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**Abbreviated title**

Effects of Phthalates on Human Fetal Testis.

**Key words**

Human fetus, phthalates, testis development

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**Abstract**

BACKGROUND

Several studies have described an increasing frequency of male reproductive disorders, which may have a common origin in fetal life and which are hypothesized to be caused by endocrine disruptors. Phthalates esters represent a class of environmental endocrine active chemicals known to disrupt development of the male reproductive tract by decreasing testosterone production in fetal rat.

OBJECTIVES

Using the organ culture system we previously developed, we investigated the effects on the development of human fetal testis of one phthalate, MEHP (mono-2-ethylhexyl phthalate), an industrial chemical found in many products, incriminated as a disruptor of male reproductive function.

METHODS

Human fetal testes were recovered during the first trimester (7-12 wk) of gestation, a critical period for testicular differentiation, and cultured for 3 days with or without MEHP in basal or in luteinizing hormone (LH)-stimulated conditions.

RESULTS

Whatever the dose, MEHP treatment had no effect on basal or LH-stimulated testosterone produced by the human fetal testis *in vitro*, whereas the testosterone production can be modulated in our culture system. MEHP ($10^{-4}$ M) did not affect proliferation and apoptosis of Sertoli cells, but it reduced the mRNA expression of AMH. Lastly, 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

This is the first experimental demonstration that phthalates alter the development of the germ cell lineage in human. However, on the contrary to the results observed in the rat, phthalates didn’t affect steroidogenesis.

## Introduction

Fetal life is a critical step in the development of male reproductive functions. Indeed, the two major functions of the testis, *i.e.* gametogenesis and steroidogenesis, take place during this period. In humans, testis formation begins by the migration of primordial germ cells (PGCs) from extraembryonic areas to the genital ridge during the $5^{th}$ week of gestation (Wartenberg 1989). Sertoli cells then differentiate in the gonadal ridge and surround the germ cells to form the seminiferous cords between the $6^{th}$ and $7^{th}$ weeks (Gondos 1980; Wartenberg 1989). At this time, the PGCs are called gonocytes. In parallel, Leydig cells differentiate from mesenchymal cells in the interstitial compartment (Habert et al. 2001). These steroidogenic cells are morphologically discernible at 8 weeks of gestation (Huhtaniemi and Pelliniemi 1992), whereas, in organ culture, testosterone secretion is detected from 6 weeks (Lambrot et al. 2006). The appropriate onset of gametogenesis and steroidogenesis is fundamental for the function of reproduction in the adult. Indeed, the number of germ cells formed during fetal life is essential for adult fertility. In mutant mice ($gcd^{-/-}$) characterized by a reduced number of PGCs (germ-cell-deficient mice), as in mice lacking the gene POG (proliferation of germ cells), the number of fetal germ cells is reduced and adult fertility is altered (Lu and Bishop 2003). In the same way, androgens and Insl3 (insulin-like factor 3) produced by fetal Leydig cells control the masculinization of the reproductive tract and genitalia (Jost et al. 1973; Kubota et al. 2002).

Several studies have described an increasing frequency of male reproductive disorders in humans, such as a low sperm count and a resulting decline in fertility, increased incidence of testicular cancer, cryptorchidism and hypospadias (review in Bay et al. 2006; Sharpe and Irvine 2004). It has been suggested that these alterations are symptoms of a single syndrome called the “testicular dysgenesis syndrome” (TDS) (review in Sharpe 2003; Skakkebaek and

Jorgensen 2005). It is currently thought that TDS is probably caused by changes in the development of the fetal testis and may result from the effect of genetic and/or environmental factors. Thus, TDS could result from exposure to environmental chemicals, which have steadily increased in diversity and concentration in the environment and food (Delbes et al. 2006; Skakkebaek et al. 2001). Several environmental chemicals are classed among the so-called “endocrine disruptors”. Many of them act on reproductive functions because of their estrogenic and/or anti-androgenic properties. The present study focuses on the effects of phthalates (phthalic acid esters), which are industrial chemicals commonly found in many consumer products regularly used by humans, such as soap, shampoo, cosmetics and hairspray. They are also used in flexible plastics, such as food and beverage packaging, children's toys and biomedical apparatus (blood transfusion bags). Di-(2-ethylhexyl) phthalate (DEHP) is one of the most abundant phthalates produced in the world (Latini et al. 2006). It has been demonstrated that phthalates, when administered orally to humans and rodents, are rapidly hydrolyzed by esterases in the gut and other tissues to produce the corresponding active monoesters (Latini 2005). For example, DEHP is metabolized to its monoester metabolite, mono-(2-ethylhexyl) phthalate (MEHP), which is a recognized active testicular toxicant (Fisher 2004). Phthalates are not covalently bound to the plastic products and therefore may leak out to contaminate blood or food products and can be ingested. In an epidemiological study, 75% of the 289 human subjects tested were positive for the presence of four different types of phthalates in their urine samples (Blount et al. 2000). In rodents, both *in vivo* and *in vitro* approaches have been used to determine the effects on testicular functions of exposure to phthalates (review in Sharpe 2006). Several studies have shown that a fetal exposure to phthalate induced by the gavage of the pregnant rat with di(*n*-butyl) phthalate (DBP) induces TDS-like effects (Barlow and Foster 2003; Fisher et al. 2003; Mylchreest et al. 2000).

