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Effect of mono-(2-ethylhexyl) phthalate on human and mouse fetal testis: *In vitro* and *in vivo* approaches

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ABSTRACT

The present study was conducted to determine whether exposure to the mono-(2-ethylhexyl) phthalate (MEHP) represents a genuine threat to male human reproductive function. To this aim, we investigated the effects on human male fetal germ cells of a 10^{-5} M exposure. This dose is slightly above the mean concentrations found in human fetal cord blood samples by biomonitoring studies. The *in vitro* experimental approach was further validated for phthalate toxicity assessment by comparing the effects of *in vitro* and *in vivo* exposure in mouse testes.

Human fetal testes were recovered during the first trimester (7–12 weeks) of gestation and cultured in the presence or not of 10^{-5} M MEHP for three days. Apoptosis was quantified by measuring the percentage of Caspase-3 positive germ cells. The concentration of phthalate reaching the fetal gonads was determined by radioactivity measurements, after incubations with 14 C-MEHP.

A 10^{-5} M exposure significantly increased the rate of apoptosis in human male fetal germ cells. The intratesticular MEHP concentration measured corresponded to the concentration added *in vitro* to the culture medium. Furthermore, a comparable effect on germ cell apoptosis in mouse fetal testes was induced both *in vitro* and *in vivo*.

This study suggests that this 10^{-5} M exposure is sufficient to induce changes to the *in vivo* development of the human fetal male germ cells.

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Introduction

Phthalate esters are a class of environmental endocrine disrupting chemicals, which are mainly used as plasticizers in PVC plastics. They are present in flexible plastics, such as food and beverage packaging, children's toys and biomedical apparatuses. As phthalate esters are not covalently bound to the plastic, overtime they can leach from these products and eventually be ingested (Silva et al., 2004). After absorption, phthalates are rapidly hydrolyzed by esterases, which are predominantly present in the gut and pancreas, into monoesters, that

are the biologically active molecules (Latini, 2005). Di-(2-ethylhexyl) phthalate (DEHP), one of the most widely used phthalates, is metabolized into its monoester metabolite, MEHP, which is a recognized testicular toxicant (Habert et al., 2009; Sharpe and Skakkebaek, 2008).

Studies carried out in animal models have established the toxic effects of phthalates on testicular function. Specifically, in the rat, *in utero* exposure to phthalates leads to male reproductive disorders, including altered seminiferous cord formation, increased germ cell apoptosis, appearance of multinucleated gonocytes (MNG), reduced anogenital distance, hypospadias, cryptorchidism (Fisher et al., 2003; Foster, 2006; Gray et al., 2006) as well as reduction of testosterone production and *Insl3* expression in fetal Leydig cells (Lehmann et al., 2004; McKinnell et al., 2005; Sharpe and Skakkebaek, 2008). Some of these effects were also recently observed *in vitro* using the organotypic culture system that has been developed and validated by our laboratory for testing the effects of specific compounds on rat, mouse and human fetal testes (Chauvigne et al., 2009; Livera et al., 2006).

Abbreviations: AMH, anti-Müllerian hormone; DAB, 3,3'-diaminobenzidine; DEHP, di-(2-ethylhexyl) phthalate; dpc, days post coitum; m/hFETA, mouse/human FETal Testis Assay; HPLC, High Pressure Liquid Chromatography; LC-MS/MS, Liquid Chromatography–Tandem Mass Spectrometry; MEHP, mono-(2-ethylhexyl) phthalate; MNG, multi-nucleated gonocyte; Mvh, mouse vasa homologue; NGS, normal goat serum; NOAEL, No Observable Adverse Effect Level; R_f , retention time.

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In the mouse, a single exposure to 500 mg/kg or repeated exposures to 250 mg/kg of di-butyl phthalate (DBP) during fetal development caused rapid and dynamic changes in testis gene expression profile. Moreover, increased number of multinucleated gonocytes per cord, and of nuclei per multinucleated gonocyte has also been reported (Gaido et al., 2007). Recently, using our *in vitro* organotypic culture system (mouse Fetal Testis Assay: mFETA) (Habert et al., 2009), specific periods of MEHP sensitivity during fetal mouse testis development were identified (Lehraiki et al., 2009). Indeed, the effects of phthalates on steroidogenesis vary with the developmental stage, whereas, the deleterious effects of phthalates on germ cells were observed consistently during the active phases of gonocyte development, thus indicating no correlation between these effects (Lehraiki et al., 2009).

On the other hand, very few experimental studies have examined the potential effects of phthalates on the development of the human fetal reproductive organs. Using our validated organotypic culture system (human Fetal Testis Assay: hFETA), we recently demonstrated that phthalates have the potential to impair the *in vitro* development of the male fetal germ cell lineage, without associated changes in testosterone production (Habert et al., 2009; Lambrot et al., 2009). Specifically, after exposure to 10^{-4} M MEHP for three days, the number of germ cells in cultured human fetal testes was reduced by 40% due to a strong increased apoptosis. This work was the first experimental demonstration of the potentially deleterious effect of phthalates on human male reproductive functions (Lambrot et al., 2009).

