



## **Human testis steroidogenesis is inhibited by phthalates.**

Christele Desdoits-Lethimonier, Océane Albert, Bruno Le Bizec, Elisabeth Perdu, Daniel Zalko, F. Courant, Laurianne Lesné, François Guille, Nathalie Dejucq-Rainsford, Bernard Jégou

### **► To cite this version:**

Christele Desdoits-Lethimonier, Océane Albert, Bruno Le Bizec, Elisabeth Perdu, Daniel Zalko, et al.. Human testis steroidogenesis is inhibited by phthalates.. Human Reproduction, 2012, 27 (5), pp.1451-9. 10.1093/humrep/des069 . hal-00876965

**HAL Id: hal-00876965**

**<https://hal.science/hal-00876965>**

Submitted on 29 May 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Human testis steroidogenesis is inhibited by phthalates

C. Desdoits-Lethimonier<sup>1,2,†</sup>, O. Albert<sup>1,2,†</sup>, B. Le Bizec<sup>3</sup>, E. Perdu<sup>4</sup>, D. Zalko<sup>4</sup>, F. Courant<sup>3</sup>, L. Lesné<sup>1,2</sup>, F. Guillé<sup>2,5</sup>, N. Dejucq-Rainsford<sup>1,2</sup>, and B. Jégou<sup>1,2,\*</sup>

<sup>1</sup>Inserm (Institut national de la santé et de la recherche médicale), IRSET, U1085, Campus de Beaulieu, F-35042 Rennes CEDEX, France

<sup>2</sup>Université de Rennes I, Campus de Beaulieu, F-35042 Rennes CEDEX, France <sup>3</sup>USC 1329 LABERCA, ONIRIS, Atlanpôle - La Chantrerie, BP 50707, F-44307 Nantes, France <sup>4</sup>UMR 1331 TOXALIM, INRA (Institut National de la Recherche Agronomique), Chemin de Tournefeuille, F-31027 Toulouse, France <sup>5</sup>Centre de Lutte contre le cancer Eugène Marquis, rue de la Bataille Flandres Dunkerque CS44229, F-35042 Rennes CEDEX, France

\*Correspondence address. Tel: +33-2-23-23-69-11; Fax: +33-2-23-23-50-55; E-mail: bernard.jegou@inserm.fr

Submitted on November 9, 2011; resubmitted on January 23, 2012; accepted on February 2, 2012

**BACKGROUND:** Phthalic acid esters are widely used in the manufacture of plastics. Numerous studies have shown that these phthalates impair testicular testosterone production in the rat. However, the scarce and contradictory data concerning humans have cast doubt over whether these compounds are also anti-androgenic in man. We therefore investigated the direct effects of di-(2-ethylhexyl) phthalate (DEHP) and mono-(2-ethylhexyl) phthalate (MEHP) on organo-cultured adult human testis and a human cell line.

**METHODS:** Adult human testis explants or NCI-H295R adrenocortical human cells were cultured with DEHP or MEHP. The effects of ketoconazole, used as a reference molecule, were also assessed.

**RESULTS:** In both models, DEHP and MEHP significantly inhibited testosterone production. The effects of both phthalates appeared to be specific for steroidogenesis, as INSL3 production by Leydig cells was not altered. Furthermore, the phthalates of interest had no effect on inhibin B production by Sertoli cells or on germ cell apoptosis. As only a small fraction of the phthalates added was found in the testis explants, and as these compounds were found to be metabolized, we estimate that the anti-androgenic effects observed occurred at concentrations of phthalates that are of the same order of magnitude as exposures reported in the literature for men.

**CONCLUSIONS:** We provide the first evidence that DEHP and MEHP can inhibit testosterone production in the adult human testis. This is consistent with recent epidemiological findings of an inverse correlation between exposure to MEHP and testosterone concentrations.

**Key words:** human testis / testosterone / phthalates / endocrine disruption / steroidogenesis

## Introduction

Phthalates are man-made chemicals widely distributed in the human environment. Numerous studies have been performed to assess the effects of phthalates, principally on the male reproductive functions of the fetal and prepubertal rat. These experiments have led to the conclusions that exposure to phthalate esters results in the inhibition of testicular testosterone synthesis (Parks *et al.*, 2000) associated with the development of abnormalities of the reproductive tract (Imajima *et al.*, 1997; Gray *et al.*, 2000; Parks *et al.*, 2000; Shono *et al.*, 2005; Lin *et al.*, 2008). In post-pubertal rats, phthalates were also found to decrease sperm production (Barlow *et al.*, 2004).