However, despite the growing body of literature on phthalate reproductive toxicity and data demonstrating extensive human exposure, very few studies have examined the effects of these chemicals on human reproductive development. Recently, an inverse correlation has been shown between the maternal urinary phthalate concentration at the end of pregnancy and the ano-genital distance at birth (Swan et al. 2005). In the same way, a dose-dependent association between phthalates in breast milk and levels of reproductive hormones in boys at 3 months of age has also been reported (Main et al. 2006). These findings are particularly important as they are the unique epidemiological studies exhibiting negative effects of phthalates at environmental concentrations. Until now, no experimental study has succeeded in demonstrating a deleterious effect of phthalates on the human testis functions or development.

In this study, we focus on phthalate effects specifically on testis. We use the organ culture system of human fetal testes that we developed previously, coupled with morphological, functional and molecular methods (Lambrot et al. 2006; Lambrot et al. 2007) to analyze the effects of one phthalate (mono-2-ethylhexyl phthalate: MEHP) on the development of testicular somatic and germ cells during the first trimester of pregnancy (7-12 weeks of gestation). This early developmental period of the testis was shown to be a critical window for the determination of the reproductive tract (Welsh et al. 2008).

## Materials and Methods

*Collection of human fetal testis.*

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 abortion in the first trimester of pregnancy, *i.e.* from the $7^{th}$ until the $12^{th}$ week of gestation, as previously described (Lambrot et al. 2006). None of the terminations was for reasons of fetal abnormality, and all fetuses appeared morphologically normal. The sex of the fetus was determined by the morphology of the gonads, and the fetal age was evaluated by measuring the length of limbs and feet (Evtouchenko et al. 1996). The fetus was dissected under a binocular microscope and testes were removed aseptically and immediately explanted *in vitro*. We found testes within the abortive material in only 12% of cases. The Antoine Béclère Hospital Ethics Committee approved this study.

*Organ cultures.*

Testes were cultured on Millicell-CM Biopore membranes (pore size 0.4 µm, Millipore, Billerica, MA) as previously described (Habert et al. 1991; Lambrot et al. 2006). The culture medium was phenol red-free Dulbecco modified Eagle medium/Ham F12 (1:1) (Gibco, Grand Island, NY) supplemented with 80 µg/mL gentamicin (Sigma, St. Louis, MO) and devoid of hormones, growth factors and serum. Mono-2-ethylhexyl phthalate (MEHP) was from TCI Europe (Antwerp, Belgium).

Each human testis was cut into small pieces, all pieces from the same testis were placed on Millicell membranes, floating on 320 µL of culture medium in tissue culture dishes and cultured for 4 days at 37°C in a humidified atmosphere containing 95% air / 5% $CO_2$. The medium was changed every 24 h. The responses to MEHP ($10^{-6}$, $10^{-5}$ and $10^{-4}$M) were

measured by comparing one testis cultured in medium containing the tested factor with the other testis from the same fetus cultured in control medium. Luteinizing hormone (LH) (100 ng/mL) from human pituitary (≥ 5000 IU/mg) (Sigma, St Louis, MO) or ketoconazole (4 µM) (Sigma, St Louis, MO) was added every 24 hours to the culture medium. BrdU (30 µg/mL) (Amersham Biosciences, Little Chalfont, Bucks, England) was added during the last 3 hours of culture for the measurement of proliferating index. At the end of the culture, explants were frozen in RLT buffer (Quiagen, Valencia, CA) at -20°C for RNA analyses, or dry frozen with liquid nitrogen for protein analyses. For cellular analyses, the explants were fixed for 2 h in Bouin's fluid, embedded in paraffin, and 5-µm sections were cut.

*Germ cell counting.*

We mounted serial sections on slides, removed the paraffin and rehydrated the sections. We then carried out immunohistochemical assays for anti-Müllerian hormone (AMH) as previously described (Lambrot et al. 2006) using an anti-AMH polyclonal antibody (1:2000) (generously provided by Dr N. Di Clemente [INSERM U782, Clamart, France]). Peroxidase activity was visualized using 3,3-diaminobenzidine as substrate. Germ cells were identified as AMH-negative cells within the seminiferous cords, whereas Sertoli cells were the AMH-positive cells. The counting was done as previously described and validated for rodents (Livera et al. 2006; Olaso et al. 1998) and humans (Lambrot et al. 2006; Lambrot et al. 2007). Briefly, we counted germ cells in one out of 10 sections for the 7-week-old human fetuses and one out of 20 sections for later stages, but never less than ten sections equidistantly distributed along the pieces of testis. All counts were done using Histolab analysis software (Microvision Instruments, Evry, France). We counted all germ cells on the section. We multiplied the sum of the values obtained for the observed sections of one testis by 10 or 20 respectively to obtain a crude count of germ cells per testis. We then used the Abercrombie formula

(Abercrombie 1946), which uses the average measured diameter of the germ cells nuclei and the thickness of sections to correct for any double counting due to single cells appearing in two successive sections. All counts were carried out blind.