The present study was conducted to analyze the relevance of this initial data, particularly in relation to the risk of MEHP exposure for human reproductive health. As concentrations as high as 10^{-4} M have never been detected in human biological fluids, we first tested the effects of a lower dose of MEHP (10^{-5} M) on human male fetal germ cell development. Then, as MEHP might strongly adsorb onto plastics, which are used in a large part of the tissue culture systems, it was important to evaluate precisely the concentration of the added MEHP that reaches the cultured human fetal testes. Finally, as the *in vitro* approach is one of the only conceivable experimental methods for assessing the effects of MEHP on human fetal testis, a mouse model was used to compare *in vitro* and *in vivo* exposures.

Materials and methods

Animals. C57Bl/6 mice were housed under controlled photoperiod conditions (lights on 08:00 h–20:00 h) with *ad libitum* access to tap water and a soy and alfalfa-free breeding diet (Global diet 2019, Harlan Teklad, Indianapolis, IN). Males were caged with females overnight and the day following overnight mating was counted as 0.5 days post coitum (dpc).

For studying *in utero* MEHP exposure, corn oil alone (vehicle) or 720 mg/kg MEHP was administered by oral gavage (360 mg/kg twice a day at 08:30 h and 18:30 h) to pregnant females at 13.5 dpc for 24 h. Pregnant mice were then killed by cervical dislocation at 14.5 dpc, fetuses quickly removed and dissected under a binocular microscope. Their gender was determined on the basis of gonad morphology. Testes were collected from male fetuses and fixed in Bouin's fluid. For *in vitro* studies, 13.5 dpc fetuses were removed from control pregnant mice and cultured for 24 h as described in the original publication (Lehraiki et al., 2009). Authorizations for manipulating animals were given by the French Ministry of Agriculture and all animal studies were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Collection of human fetal testes. Human fetal testes were obtained from pregnant women referred to the Department of Obstetrics and Gynecology at the Antoine Béclère Hospital, Clamart (France) for legally induced abortions in the first trimester of pregnancy (*i.e.* from the 7th until the 12th week post conception), as previously described

(Angenard et al., 2010; Lambrot et al., 2006b). None of the terminations was for reasons of fetal abnormality and all fetuses appeared morphologically normal. Fetal gender was determined by gonad morphology and fetal age was evaluated by measuring the length of limbs and feet (Evtouchenko et al., 1996). Male fetuses were dissected under a binocular microscope and gonads removed aseptically and immediately explanted *in vitro*. Testes within the abortive material were retrieved in approximately 30% of cases. The Antoine Béclère Hospital Ethics Committee approved this study.

Organ cultures. The organotypic culture system used here was first set up for rat fetal testis experiments (Habert et al., 1991; Livera et al., 2000; Olasso et al., 1998) and then improved and adapted to mouse (mFETA) (Lambrot et al., 2006b; Livera et al., 2006) and human testes (hFETA) (Lambrot et al., 2006a, 2006b). This culture system has been validated for toxicological studies (Habert et al., 2009; Lambrot et al., 2006a, 2009; Livera et al., 2006). Briefly, human and mouse testes were cultured on Millicell-CM Biopore membranes (pore size 0.4 μ m, Millipore, Billerica, MA) as previously described. The culture medium was phenol-red free Dulbecco modified Eagle medium/Ham F12 (1:1) (Gibco, Grand Island, NY), supplemented with 80 μ g/mL gentamicin (Sigma, St. Louis, MO). MEHP was obtained from TCI Europe (Antwerp, Belgium). Gonad explants were placed on membranes, floating on 320 μ L of culture medium in tissue culture dishes, and cultured at 37 °C in a humidified atmosphere containing 95% air/5% CO₂. Polystyrene culture dishes that do not contain phthalates were used. Each human gonad was cut into small pieces. Three random pieces from an individual gonad were placed on a membrane and cultured for a total of 4 days of which the last three in the presence of MEHP. An equal number of explants from the same gonad were cultured in control medium containing DMSO (vehicle, 1/4000). Culture medium was changed every 24 h. At the end of the culture period, each explant was fixed in Bouin's fluid for 1 h, dehydrated and embedded in paraffin blocks.