Studies on the effects of phthalates in human are few and often contradictory. The concentrations of phthalates in mother's urine have been found to be associated with a shorter anogenital distance in newborn boys (Swan *et al.*, 2005; Swan, 2008), but such an association was not found by others (Huang *et al.*, 2009). Significant correlations between phthalate monoesters in breast milk samples and several infant hormones have also been demonstrated (Main *et al.*, 2006). However, two recent *in vitro* studies did not find any suppressive effect of mono-(2-ethylhexyl) phthalate (MEHP) or of mono-*n*-butyl phthalate (MBP) on fetal testosterone levels (Hallmark *et al.*, 2007; Lambrot *et al.*, 2009). Furthermore, a recent study in healthy adult men has failed to demonstrate any association between MEHP and reproductive biomarkers (Jonsson *et al.*, 2005).

<sup>†</sup> These authors equally contributed to this work.

In contrast, Pan *et al.* (2006) reported that workers exposed to MBP and MEHP had significantly lower free testosterone levels than unexposed men. A more recent study also showed that urinary MEHP concentrations were negatively correlated with free testosterone and estradiol levels in infertile men (Meeker *et al.*, 2009). The latter observations argue in favour of phthalates having anti-androgenic effects in the adult human testis. However, no direct effect on human testicular steroidogenesis has yet been shown.

Here, we report a study investigating the potential direct effects on human testicular function of both di-(2-ethylhexyl) phthalate (DEHP), the most commonly used and widely distributed phthalate, and its major metabolite, MEHP.

## Materials and Methods

### Organotypic cultures of human testis explants (TEXAS)

The study protocol was approved by the local ethics committee, and informed consent was obtained from all donors. Testes were obtained from prostate cancer patients and processed as previously described (Roulet *et al.*, 2006). Treatments involved culturing two testis explants of about 3 mm<sup>3</sup> onto a PET insert (Falcon Labware; Becton Dickinson, Lincoln Park, NJ, USA) at the interface of air in 1 ml medium (Dulbecco's modified Eagle's medium; Sigma-Aldrich, Saint Quentin Fallavier, France) containing 0.1% dimethylsulphoxide (DMSO) as a control, or 10<sup>-5</sup>–10<sup>-4</sup> M of DEHP (purity > 99%; Interchim, Montluçon, France) or of MEHP (purity > 99%; Interchim) in 0.1% DMSO. Ketoconazole (Sigma-Aldrich) was also used at 10<sup>-5</sup> M in 0.1% DMSO. For each condition, a minimum of four wells (i.e. eight explants) was analysed. This culture system was incubated for 24 or 48 h in a humidified atmosphere containing 5% CO<sub>2</sub> at 34°C. The culture media were fully collected after either 24 or 48 h and stored at -20°C. On the day of collection, four testis explants were fixed in either neutral buffer 4% formaldehyde or Bouin's fixative for 2 h at +4°C, embedded in paraffin, sliced into 5.0 µm thick sections and stored at +4°C until immunostaining.

### Immunostaining

Immunocytochemistry with formaldehyde-fixed, paraffin-embedded tissues was used to estimate the number of cells undergoing apoptosis. Testis explant sections (5 µm thick) were deparaffinized, rehydrated, antigen-retrieved for 20 min at +90°C in boiled EDTA buffer (pH 6), and washed three times in 0.05 mol/l phosphate-buffered saline (PBS, pH 7.6). Endogenous peroxidases were inactivated with a 5 min treatment in PBS–3% H<sub>2</sub>O<sub>2</sub>. Non-specific binding sites were blocked in PBS supplemented with 3% fetal calf serum (FCS; Eurobio, Courtaboeuf, France) and the samples were incubated overnight at +4°C with the primary rabbit antibody directed against cleaved caspase-3 (Cell signalling, Ozyme, Saint Quentin en Yvelines, France) diluted 1/100 in PBS–3% FCS. The slides were rinsed several times in PBS and incubated for 1 h with a biotinylated goat anti-rabbit secondary antibody (1/200; DAKO, Trappes, France) then for 30 min with a Vectastain Avidin–Biotinylated enzyme Complex (Vector, Burlingame, USA). Bound antibody was visualized with 3,3'-diaminobenzidine (DAKO). The reaction was stopped by immersion in water; sections were then counterstained with hemalin and mounted. We counted the caspase-3 immunopositive cells using the Computer-Assisted Stereology Toolbox (CAST) Grid system (Olympus, Copenhagen, Denmark) with a light microscope (Olympus BX S1). The number of apoptotic germ cells was divided by seminiferous tubule area and the results were expressed as percentages of the control values.