*Immunohistochemical staining for cleaved caspase-3.*

Caspase-3 is involved in most of the 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. 2006). We mounted six sections on a single slide and heated the slide for 30 min in a permeabilization solution (0.05 M Tris, pH 10.6). The procedure was then the same as for detection of AMH, except that the primary antibody was the anti-cleaved caspase-3, (1:50, Cell Signaling Technology, Beverly, MA). Stained and unstained germ and Sertoli cells were counted in all six sections. For all immunohistochemical staining, negative controls were done by omitting the primary antibody.

*Measurement of BrdU incorporation index.*

Testes were labeled with BrdU (labeling reagent diluted 1:100 according to the instructions of the cell proliferation kit; Amersham, Amersham Biosciences, Little Chalfont, Bucks, England) during the last 3 h of culture. BrdU incorporation into proliferating cells was detected by immunocytochemistry according to the manufacturer's recommendations, as previously described (Lambrot et al. 2006; Livera et al. 2000). The BrdU incorporation index was obtained by a blind counting of stained and unstained germ or Sertoli cell nuclei in all sections.

*Testosterone radioimmunoassay.*

We measured the testosterone secreted into the medium in duplicate by radioimmunoassay as previously described (Habert et al. 1991). No extraction or chromatography was performed because 17β-hydroxy-5α-androstan-3-one (DHT), the only steroid which significantly cross-reacts with testosterone (64%), is secreted in minute amounts by the fetal testis (George et al. 1987).

*Reverse Transcription and Real-Time Polymerase Chain Reaction.*

RNA expression in the fetal testis was performed by RT with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Courtabeuf, France), followed by real-time PCR as previously described (Lambrot et al. 2007). Primers and probes used were designed by Applied Biosystems (sequences not provided: *β–Actin*, NM_001101.2; *AMH*, Hs00174915_m1; *Insl3*, Hs01394273_m1; *P450c17*, Hs00164375_m1; *P450scc*, Hs00167984_m1; *RPLPO*, NM_053275.3; *StAR*, Hs00264912_m1; *Wt1*, Hs01103749_m1). Reactions were carried out and fluorescence was detected using an ABI Prism 7000 apparatus (Applied Biosystems, Courtabeuf, France). Each sample was run in triplicate. Negative controls were run for every primer/probe combination. The measured amount of each cDNA was normalized using β-actin and RPLPO (large ribosomal protein) or Wt1 for AMH.

*Protein extraction and Western blotting.*

One testis was lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4O_7P_2$, 1 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, and 1 µg/mL leupeptin. Protein in total cell lysates (5 µg) was resolved by SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane (Amersham), and probed with the AMH (same as for immunohistochemistry) and

β-actin (Sigma) antibodies. Cy5 coupled anti-rabbit and Cy3 coupled anti-mouse secondary antibodies (Amersham) were used, and the blot was revealed under fluorescence in a Typhoon 9400 scanner (Amersham). The bands were quantified by the volumetric method with the software ImageQuant (Molecular Dynamics, Sunnyvale, CA).

*Statistical analysis.*

All values are expressed as means ± SEM. For all mRNA expression analysis and studies on proliferation or apoptosis, the significance of the differences between mean values for the treated and untreated testes from the same fetus was evaluated using Wilcoxon's non-parametric paired test (for small samples). For total germ cell number counting, Student's paired t-test was used because of the high variability in the number of germ cell between ages. Concerning testosterone secretion analysis, one-way ANOVA was used to assess the significance of the differences for secretion evolution between control and treated testes during the 3 days of culture.

## Results

*Effect of MEHP on Leydig cell function*

We cultured testes from fetuses at 7 to 12 weeks of development with or without $10^{-6}$, $10^{-5}$ or $10^{-4}$ M MEHP for four days. Daily testosterone production was unaffected by the addition of MEHP to the medium (Figure 1A). In order to check the lack of effect of MEHP, we analyzed the mRNA expression of various enzymes involved in steroidogenesis. MEHP treatment did not affect the mRNA expression of P450scc, P450c17 or StAR (Figure 1B). We also analyzed the mRNA expression of Insl3 produced by fetal Leydig cells which is known to be involved in testicular descent. MEHP did not modify mRNA expression of Insl3 (Figure 1C).

In order to assay the ability of testosterone secretion to be modulated in our organotypic culture system, cultures were performed with LH for testosterone stimulation and ketoconazole (KTZ) for testosterone inhibition. With 100 ng/mL LH the relative testosterone secretion was increased 5-fold at day 3 (Figure 2B). On the other hand, treatment with 4 µM KTZ (a CYP inhibitor), which we have determined to be a nontoxic concentration for the testis (Figure 2A), induced very strong inhibition of testosterone production from day 2. Using one-way ANOVA, the change in testosterone secretion with both LH and KTZ treatments differed significantly from their respective controls. These results strengthen the validity of testosterone measurement in this model.