¹⁴C-MEHP preparation and purification. [U-ring-¹⁴C]-DEHP (radiochemical purity 99.0% checked by radio-HPLC; specific activity 244.2 MBq/mmol) was purchased from Sigma-Aldrich (St. Louis, MO). Flo-Scint II and Ultima Gold liquid scintillation cocktails were from Perkin Elmer Life Sciences (Waltham, MA). All solvents were of analytical grade and were purchased from Scharlau (Barcelona, Spain). Milli-Q ultrapure water (Millipore, Billerica, MA) was used for the HPLC mobile phases. Standard MEHP was from Sigma-Aldrich. ¹⁴C-MEHP was produced by hydrolysis of ¹⁴C-DEHP by incubating 1.5 kBq ¹⁴C-DEHP with 2.9 units of pig liver esterase in ammonium sulfate suspension (Sigma-Aldrich, St. Louis, MO) in 0.5 mL of 0.1 M Tris buffer (pH 7.4) with shaking at 37 °C for 30 min. The reaction was stopped by adding one volume of methanol and the solution was centrifuged for 10 min at 8000 g. Radio-HPLC analysis of an aliquot of the supernatant showed that more than 90% of the radioactivity was associated with a compound, that had the retention time (R_T) of MEHP. The identity of MEHP was confirmed by LC-MS/MS analysis in negative-ion mode using an electrospray source (data not shown). All produced ¹⁴C-MEHP (n = 10 reactions) was analyzed on a HP1100 apparatus (Hewlett-Packard, Waldbronn, Germany) connected to a Flo-one 610TR radiometric flow scintillation analyzer (Perkin-Elmer Life Sciences, Courtabœuf, France) for radioactivity detection. Flow-Scint II was used as the scintillation cocktail. The HP1100 apparatus was connected to a Gilson model 201/202 fraction collector (Gilson France, Villiers-Le-Bel, France) for ¹⁴C-MEHP isolation. The reverse phase HPLC system consisted of a C18 guard pre-column coupled to a Kromasil C18 column (250 mm × 4.6 mm, 5 μ m) (Interchim, Montluçon, France). Mobile phases were as follows: A = 20 mM ammonium acetate buffer (adjusted to pH 2.8) and methanol (90:10 v/v), and B = methanol. The flow rate was 1 mL/min at 35 °C. The gradient was as follows: 0–35 min linear gradient from

100% A to 100% B, 35–50 min 100% B. In such conditions, MEHP R_T was 32 min whereas DEHP R_T was 40 min. The radiochemical purity of purified ^{14}C -MEHP, checked by radio-HPLC, was greater than 99.0%.

Distribution of ^{14}C -MEHP. In order to evaluate the concentration of MEHP in the gonads, the testis volume was determined by measuring the length, width and depth of each testis on a micrograduated ruler using a binocular microscope. Testis cultures were performed in the presence of 10^{-4} M unlabelled MEHP for 48 h and then for 24 h with 1.7 kBq ^{14}C -MEHP mixed with unlabeled MEHP to obtain a final concentration of 10^{-4} M radiolabeled MEHP. Gonads were then dissolved in Soluene for 6 h in the dark and transferred into scintillation liquid. The radioactivity of the culture medium and the gonads was quantified using a Tricarb analyzer (Packard Instruments, Downer Grove, IL). Each sample was measured for 30 min and sample quenching was compensated by the use of quench curves and external standardization. An aliquot of the medium was stored at -20°C for subsequent radio-HPLC analysis.

Germ cell counting. Serial slide-mounted sections of fetal testis explants were deparaffinized and rehydrated. For human testes, immunohistochemical assessment of anti-Müllerian hormone (AMH) expression was performed as previously described (Lambrot et al., 2006b) using a rabbit anti-AMH polyclonal antibody (generously provided by Dr N. Di Clemente [INSERM U782, Clamart, France]). Germ cells were identified as AMH-negative cells within the seminiferous cords (Lambrot et al., 2006b, 2007). As the mouse vasa homologue (Mvh) is a specific marker of mouse germ cells, Mvh staining was performed using a rabbit polyclonal anti-Mvh antibody (1/500; Abcam, Cambridge, UK).

For mouse gonads, all germ cells in every third section were counted. The sum of the values obtained for the counted sections was multiplied by the total number of sections/testis to obtain a crude count of germ cells per gonad. To correct for any over-counting due to single cells appearing in two successive sections, the Abercrombie's formula (Abercrombie, 1946), which is based on the mean diameter of cell nuclei and section thickness, was applied (Livera et al., 2001, 2006; Olaso et al., 1998). All counts were carried out blind and were done using the Histolab analysis software (Microvision Instruments, Evry, France).

Immunohistochemical analysis of cleaved Caspase-3 expression. Activated Caspase-3 is involved in most apoptotic pathways (Omezzine et al., 2003) and its immunodetection was used to quantify the rate of apoptosis as previously described (Delbes et al., 2004; Lambrot et al., 2006a). Six sections were mounted onto a single slide and placed under high pressure at 120°C for 30 min in a permeabilization solution (0.05 M Tris, pH 10.6) using the Retriever 2100 apparatus (Proteogenix, Oberhausbergen, France). The primary anti-cleaved Caspase-3 Asp 175 antibody (1/100, Cell Signaling, Beverly, MA) was detected using a biotinylated goat anti-rabbit secondary antibody (1/200) in 5% NGS and an avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized using 3,3'-diaminobenzidine (DAB) as substrate. For all immunohistochemical experiments, negative controls were without the primary antibody.