### Culture of the NCI-H295R cell line and treatment (CELIAS)

The NCI-H295R human adrenocortical cell line, which expresses steroidogenic enzymes, has previously been used to study anti-androgenic compounds (Sanderson *et al.*, 2000; Gracia *et al.*, 2006) and was generously provided by Dr Feige (Inserm U878, Grenoble, France). Cells were grown for 1–10 passages in 75 cm<sup>2</sup> flasks with 10 ml of supplemented medium at 37°C in a 5% CO<sub>2</sub> atmosphere. The supplemented medium was RPMI medium 1640 (Sigma-Aldrich) with 1% ITS plus (Becton Dickinson, Le Pont de Claix, France), 2% fetal bovine serum (Eurobio) and antibiotics. The cells were then sub-cultured at a density of 10<sup>6</sup> cells/well in a 12-well culture plate, and were incubated for 5 days. The medium was then replaced with serum-free RPMI 1640 containing 0.01% bovine serum albumin and 0.1% DMSO or 10<sup>-5</sup> M ketoconazole, or 10<sup>-6</sup>–10<sup>-4</sup> M of DEHP or MEHP in 0.1% DMSO and the culture was continued for 24 or 48 h. The media were collected and stored at -20°C. No DEHP, MEHP or ketoconazole-induced cytotoxicity was observed using the methylthiazolyldiphenyl-tetrazolium bromide cell viability assay (Denizot and Lang, 1986).

### DEHP and MEHP distribution in the TEXAS

The explants were incubated with 10<sup>-5</sup> M radiolabelled DEHP or MEHP as previously described (Chauvigne *et al.*, 2009). We used ring UL <sup>14</sup>C-DEHP (<sup>14</sup>C-DEHP; 244.2 MBq/mmol; purity > 98%; Sigma-Aldrich) or <sup>14</sup>C-MEHP (432.9 MBq/mmol) obtained by incubating radiolabelled DEHP (1.5 KBq) with esterase from porcine liver (2.9 Units) in 0.5 ml 0.1 M Tris buffer (pH 7.4) for 30 min under shaking at 37°C. At the end of the culture, explants were separated from filters and both were digested in 1 ml Soluene-350 (PerkinElmer, Courtaboeuf, France) and then incubated at 55°C (2 h for the testes, overnight for the filters). The digested explants were dissolved in 10 ml Ultima Gold scintillating liquid (PerkinElmer), and radioactivity was counted using a Tricarb 2100 TR scintillation counter (PerkinElmer).

### DEHP biotransformation

Unlabelled DEHP and MEHP standards were purchased from Fluka/Riedel-de-Haën (Sigma-Aldrich, L'Isle d'Abeau Chesnes, France). <sup>14</sup>C-DEHP (1591 Bq/incubation) fortified when necessary with unlabelled DEHP were added in 5 µl ethanol to the culture medium in order to reach final concentrations of 0.5, 1, 2, 5, 10, 15 or 20 10<sup>-5</sup> M of DEHP. All incubations were carried out in triplicate and lasted 24 h for TEXAS (0.1 g tissue/well) or 48 h for CELIAS (1.10<sup>6</sup> cells/well). At the end of the incubation, media were stored at -20°C. Explants were collected in 0.5 ml medium and homogenized using an Ultra-turrax disperser (IKA, Germany). Cells were collected in 0.5 ml trypsinated buffer before homogenization. The radioactivity in aliquots (50 µl) of each incubation medium and explant or cell homogenates was measured by counting using a Packard Tricarb 4430 counter with Ultima Gold (Perkin Elmer, France). High-performance liquid chromatography (HPLC, HP 1100) coupled to an online radioactivity detection system (Flo-One A500), with Flo-scint II (Perkin Elmer, France) was used for metabolite profiling. The HPLC system consisted of a Kromasil C18 column (250 × 4 mm, 5 µm) coupled to a Kromasil C18 guard precolumn. Mobile phases consisted of acidified water (pH 2.8) and methanol (90:10 v/v) in A, and 100% methanol in B. The flow rate was 1 ml/min at 30°C; gradient: 0–35 min: linear gradient from 100% A to 100% B; 35–55 min: 100% B. Metabolites were identified by comparison of radio-HPLC retention time with those of authentic standards, except for MEHP whose structure was confirmed using liquid chromatography–mass spectrometry (MS)/MS.