To investigate the effect of MEHP on stimulated testosterone secretion, testes were cultured in the presence of LH (100 ng/mL) with or without $10^{-4}$ M MEHP. Relative LH-stimulated testosterone production was unaffected by the addition of MEHP to the medium (Figure 3).

*Effect of MEHP on Sertoli cell development*

First, we studied the ratio of proliferative (BrdU-positive, Figure 4A) and apoptotic (cleaved caspase-3-positive, Figure 4B) Sertoli cells after MEHP treatment for 3 days and observed that MEHP had no significant effect on these two activities.

Second, we analyzed by real-time RT-PCR (Figure 5A) and by fluorescent Western blotting (Figure 5B) the effect of MEHP on AMH expression. Whatever the housekeeping gene (β-actin or RPLPO) or specific Sertoli cell marker used (Wt1, which is not significantly different in control and treated samples if standardized to β-actin), MEHP significantly decreased the mRNA level of AMH. However, the level of AMH protein standardized to β-actin was not modified by MEHP treatment.

*Effect of MEHP on fetal germ cell development*

Addition of $10^{-6}$ M, $10^{-5}$ M or $10^{-4}$ M MEHP for 3 days had no effect on the organization of the testis at the end of the culture (data not shown). Interestingly, whatever the age of the fetus at explantation (from 7 to 12 gestational weeks), the higher dose of MEHP ($10^{-4}$ M) significantly reduced the number of germ cells. Therefore, we expressed the results as a percentage of control and pooled the results from different ages (Figure 6A). However, the $10^{-6}$ M concentration had no effect.

$10^{-4}$ M MEHP treatment significantly increased the number of cleaved caspase-3-positive germ cells (Figure 6C and 6D) without altering their proliferation (Figure 6B). $10^{-5}$ M MEHP increased, but not significantly, the number of cleaved caspase-3 positive germ cells (3.4% in the treated versus 2% in the control) (Figure 6D).

**Discussion**

In this study we investigated the effect of one metabolite of phthalate ester, MEHP, on the development of human fetal testes, using our previously developed and validated organ culture system (Lambrot et al. 2006; Lambrot et al. 2007). In this organ culture system, the testicular architecture and intercellular communications are preserved enough to allow the development of the main fetal testicular cell types *in vitro*, without any added factor (Livera et al. 2006).

This approach allowed us to present here the first experimental demonstration that phthalates impair the development of the male fetal germ cell lineage in the human species. After three days of treatment, MEHP reduced by 40% the number of germ cells in cultured human fetal testis. This effect was due to a large increase in their apoptosis without modification of their proliferation. A negative effect of phthalates on gonocyte number has also been reported in rodents both *in vivo*, after gavage (Ferrara et al. 2006) and *in vitro*, in organ culture (Chauvigné et al. 2008; Lehraiki et al. 2008; Li and Kim 2003). It is interesting to note that, in rodents, the androgen pathway does not seem to be involved in germ cell number, as phthalates are distinct from flutamide in their ability to induce primordial germ cell degeneration (Mylchreest et al. 1999).

Phthalates induce the appearance of multinucleated gonocytes in rodents (Ferrara et al. 2006; Kleymenova et al. 2005). No multinucleated gonocytes were observed here in response to MEHP treatment. It may be due to a species characteristic. But appearance of multinucleated gonocytes depends on the age of the fetus in rodents. For example, Ferrara observed multinucleated gonocytes after DBP gavage only from day 19.5 post conception (Ferrara et al. 2006). Thus, it is possible that the sensitive window in humans occurs later than the period studied here.

Phthalates are known as Sertoli cell toxicants in rodents. Some studies have reported a decrease in Sertoli cell number or proliferation (Hutchison et al. 2008; Li and Kim 2003; Li et al. 2000), interference with cytoskeleton (Kleymenova et al. 2005) and decrease in the expression of Sertoli cell markers (AMH, GATA4, inhibin) and of FSH-stimulated cAMP production (Fisher et al. 2003; Heindel and Chapin 1989; Hutchison et al. 2008; Li and Kim 2003). In this study, we observed that MEHP did not affect the proliferation or apoptosis rate of Sertoli cells. On the other hand, MEHP decreased the mRNA expression of AMH whether relative expression was normalized to ubiquitous or to the Sertoli gene. Surprisingly however, the intracellular AMH protein level was not modified by MEHP treatment when analyzed by Western blot. To explain this discrepancy, we can hypothesize that MEHP affects RNA expression earlier than protein expression.