Statistical analysis. All values were expressed as the mean \pm SEM. For experiments with human samples, the significance of the differences between mean values for treated and untreated testes was evaluated using the Wilcoxon's non-parametric paired test due to the little number of samples. The Student's *t*-test was used for mouse experiments.

Results

In vitro exposure to 10^{-5} M MEHP apoptosis of human male fetal germ cells

Explants of human fetal testes (7 to 12 weeks of gestation) were cultured in control medium for 24 h and then in the presence or not (controls) of 10^{-5} M MEHP for 72 h. The apoptosis rate in fetal germ cell was assessed using cleaved Caspase-3 immunohistological staining. The number of cleaved Caspase-3 positive germ cells was significantly higher in treated fetal testes than in control (Figs. 1A and B). Quantification of the data indicated that the apoptosis rate was almost doubled in MEHP-exposed testes compared to control samples (Fig. 1C). Comparisons with previous results suggests a dose-response relationship, as 10^{-4} M MEHP induced a 3-fold increase in the rate of apoptosis and 10^{-6} M MEHP had no deleterious effects (Lambrot et al., 2009).

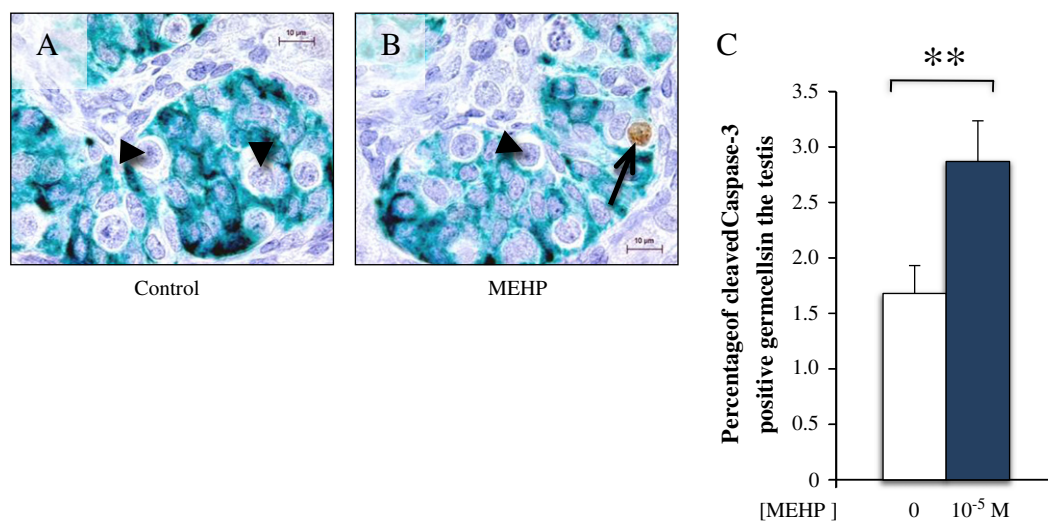


Fig. 1. Evaluation of germ cell apoptosis in human fetal testes (7 to 12 weeks of gestation) following incubation with 10^{-5} M MEHP for 72 h. (A and B) Immunohistological analysis of the expression of AMH (green) and cleaved Caspase-3 (brown) in control (A) and MEHP treated testis explants (B). (C) Quantification of the percentage of cleaved Caspase-3 positive

Distribution of MEHP in the human fetal testis organ culture system

To determine how much of the MEHP added in the culture medium effectively reach the fetal testis in our culture system, MEHP distribution was traced in the hFETA model.

^{14}C -MEHP was added to the culture media and incubated 24 h the last day of culture. ^{14}C distribution was then measured by the scintillation method. The sum of the ^{14}C amount detected in the culture medium and the gonad only represented 70% of the entire amount of ^{14}C -MEHP added to the culture. Thus, about 30% of the MEHP was adsorbed during the culture onto the plastic elements of the culture system (Fig. 2A). Moreover, when the ^{14}C amount detected in testes and culture medium was normalized to their respective volumes, MEHP concentration in fetal testis explants was slightly increased ($2.4 \times 10^{-4} \text{ M}$), whereas concentration in the culture medium showed a slight decrease ($6.4 \times 10^{-5} \text{ M}$) in comparison to the initial concentration (10^{-4} M) (Fig. 2B).

Radio-HPLC analyses were performed to investigate whether the observable effects on germ cells were due to MEHP alone or also to metabolites arising from secondary MEHP biotransformation. Analyses of the culture medium samples were characterized by a single peak corresponding with the retention time of MEHP, indicating that

MEHP was not further metabolized by cultured human fetal testes (Fig. 2C).

Comparison of the *in vitro* and *in vivo* experimental approaches using the mouse model

In humans the *in vitro* approach is the only available way to investigate the toxicological effects of compounds on fetal testes. Therefore, it is very important to perform a similar series of experiments in an animal model that allows the comparison between both *in vitro* and *in vivo* approaches.