## Measurement of testosterone production

Testosterone was assayed in duplicate using a specific radioimmunoassay (RIA; Immunotech, Beckman Coulter, Villepinte, France). The intra- and inter-assay coefficients of variation were  $\leq 8.6$  and  $11.9\%$ , respectively.

## Measurement of insulin-like factor 3 production

Insulin-like factor 3 (INSL3) was assayed in duplicate using a commercial RIA (RK-035-27, Phoenix France, Strasbourg, France). The intra- and inter-assay coefficients of variation were  $\leq 15$  and  $7\%$ , respectively.

## Measurement of inhibin B production

Inhibin B was measured using a commercial ELISA kit (DSL-10-84100 Active, Beckman Coulter, Villepinte, France). The intra- and inter-assay coefficients of variation were  $\leq 5.6\%$  and  $7.6\%$ , respectively.

## Analysis of the pathways of androgen production

Solid phase extraction (SPE) C18 cartridges, reagents and solvents were of analytical or HPLC grade quality from Solvent Documentation Synthesis (SDS, Peypin, France). Standard reference steroids were from Sigma (St Louis, MO, USA). Deuterated internal standards were from Steraloids (Wilton, NY, USA). Quantification was performed by isotopic dilution. Samples were spiked with  $400\text{ pg}$  of internal standards (etiocholanolone- $d_5$ ,  $17\alpha$ -testosterone- $d_3$ , dihydrotestosterone- $d_3$ ,  $19$ -androstenedione- $d_3$ , progesterone- $d_9$ ,  $17\alpha$ -methyltestosterone- $d_3$  and  $17\beta$ -estradiol- $d_3$ ). Samples were applied on a C18 SPE column (2 g stationary phase) previously conditioned with  $10\text{ ml}$  methanol and  $10\text{ ml}$  water. The column was washed with water ( $5\text{ ml}$ ), followed by cyclohexane ( $5\text{ ml}$ ), and steroids were eluted with methanol ( $10\text{ ml}$ ). The extracts were dried ( $N_2$ ,  $45^\circ\text{C}$ ) and  $400\text{ pg}$  of external standard (norgestrel) was added. Derivatization procedure and measurements in gas chromatography (GC)–MS/MS in electronization mode were performed as already described (Prevost *et al.*, 2004; Courant *et al.*, 2008).

## Statistical analysis

Values are means  $\pm$  SEM, and expressed as percentages of the control value. They were analysed with the non-parametric signed rank Mann–Whitney test. A  $P$ -value inferior to  $0.05$  was considered statistically significant.

## Results

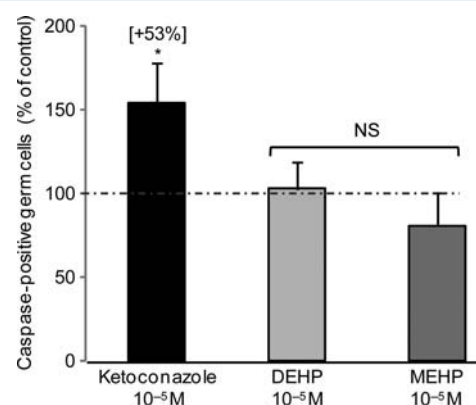
### Effect of the treatments on testis explant morphology

Exposure of the testicular explants to  $10^{-5}\text{ M}$  DEHP, MEHP did not reveal any alteration in their morphology. In contrast, ketoconazole caused an increase in the number of apoptotic germ cells (Fig. 1).