In rat, it is currently thought that phthalates act mainly on fetal Leydig cells. Thus, the main effects of phthalates are the suppression of testosterone production, an abnormal Leydig cell aggregation and the presence of intratubular Leydig cells (Culty et al. 2008; Fisher et al. 2003; Parks et al. 2000). Jegou's team (Chauvigné et al. 2008) have also observed a similar decrease in testosterone production in organ culture of rat fetal testis and we confirmed this result (unpublished data). Nevertheless, Hallmark et al. have shown that, MBP (monobutyl phthalate) reduces hCG-stimulated but not basal steroidogenesis in rat fetal testis explants (Hallmark et al. 2007). In human, we observed that MEHP changes neither the basal nor the LH-stimulated production of testosterone by the fetal testis in culture. This was confirmed by the absence of modification of the mRNA levels of steroidogenic enzymes after MEHP treatment. Sharpe and collaborators also found no *in vitro* effect of phthalates (MBP and DBP) on steroidogenesis of human fetal testis explants recovered during the second (15-20 wk) trimester of pregnancy (Hallmark et al. 2007). So, steroidogenesis of the human fetus during the first and second trimesters seems not to be sensitive to phthalates. This period in

humans corresponds to the window studied in the rat model. It is interesting to note that, in mice, MEHP stimulates testosterone production both in the fetal testis (Gaido et al. 2007; Lehraiki et al. 2008) and in Leydig tumor cells (Gunnarsson et al. 2008). This lack of effect on testosterone production in humans suggests differences between species.

The results presented here contrast with epidemiological data in humans (Main et al. 2006; Swan et al. 2005). We can formulate two hypotheses to explain this apparent discrepancy: first, the studies differ in phthalate concentration, nature and duration of exposure. We chose to investigate the effect phthalate concentrations ranging from $10^{-6}$ M to $10^{-4}$ M. In a prospective study on cryptorchidism carried out on the phthalate monoester contamination of human breast milk, many phthalate monoesters were found over a large concentration range, from 1.5 to 1410 μg/L, which corresponds to about $10^{-9}$ M to $10^{-6}$ M for MEHP (Main et al. 2006). In our study, no effect was observed at the lower concentration, either on steroidogenesis or on gametogenesis. However, it is important to note that the mother and thus the fetus are exposed to a combination of multiple phthalates (Swan et al. 2005) which could explain the need for a greater dose of MEHP to show a potent effect *in vitro.* Second, in our study we focus on the effect of MEHP specifically on testis and we can not rule out that the observed effects in epidemiological studies (cryptorchidism and ano-genital distance) are due to a direct effect of phthalates on the reproductive tract.

In the human fetus, intra-abdominal testicular descent to the inner inguinal ring is initiated at about 10-14 weeks of gestation (Barteczko and Jacob 2000; Klonisch et al. 2004). A role for Insl3 secreted by differentiated Leydig cells, in development of the gubernaculum and this first phase of testicular descent, has emerged after analysis of mice genetically modified for Insl3 expression (Adham et al. 2002; Ivell and Bathgate 2002; Nef and Parada 1999; Zimmermann et al. 1999). Moreover, underdeveloped gubernaculum (Barlow and Foster 2003) and reduced Insl3 expression have been observed following fetal exposure to

several different phthalates in male rats (Lehmann et al. 2004; McKinnell et al. 2005; Wilson et al. 2004). We observed no effect of $10^{-4}$ M MEHP (highest concentration) on the Insl3 mRNA in human testis culture, even though our study took place during the set up of the testicular descent. Lague and Tremblay have recently demonstrated that MEHP represses Insl3 transcription by antagonizing testosterone action in Leydig cells (Lague and Tremblay 2008). Thus, the absence of effect on Insl3 expression observed here can be explained by the lack of effect of MEHP on testosterone production in our model. Nevertheless, androgen receptor antagonists seem to have no effect on Insl3 expression (McKinnell et al. 2005; Wilson et al. 2004).

In conclusion, this is the first experimental demonstration that phthalates, a family of compounds known as endocrine disruptors, widely distributed in the environment, are able to alter the development of male germ cell lineage in human. This effect is not mediated by a decrease in the testosterone produced by the Leydig cells, which is unchanged. Furthermore, this study shows the efficiency of our organ culture system in investigating the effects and mechanisms of action of environmental disruptors on the development of the human fetal testis. Lastly, our work provides important insight into the potential role of exposure to environmental pollutants during fetal testicular development and their potential deleterious effects on male fertility in adulthood.

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**Figure legends**

**Figure 1. Effect of MEHP on Leydig cell function in the human fetal testis in culture.**

Testes from 7- to 12-week-old fetuses were cut into pieces and cultured for four days on Millicell membranes floating on a synthetic medium. The medium was changed every 24 h. Testicular explants were cultured for the first 24 h (Day 0) in control medium and for three more days (D1 to D3) in the presence or absence of different concentrations of MEHP. Panel A represents the testosterone production. Testosterone produced on each day was measured by radioimmunoassay and referred to the production on Day 0. This value was then expressed as the percentage of the value obtained with the control testis taken every day as the 100% reference. Values are means ± SEM: MEHP $10^{-6}$ M (n=4); MEHP $10^{-5}$ M (n=3); MEHP $10^{-4}$ M (n=15). Panel B represents the levels of mRNA expression of specific Leydig cells markers. At the end of the culture period, with or without $10^{-4}$ MEHP, total RNA was extracted and RT-qPCR with specific primers was performed to analyze expression of genes encoding P450scc, P450c17, StAR and Insl3. Results were standardized to either β-actin (gray bars) or RPLPO (large ribosomal protein) (striped bars) as endogenous control, and are presented as a percentage of the value obtained with control testis. Means ± SEM of 3 different determinations are shown.