Data published by our group concerning the *in vitro* effects of MEHP on germ cells in 13.5 dpc cultured mouse fetal testes exposed to $2 \times 10^{-5} \text{ M}$ MEHP for 24 h (Lehraiki et al., 2009) were compared with the effects of MEHP on fetal germ cells following its administration to pregnant mice in comparable experimental conditions (exposure period and developmental stage). *In vivo*, exposure to 720 mg/kg/day MEHP between 13.5 dpc and 14.5 dpc induced the loss of about 30% of germ cells (from 5600 germ cells/testis in control fetuses to 3800 in treated ones) (Fig. 3A) as indicated by the reduction of Mvh stained cells in treated testes compared to controls (Figs. 3C and D). Histological analysis of the cleaved Caspase-3 positive germ cell indicated that the

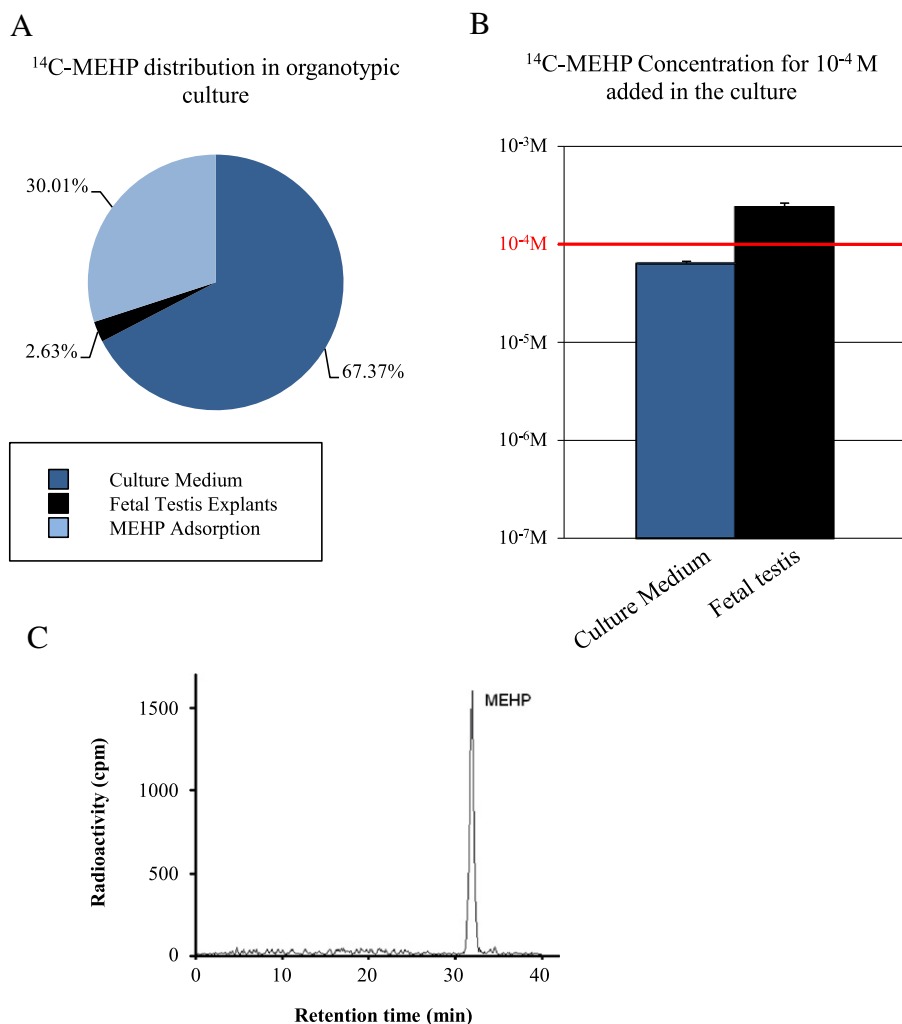


Fig. 2. MEHP distribution in the *in vitro* organotypic culture system for human fetal testes (hFETA). Testis explants were culture in the presence of 10^{-4} M unlabeled MEHP for 48 h and then ^{14}C -MEHP was added to the culture medium for 24 h (last day of culture). (A) Distribution of ^{14}C -MEHP in the different compartments of the culture system. (B) ^{14}C -MEHP normalized concentrations in the culture medium and fetal testis explants represented on a logarithmic scale. The red line represents the MEHP concentration initially added in the culture medium. (C) HPLC analysis of the different ^{14}C -labeled compounds in the culture medium. 32 min corresponds to the retention time of MEHP. The histograms represent the mean \pm SEM of 3 different fetuses.