### DEHP, MEHP and ketoconazole effects on human testosterone production

Incubation of testicular explants with  $10^{-5}\text{ M}$  ketoconazole resulted in an reduction in testosterone of  $53\%$  after  $24\text{ h}$  of exposure and  $59\%$  after  $48\text{ h}$  (Fig. 2A).

After  $24\text{ h}$  of culture with  $10^{-5}\text{ M}$  DEHP, testosterone levels were reduced by  $29\%$ , whereas  $10^{-4}\text{ M}$  DEHP had no significant effect



**Figure 1** Effects of  $10^{-5}\text{ M}$  of DEHP, MEHP and ketoconazole on the number of apoptotic germ cells. Values are means  $\pm$  SEM of caspase-positive cells in three independent experiments from different donors. \* $P < 0.05$  by non-parametric signed rank Mann–Whitney test on unpaired data. NS, not significant.

(Fig. 2A). Exposure to either  $10^{-5}$  or  $10^{-4}\text{ M}$  MEHP resulted in a significant reduction in testosterone (Fig. 2A). Neither DEHP nor MEHP had any significant effect on testosterone after  $48\text{ h}$  (Fig. 2A).

### Distribution of DEHP and MEHP in the TEXAS

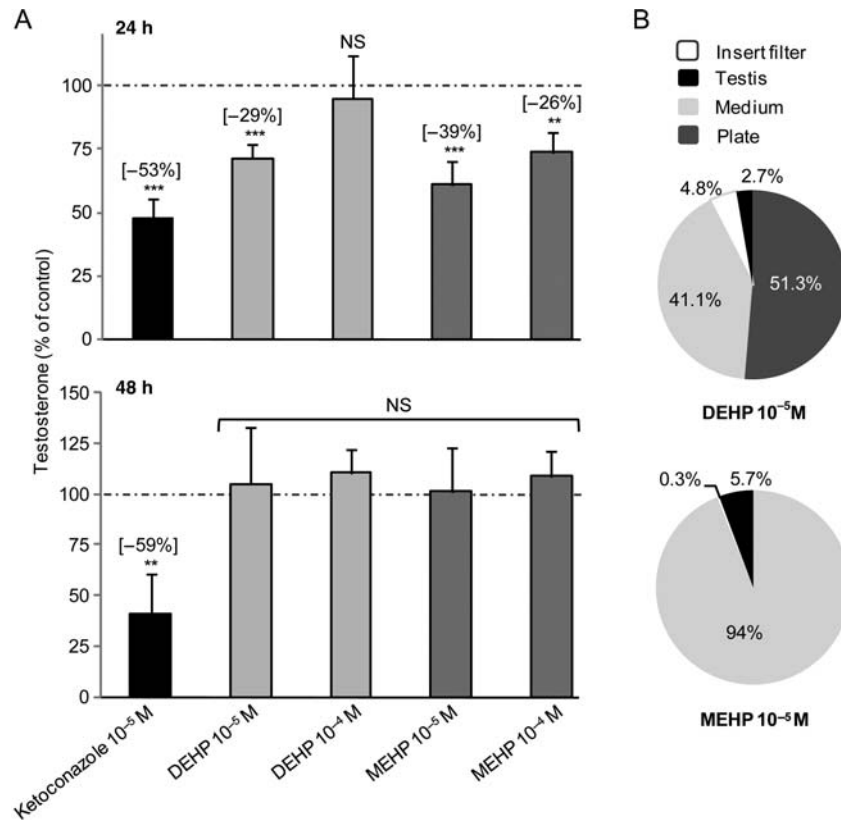
After  $24\text{ h}$ ,  $<3\%$  of the  $10^{-5}\text{ M}$  of DEHP and  $<6\%$  of the MEHP added to the cultures were found in the explants, and the rest was either found in the culture medium ( $41$  and  $94\%$  for DEHP and MEHP, respectively), the PET insert ( $<5$  and  $1\%$  for DEHP and MEHP, respectively), or bound to the plastic culture plate ( $51\%$  for DEHP; Fig. 2B).