**Figure 2. Effects of LH or ketoconazole on testosterone production by human fetal testis *in vitro*.**

Testes from 7- to 12-week-old fetuses were cultured for the first 24 h (Day 0) in control conditions and in the presence or absence of 100 ng/mL LH or 4 µM ketoconazole (KTZ) for the next 3 days (D1 to D3). As shown in panel A, 4 µM ketoconazole was not toxic for testis (scale bar corresponds to 30 µm). Testosterone secreted in the medium at the end of each day of culture was radioimmunoassayed and the results were expressed as percentages of the

secretion measured on first day of culture (day 0). Values presented in panel B are the means ± SEM of 3 determinations.

**Figure 3. Effect of MEHP on LH-stimulated testosterone secretion by the human fetal testis *in vitro.***

Testes from 7- to 12-week-old were cultured for the first day (Day 0) in control medium and for the next 3 days with 100 ng/mL LH and in the presence or absence of $10^{-4}$ M MEHP as described in the legend of Figure 1. Testosterone production, measured by radioimmunoassay, was referred to the production on Day 0. Values are means ± SEM of 7 determinations.

**Figure 4. Effect of MEHP on the proliferative and apoptotic activities of Sertoli cells in human fetal testis in culture.**

Testes from 7- to 12-week-old fetuses were cultured for 4 days with or without $10^{-4}$ M MEHP added after the first 24 h of culture as described in the legend of Figure 1. BrdU (30 µg/mL) was added for the last 3 hours of culture. Sections of the cultured testes were immunostained for AMH to identify Sertoli cells. Panel A shows the percentage of Sertoli cells in proliferation, determined by immunohistochemical detection of BrdU incorporation into the nuclei (n=3). Panel B shows the apoptosis of the Sertoli cells measured by immunodetection of the cleaved caspase-3 (n=4).

**Figure 5. Effect of MEHP on AMH expression in human fetal testis culture.**

Testes from 7- to 12-week-old fetuses were cultured during 4 days with or without $10^{-4}$ M MEHP added after the first 24 h of culture as described in the legend of Figure 1. At the end of the culture period, total RNA or proteins were extracted. In the first case, presented in panel A, RT-qPCR with AMH specific primers was performed. The results are normalized to

either β-actin (gray bar) or RPLPO (black striped bar) or Wt1 (white striped bar) as Sertoli endogenous control, and are expressed as a percentage of the control. Values are means ± SEM of 3 independent samples. * P<0.05 in the paired comparison with the corresponding control values (Wilcoxon paired test). Panel B shows a representative fluorescent Western blot revealing AMH protein (green) and β-actin (red).

**Figure 6. Effect of MEHP on the number and the proliferative and apoptotic activities of germ cells in human fetal testis in culture.**

Testes from 7- to 12-week-old fetuses were cultured for 4 days with or without $10^{-4}$ M MEHP added after the first 24 h of culture as indicated in the legend of Figure 1. At the end of the culture period, morphometric analyses were performed. The cytoplasm of the Sertoli cells was immunostained with AMH to identify germ cells as the cells with unlabeled cytoplasm inside the seminiferous cords. Panel A shows the total number of germ cells per testis. The result is expressed as a percentage of the value obtained with MEHP-treated testis compared with the contralateral testis cultured in the absence of MEHP and used as control. Values are means ± SEM of 4 experiments. * P<0.05 in the paired comparison with the corresponding control values (Student t-test). Panel B shows the proliferation of the germ cells determined by immunohistochemical detection of BrdU incorporation into the nuclei during the last 3 hours of culture. Values are means ± SEM of 3 experiments. Panel C shows a micrograph after immunohistochemical detection of cleaved caspase 3, a marker of apoptosis, after culture with or without MEHP. Arrowheads represent cleaved caspase 3-positive gonocytes and arrows cleaved caspase 3-negative gonocytes. Bars represent 10 µm. Panel D give the percentage of labeled gonocytes for cleaved caspase 3. Values are means ± SEM of 3 or 4 experiments. * P<0.05 in the paired comparison with the corresponding control values (Wilcoxon paired test).