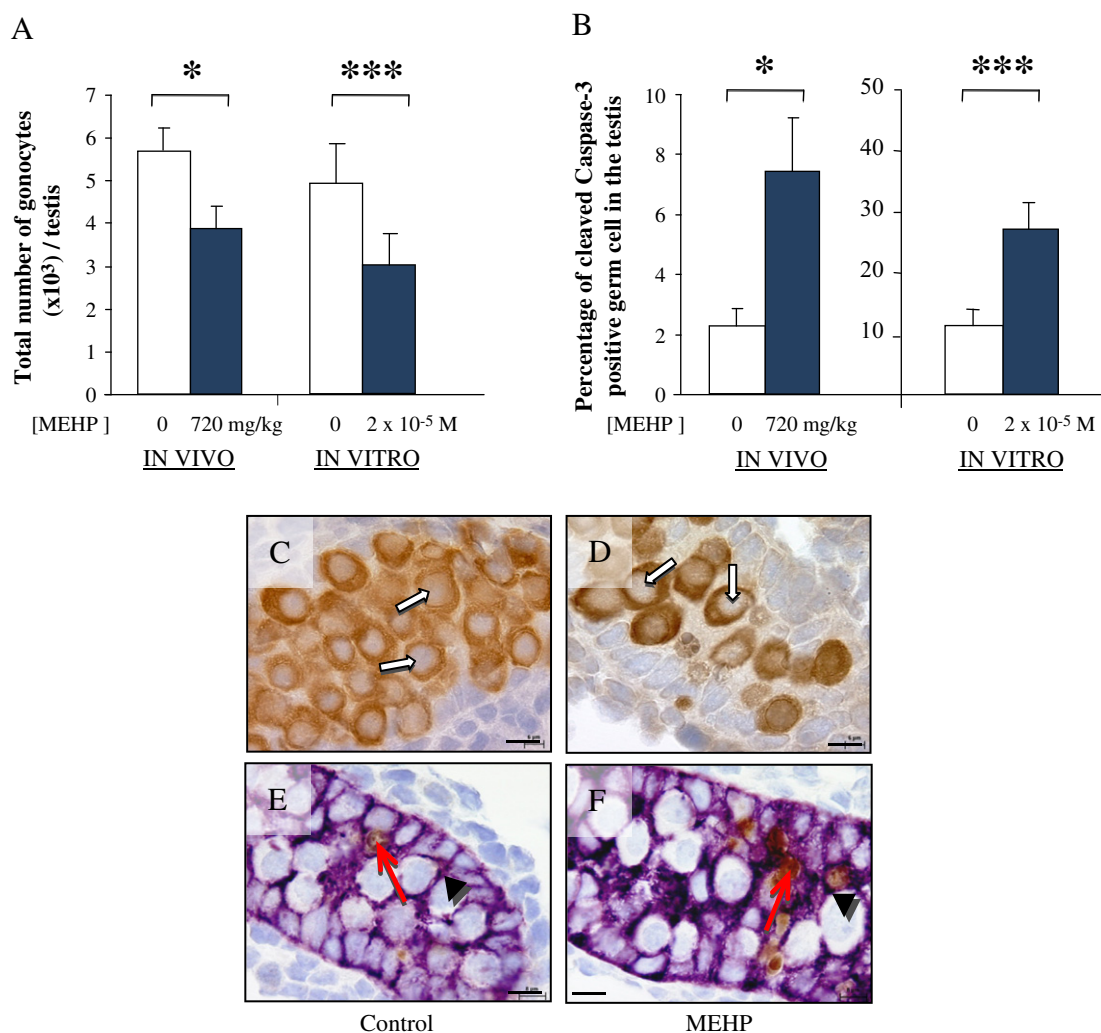


Fig. 3. Comparison of the *in vivo* and *in vitro* MEHP effects on fetal mouse testes. C57Bl/6 pregnant females were fed with 720 mg/kg MEHP at 13.5 dpc for 24 h and euthanized at 14.5 dpc. *In vitro* data in (A) and (B) originate from Lehraiki et al. (2009). (A) Total number of gonocytes per testis determined by counting germ cells in histological sections after *in vivo* and *in vitro* exposure, or not, to MEHP. (B) Percentage of cleaved Caspase-3 positive gonocytes in mouse fetal testes following *in vitro* and *in vivo* exposure, or not, to MEHP. Immunodetection of Mvh (C and D) or cleaved Caspase-3 (E and F) in sections of mouse fetal testes (14.5 dpc) following *in vivo* exposure, or not, to MEHP. White arrows indicate Mvh positive gonocytes, black arrowheads show cleaved Caspase-3 negative gonocytes and red arrows indicate cleaved Caspase-3 positive gonocytes. Scale bar = 10 μ m. Histograms represent the mean \pm SEM of 5 to 6 different fetuses from three different litters. * $p < 0.05$ and *** $p < 0.001$ by Student's paired *t*-test.

germ cell loss was correlated to a significant 4-fold increase in the rate of apoptosis, increasing from 2% in controls (Figs. 3B and E) to 8% in testes exposed to MEHP (Figs. 3B and F). These results mirror the *in vitro* data we previously reported in Lehraiki et al., 2009 (Figs. 3A and B *in vitro* charts).

