal urinary concentrations of MBP and 3 other monoester phthalate metabolites measured at the end of pregnancy [38]. Interestingly, no correlation was observed for MEHP. In the same way, a concentration-dependent association between phthalates in breast milk and levels of reproductive hormones in boys at 3 months of age was also reported [39]. However, a recent paper by Huang *et al* 2009 reported no association between the anogenital distance measured in boys at birth and the concentration of any phthalates in the amniotic fluid or maternal urine during pregnancy. Taken together, these epidemiological studies suggest that some phthalates at environmental concentrations have antiandrogenic effects during neonatal life. Their antiandrogenic effects during foetal life are not demonstrated.

Recently our team reported the first experimental demonstration of the potential deleterious effect of phthalates on human testis function or development [76]. In this study, we used the organ culture system of human foetal testes that we developed previously coupled with morphologic, functional, and molecular methods [68-70] to analyse the effects of MEHP on the development of testicular somatic and germ cells during the first trimester of pregnancy (Fig 1). This early developmental period of the testis has been shown to be a critical window for the determination of the reproductive tract [77].

Folia Histochem Cytobiol. 2009:47(5): S70 (S67-S74)
10.2478/v10042-009-0056-5

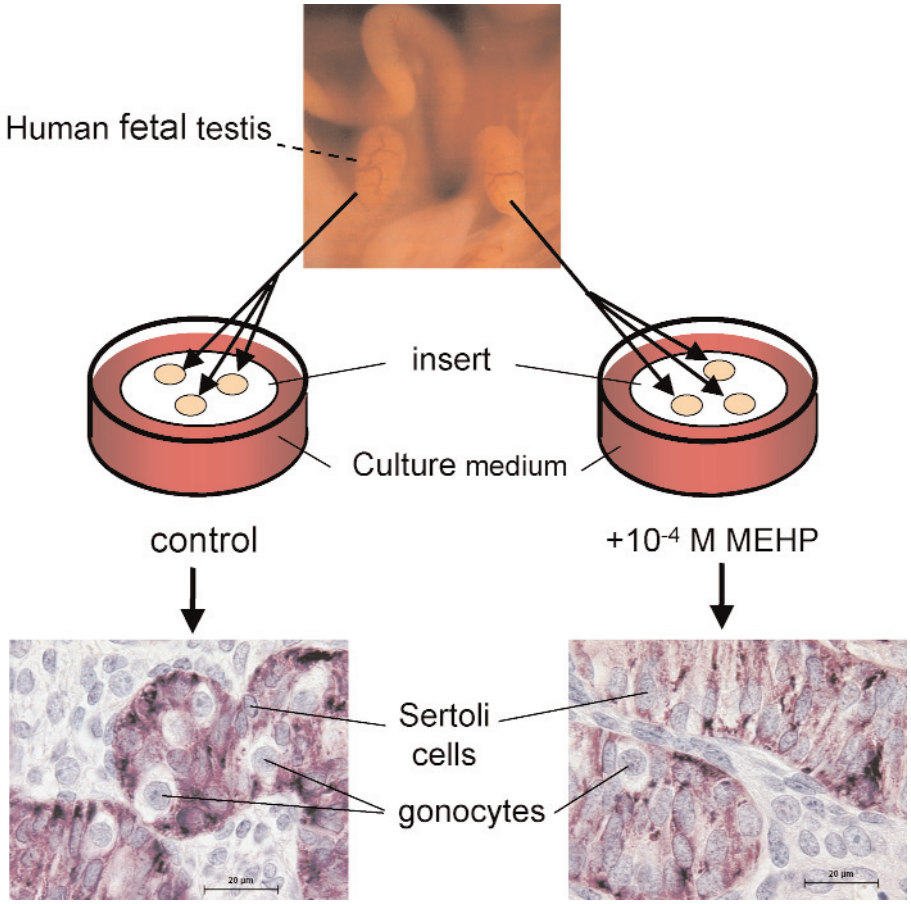

Fig. 1. Human foetal testis assay in humans (hFETA): One testis is cultured after fragmentation in the presence of the test compound and the other one from the same foetus is cultured in the absence of the test compound and served as control. MEHP was assayed and microphotographs show the reduction of the number of gonocytes after 3 days of culture in the presence of 10^{-4} M MEHP [76]. Gonocytes are identified as white cells inside the seminiferous cords, while the Sertoli cells have been immunostained for anti-Müllerian hormone.

Human foetal testes were recovered during the first trimester (7-12 weeks) of gestation and cultured for 3 days with or without MEHP in basal conditions or stimulated with luteinizing hormone (LH). Whatever the dose, MEHP treatment had no effect on basal or LH-stimulated testosterone produced by the human foetal testis *in vitro*. MEHP treatment did not affect the mRNA expression of P450c17, P450scc, or StAR, or that of Insl3 produced by foetal Leydig cells, which is known to be involved in testicular descent and the expression of the steroidogenic enzymes. MEHP (10^{-4} M) did not affect proliferation or apoptosis of Sertoli cells, but it reduced the mRNA expression of anti-Müllerian hormone. Interestingly, MEHP (10^{-4} M) reduced the number of germ cells by increasing their apoptosis, measured by the detection of caspase-3-positive germ cells, without modification of their proliferation.

Conclusions

We review here arguments suggesting that the alterations of the male reproductive functions observed during the last decades are linked to an increasing exposure to endocrine disruptors with a focus on the phthalates. Our laboratory recently here the first experimental evidence for the potential of phthalates to impair the development of foetal testis in the human species [76]. Interestingly, the main disruption appears to be of the gametogenic function of the foetal testis, and no effect was observed on Leydig cell development and function. In the same way, epidemiological studies suggest that the antiandrogenic effects of environmental phthalates occur in the baby or the young child and not during foetal life. In rats, as in humans, phthalates act on foetal/neonatal gametogenesis, but, unlike in humans, they induce large antiandrogenic effects. Thus, the relevance of the rat model for toxicological studies related to phthalates must be debated. A recent paper reports that *in utero* exposure to phthalates impairs the development of gametogenesis without alterations of steroidogenesis of the foetal testis in mouse [78]. Using mouse foetal testis in culture, we also observed impairment of gametogenesis and no decrease of steroidogenesis [79]. Thus it will be important to establish whether the mouse could be an interesting model for studying the effects and mechanisms of action of phthalates in human foetal testicular development.

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