**Figure 1**

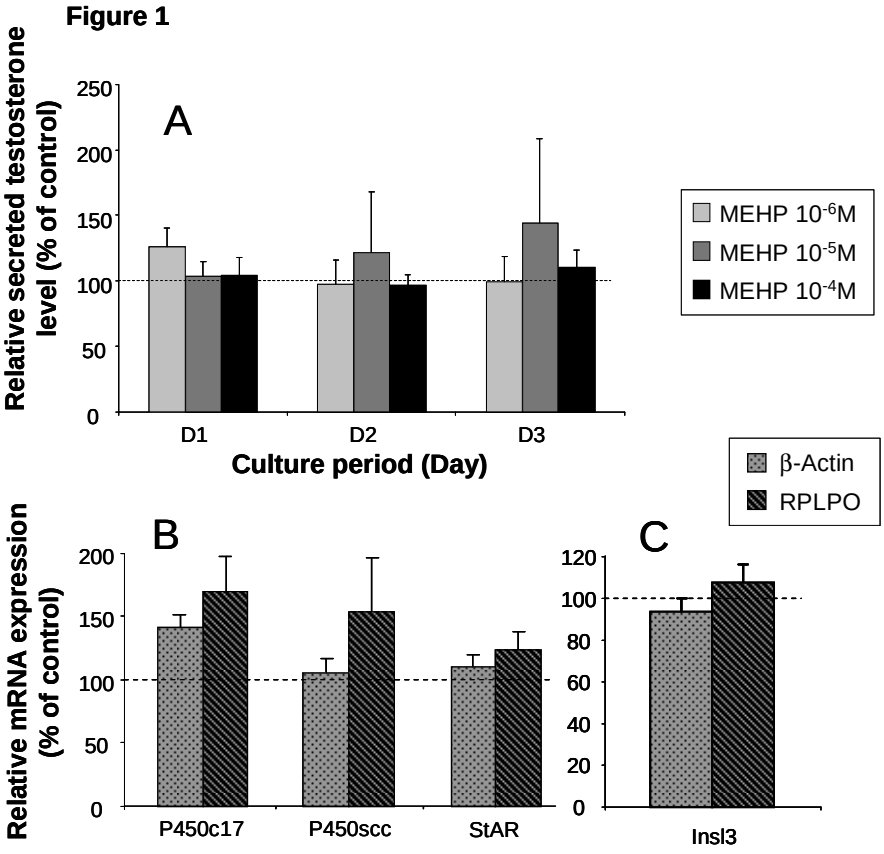



**Figure 2**

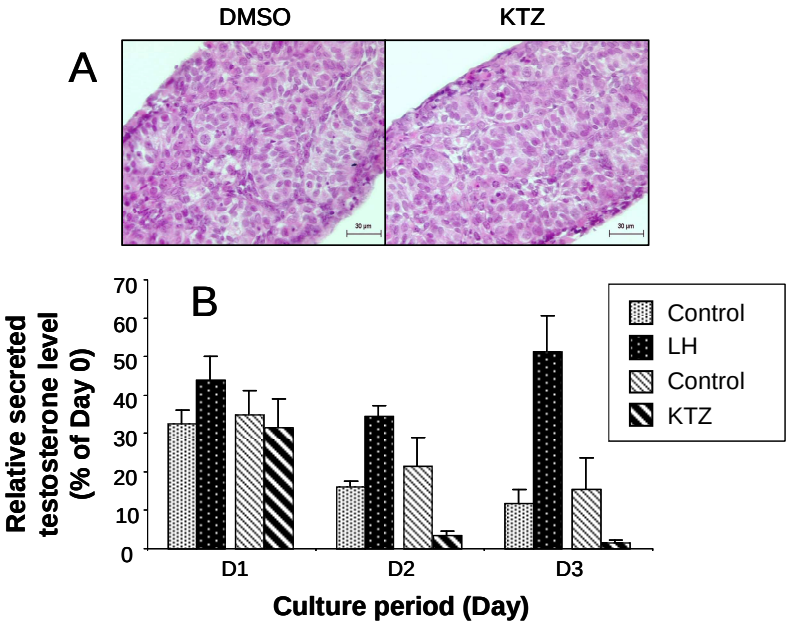


**Figure 3**

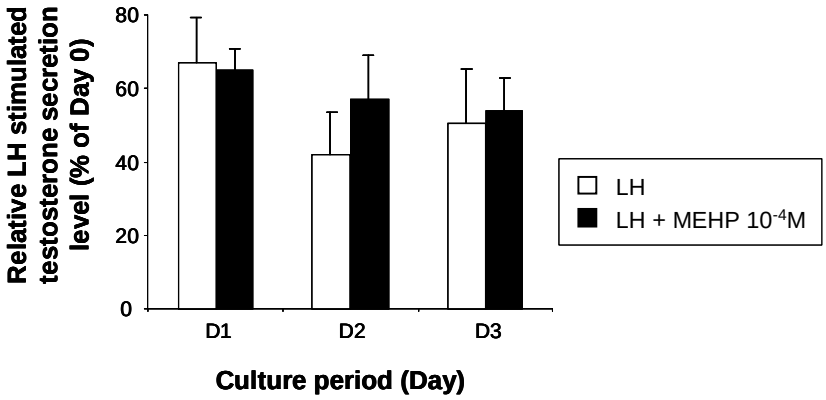
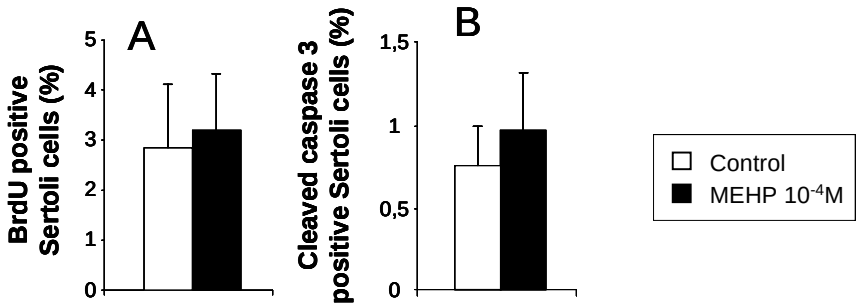