Discussion

We previously showed that 10^{-4} M MEHP disrupts the *in vitro* development of the fetal male germ cell lineage in humans. The present study was conducted to assess whether MEHP represents a real toxicological risk for human reproduction.

First, we show that 10^{-5} M MEHP significantly increased the apoptosis rate of human gonocytes in our *in vitro* culture system. As we previously demonstrated that 10^{-6} M MEHP does not have any detectable effect (Lambrot et al., 2009), we conclude that the No Observable Adverse Effect Level (NOAEL) of MEHP for human germ cells ranges between 10^{-6} M and 10^{-5} M (278 to 2780 μ g/L). This new information is relevant for the assessment of MEHP associated risk. No data presently exists on the concentration of phthalates

present in human fetuses during the first trimester of intrauterine life. Reported MEHP concentrations in human biological fluids during the fetal and neonatal periods can be used to estimate the concentration of MEHP to which fetal testes may be exposed (Table 1). However, the use of body fluids for biomonitoring phthalate exposure can be subjected to extensive variations from person to person and in the various fluids (Koch and Calafat, 2009; Swan et al., 2005). Indeed, in a prospective study on cryptorchidism, MEHP concentrations in human breast milk ranged from 5×10^{-9} M to 5×10^{-6} M (Main et al., 2006), whereas the highest concentration detected in maternal milk by Hogberg et al. (2008) was 2×10^{-8} M. The levels of MEHP in amniotic fluid are also always lower than the NOAEL determined in this study (Table 1). On the other hand, measuring MEHP level in fetal blood might be a better approach to ascertain the potential concentration to which testes are exposed *in utero*. Very few studies on MEHP levels in cord blood samples are available. Latini et al. (2003) reported values ranging from undetectable concentrations to 10^{-5} M in neonate cord blood samples, with a mean value of 2×10^{-6} M. More recently Lin et al. (2008) found a mean concentration of about 3.6×10^{-5} M in fetal cord blood samples but a more recent study reported a mean

Table 1
Concentrations of different phthalate monoesters detected in various human biological fluids during pregnancy and lactation.

Biological fluid	Phthalate	Minimal concentration	Maximal concentration	References
Urine from pregnant women	MEP	10^{-7} M	10^{-5} M	Silva et al., 2004
	MBP	3.10^{-8} M	8.10^{-7} M	
	MEHP	5.10^{-9} M	8.10^{-8} M	
Urine from pregnant women	MEP	3.10^{-7} M	2.10^{-6} M	Swan et al., 2005
	MBP	3.10^{-8} M	1.10^{-7} M	
	MEHP	5.10^{-9} M	3.10^{-8} M	
Urine from pregnant women	MEP	2.10^{-8} M	7.10^{-7} M	Huang et al. 2009
	MBP	9.10^{-8} M	2.10^{-6} M	
	MEHP	2.10^{-8} M	5.10^{-7} M	
Amniotic fluid	MEP	$<6.10^{-9}$ M	5.10^{-8} M	Silva et al., 2004
	MBP	$<4.10^{-9}$ M	1.10^{-6} M	
	MEHP	$<3.10^{-9}$ M	1.10^{-8} M	
Amniotic fluid	MEP	$<5.10^{-9}$ M	4.10^{-8} M	Huang et al. 2009
	MBP	1.10^{-7} M	7.10^{-7} M	
	MEHP	$<3.10^{-9}$ M	4.10^{-7} M	
Umbilical cord blood	MEHP	Not detectable	10^{-5} M	Latini et al., 2003
Human milk	MEP	4.10^{-10} M	2.10^{-7} M	Main et al., 2006
	MBP	3.10^{-9} M	5.10^{-5} M	
	MEHP	5.10^{-9} M	5.10^{-6} M	
Human milk	MEP	3.10^{-9} M	10^{-8} M	Hogberg et al., 2008
	MBP	2.10^{-9} M	3.10^{-8} M	
	MEHP	2.10^{-9} M	2.10^{-8} M	
Serum samples from nursing mothers	MEP	3.10^{-9} M	7.10^{-8} M	Hogberg et al., 2008
	MBP	2.10^{-9} M	9.10^{-8} M	
	MEHP	2.10^{-9} M	2.10^{-8} M	
Urine from nursing mothers	MEP	3.10^{-9} M	4.10^{-6} M	Hogberg et al., 2008
	MBP	2.10^{-8} M	9.10^{-7} M	
	MEHP	10^{-8} M	2.10^{-7} M	

concentration of only 1.1×10^{-8} M (Lin et al., 2011). This suggests that human fetuses could be exposed to MEHP concentrations close to the NOAEL estimated in this study. Moreover, MEHP is not the only phthalate that can be detected in biological fluids. Indeed, several studies have identified many other phthalates monoesters (mainly mono-ethyl phthalate and mono-butyl phthalate) in biological fluids (see Table 1 for their concentrations), suggesting the possibility of additive or synergistic effects, due to cumulative doses of different phthalates and/or in combination with other endocrine disruptors (Koch and Calafat, 2009).