### Biotransformation of DEHP

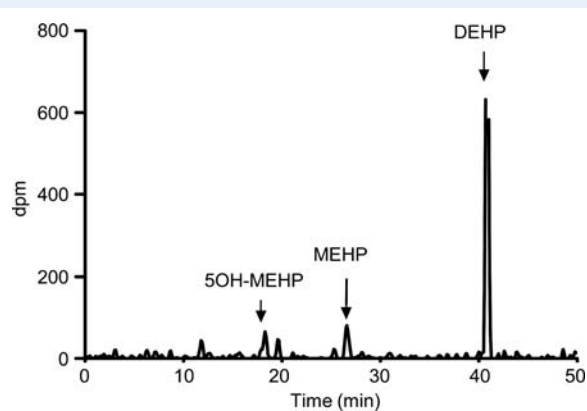
DEHP has to be metabolized into MEHP to be bioactive (Gray and Gangolli, 1986) and metabolites of MEHP, including  $5\text{OH-MEHP}$  (Koch *et al.*, 2005), also display anti-androgenic properties (Chauvigne *et al.*, 2009). A typical radio-HPLC chromatogram showing the biotransformation of DEHP in the TEXAS is shown in Fig. 3. We found that DEHP was metabolized into MEHP, and that this MEHP was processed into  $5\text{OH-MEHP}$ . Qualitatively, similar results were obtained when using the CELIAS system. The use of seven different DEHP concentrations showed that the formation of MEHP and  $5\text{OH-MEHP}$  peaked at  $7.8 \pm 2.7\text{ nmol}/24\text{ h}/0.1\text{ g tissue}$  and  $5.8 \pm 0.78\text{ nmol}/24\text{ h}/0.1\text{ g tissue}$ , respectively, in the TEXAS, and at  $24.66 \pm 9.97\text{ nmol}/48\text{ h}/1.10^6\text{ cells}$  and  $9.97 \pm 0.04\text{ nmol}/48\text{ h}/1.10^6\text{ cells}$ , respectively, in the CELIAS.

### Steroidogenic pathways of testosterone production

The global GC–MS/MS analysis of testosterone pathways confirmed that both ketoconazole and MEHP inhibited testosterone ( $-48$  and  $-40\%$ , respectively; Fig. 4). We also found that  $4$ -androstene- $3,17$ -dione, the precursor of testosterone, was significantly inhibited by ketoconazole ( $-47\%$ ) and by MEHP ( $-33\%$ ; Fig. 4A). Likewise, ketoconazole



**Figure 2** (A) Time- and dose-dependent effects of  $10^{-5}$  and  $10^{-4}$  M DEHP or MEHP, and  $10^{-5}$  M ketoconazole on testosterone in human testicular explants. Each bar represents the mean  $\pm$  SEM of seven (24 h) or four (48 h) independent experiments from different donors. Values between brackets indicate the percentage decrease compared with control. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  by non-parametric signed rank Mann–Whitney test on unpaired data. NS, not significant. (B) Distribution of DEHP and MEHP in human testis organotypic culture. Human testis explants were incubated with  $^{14}$ C DEHP or  $^{14}$ C MEHP for 24 h. Radioactivity was counted in each compartment.



**Figure 3** Typical radio-chromatographic profile obtained from the incubation of  $10^{-5}$  M  $^{14}$ C-DEHP with human testicular explants in culture for 24 h. dpm, disintegrations per minute.

decreased the levels of 17OH-pregnenolone and DHEA. In contrast, the production of pregnenolone, which is the substrate of the CYP17,20 hydroxylase enzyme, was not decreased by ketoconazole (Fig. 4A).

Production of all the precursors of testosterone of the  $\Delta 5$  pathway was inhibited after exposure of the explants to MEHP (Fig. 4A). In the  $\Delta 4$  pathway, ketoconazole decreased 17OH-progesterone and downstream testosterone precursor levels to a similar extent as testosterone (Fig. 4B). However, ketoconazole did not affect pregnenolone production, and actually caused a progesterone accumulation (+163%). Similar to the  $\Delta 5$  pathway, MEHP significantly decreased all the tested steroids in the  $\Delta 4$  pathway (Fig. 4B).