**Figure 4**

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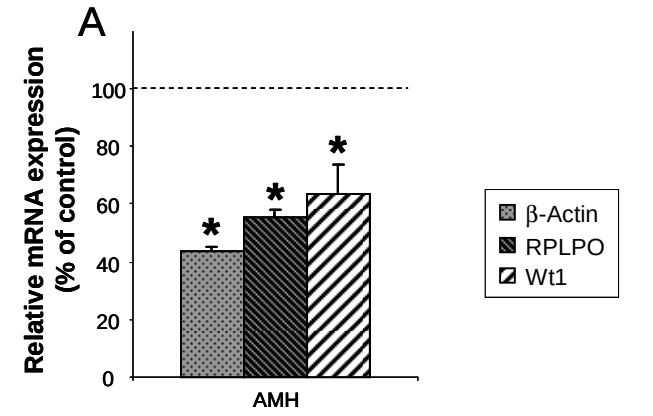


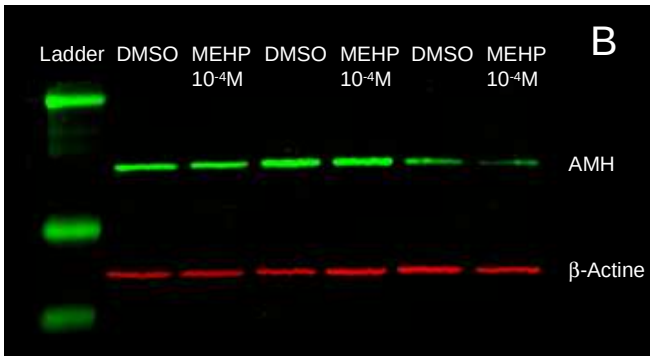
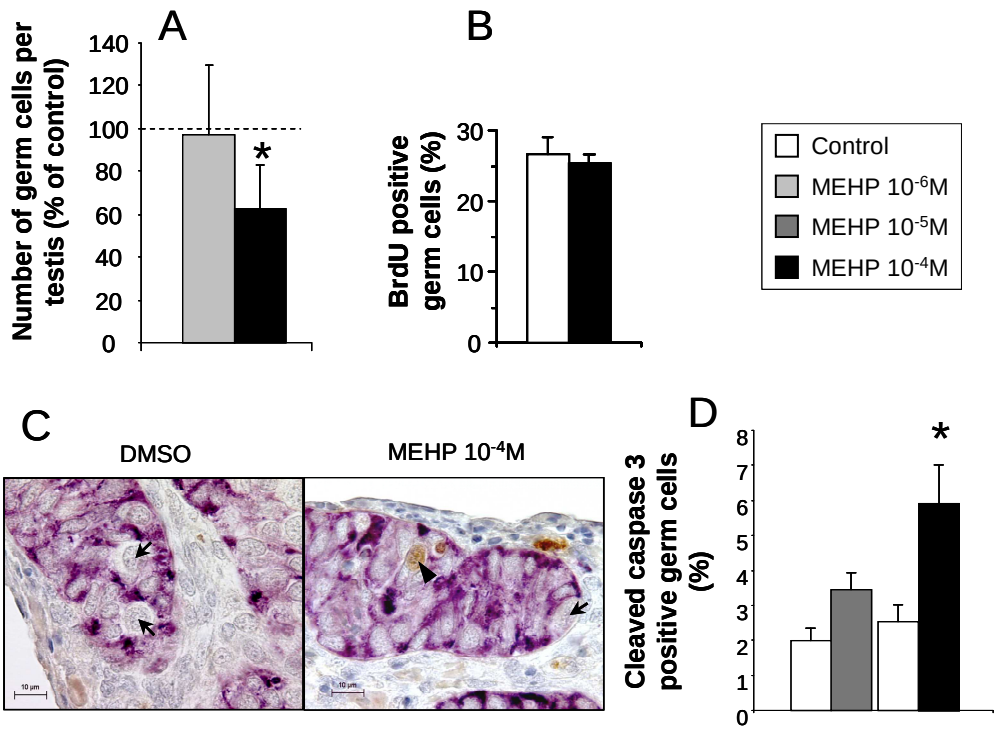



**Figure 6**


A
Number of germ cells per testis (% of control)
140
120
100
80
60
40
20
0
*
B
BrdU positive germ cells (%)
30
20
10
0
Control
MEHP 10-6M
MEHP 10-5M
MEHP 10-4M
C
DMSO
MEHP 10-4M
D
Cleaved caspase 3 positive germ cells (%)
8
7
6
5
4
3
2
1
0
*