To validate the estimated NOAEL for MEHP it was necessary to determine the amount of MEHP added in the medium that effectively reached the testes in our culture system. The volume of the explanted testes and the concentration of ^{14}C -MEHP in both culture medium and testicular explants were measured. The concentration of MEHP recovered in the testicular explants was close to the expected concentration and 30% of the added phthalate was adsorbed onto the plastic of culture dishes and inserts. Chauvigne et al. (2009) also analyzed the distribution of MEHP in the different compartments of the organotypic culture system using the rat fetus model. They observed that 0.3 to 0.4% of the MEHP was recovered in the cultured fetal testes. When normalizing this value to the testis volume (Habert and Picon, 1984) the intratesticular concentration of MEHP was, here also, relatively close to the expected concentration.

The use of radiolabeled MEHP allowed us to analyze the fate of this phthalate in cultured human fetal testes as well. No MEHP metabolites were found in these experimental conditions, suggesting that the all MEHP remained in this molecular state. In mammals, MEHP is mainly metabolized in the liver by the cytochrome P450 enzyme family, via metabolite oxidation mainly at the terminal (ω position) or penultimate carbon ($\omega-1$ position) of the alkyl ester side chain (SCENIHR, 2007). Although it is not known which specific cytochrome P450 is involved in MEHP oxidation, it can be speculated that the CYP4A subfamily, which governs the ω and $\omega-1$ oxidation of fatty acids and alkyl chains (Fer et al., 2008; Muller et al., 2007) and is induced by DEHP (Okita and Okita, 1992), could play a role in this reaction. Testis contains different cytochrome P450 enzymes. Some

of these enzymes are involved in testosterone biosynthesis (e.g., CYP11A1 and CYP17A1) or prostaglandin hydroxylation (CYP4F8), while others, such as CYP1A1 or CYP1B1, could play a role in the biotransformation of hydrophobic xenobiotic compounds to more water-soluble metabolites (Leung et al., 2009; Schuppe et al., 2000; Shan et al., 1993). However, CYP4A isoforms in the testis have not been detected so far. In addition, the absence of oxidized metabolites in our biological model may be explained by the presence of immature forms or lack of expression of these enzymes and is consistent with the very low concentration of oxidized MEHP metabolites found in the amniotic fluid (Wittassek et al., 2009).

As only *in vitro* approaches can be used to experimentally assess the toxicology risk in humans, we used an animal model to compare *in vitro* findings obtained with this organotypic culture system with *in vivo* exposure. For this purpose, we used the mouse model because the *in vitro* developments of human and mouse fetal testes are comparable (Lambrot et al., 2006b; Livera et al., 2006). For instance, in organotypic cultures, the basal testosterone secretion slightly increases or remains stable with time in very young mouse and human testes (12.5 dpc for mouse and 7 weeks of gestation for human testes), whereas it decreases if human or mouse testes are explanted at later stages. Thus, this organotypic culture system is a suitable model when applied to young testes development but only allows short- or medium-term studies. As the *in vitro* effects of MEHP on mouse fetal testes were already demonstrated by our group (Lehraiiki et al., 2009), pregnant mice were force-fed MEHP in similar conditions. For this comparison, MEHP doses about 20-fold higher than the NOAEL were used for both *in vivo* and *in vitro* studies (Lehraiiki et al., 2009; McKee, 2004). Here in the mouse, MEHP induces a comparable diminution of the number of male germ cells, associated with a similar relative increase in the rate of apoptosis, both *in vivo* and *in vitro*. Moreover, in the study by Stroheker et al. (2006), pregnant rats were treated by gavage with 750 mg/kg/day of DEHP at 14 dpc. The day after, radio-labeled DEHP was administered and 2 h later the radioactivity content was measured in the amniotic fluid. The radioactivity concentration was 25.1 nmol/g, which approximately corresponds to 2.5×10^{-5} M of DEHP equivalent

concentration (Stroheker et al., 2006). These data strengthen the legitimacy of our comparison between *in vivo* and *in vitro* approaches.

Conclusion

This study reported the deleterious effects of 10^{-5} M MEHP on the *in vitro* development of the human male germ cell lineage. Although the average MEHP concentration in human fetal cord blood is lower, the use of 10^{-5} M reflects exposures to some of the highest concentrations reported. These data therefore constitute a significant evaluation of the toxicological risk of MEHP to human health. Particularly, we determined the actual MEHP concentration in cultured human fetal testes after exposure and we demonstrated that the toxicological effects on germ cell development observed *in vitro* might cautiously be extrapolated to *in vivo* exposure.

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Conflict of interest statement

All authors declare not to have any conflict of interest in the following work and approve the manuscript.

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