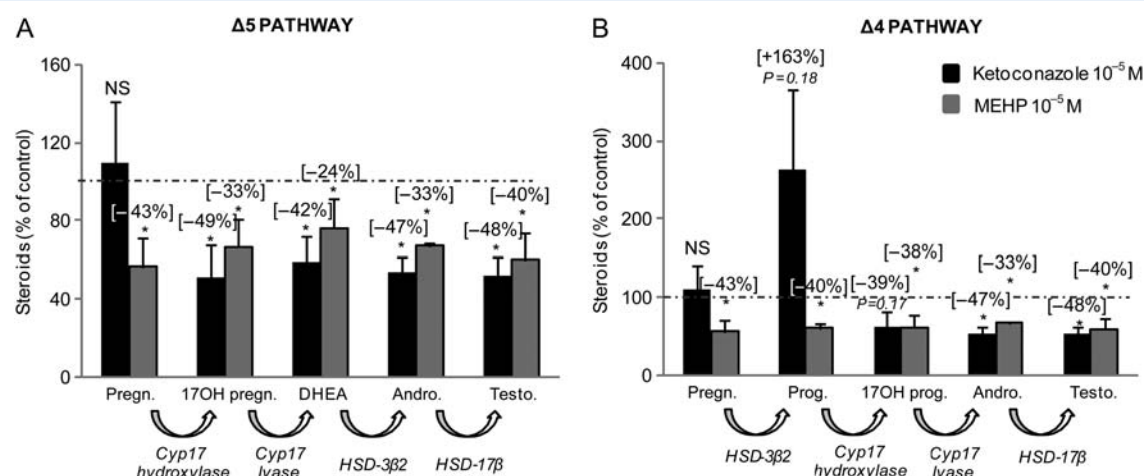
### DEHP, MEHP and ketoconazole and INSL3 production

None of the treatments were found to affect Leydig cell INSL3 concentrations (Fig. 5).

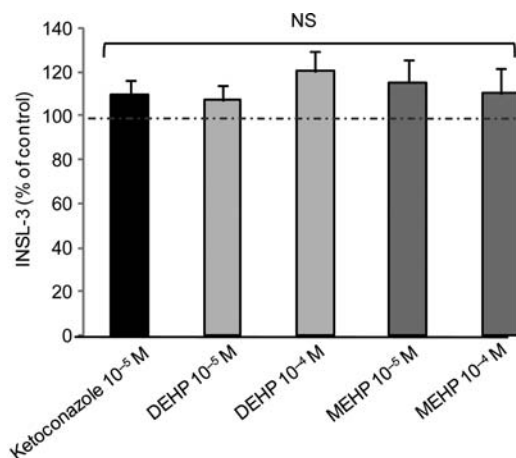
### DEHP, MEHP and ketoconazole and inhibin B production

In contrast to ketoconazole, which significantly increased inhibin B (+23%), neither DEHP nor MEHP had any effect on this Sertoli cell product (Fig. 6).

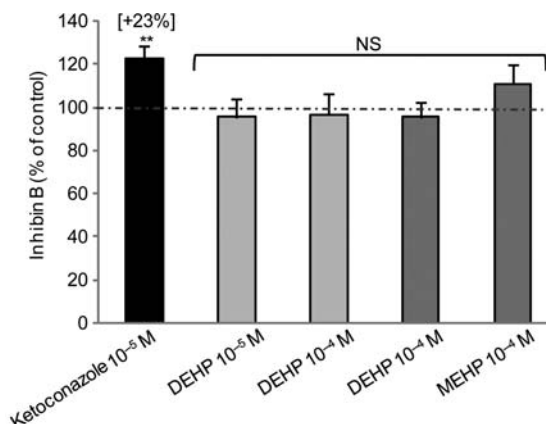




**Figure 4** Effects of ketoconazole and MEHP on the  $\Delta 5$  and  $\Delta 4$  pathways of testosterone production. Human testis explants were incubated for 24 h with  $10^{-5}$  M ketoconazole or MEHP. Steroid precursors of the  $\Delta 5$  (A) and  $\Delta 4$  pathways (B) were measured by GC–MS/MS. Values are means  $\pm$  SEM of four independent experiments from different donors. Values between brackets indicate the percentage of variation compared with control. \* $P < 0.05$  by non-parametric signed rank Mann–Whitney test on unpaired data. NS, not significant. Pregn. = pregnenolone; 17OH pregn. = 17-OH-pregnenolone; Prog. = progesterone; 17OH prog. = 17OH-progesterone; Andro. = 4-androstene-3,17-dione; Testo. = testosterone.



**Figure 5** Effects of ketoconazole, DEHP and MEHP on INSL-3 levels. INSL-3 was measured by RIA. Values are means  $\pm$  SEM of four independent experiments from different donors. NS, not significant.



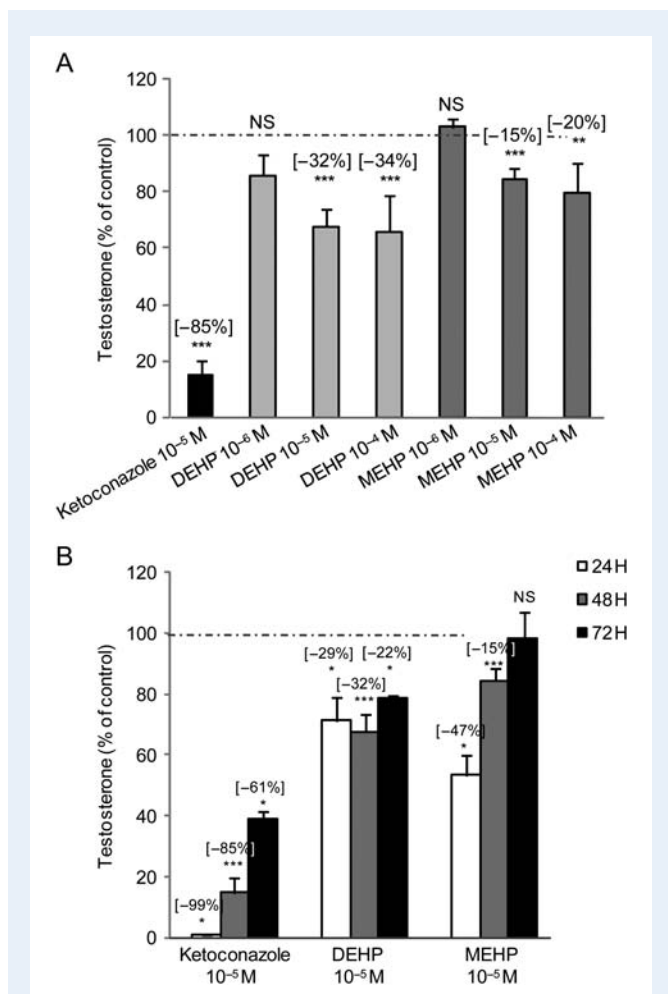
**Figure 6** Effects of DEHP, MEHP and ketoconazole on inhibin B production. Values are means  $\pm$  SEM of three independent experiments from different donors. The value between brackets indicates the percentage increase compared with control. \*\* $P < 0.01$  by non-parametric signed rank Mann–Whitney test on unpaired data.

## DEHP, MEHP and ketoconazole effects on testosterone in the CELIAS

DEHP, MEHP and ketoconazole all inhibited testosterone production by NCI-H295R cells in a dose-dependent manner (Fig. 7A). The effects of ketoconazole on testosterone decreased as the culture time was extended from 24 to 72 h (Fig. 7B). Both DEHP and MEHP inhibited testosterone after 24 h of exposure (–29 and –47%, respectively; Fig. 7B), and only a small difference was observed for DEHP between 24, 48 and 72 h (–32 and –22%, respectively; Fig. 7B); but the inhibitory action of MEHP declined between 48 and 72 h.

## Discussion

This study is the first to provide direct evidence that phthalates have the ability to suppress human testicular steroidogenesis, and notably androgen synthesis. Previous studies have failed to provide evidence of any direct anti-androgenic effect of either MBP (Hallmark *et al.*, 2007) or MEHP (Lambrot *et al.*, 2009) in human fetal testis. This may be due to the differences between fetal and adult-type Leydig cells (Jackson *et al.*, 1986; De Kretser and Kerr, 1988; Svechnikov *et al.*, 2010), or to the differences in the culture conditions between the model systems. It cannot also be totally ruled out that the use



**Figure 7** (A) Effects of DEHP, MEHP and ketoconazole on testosterone production by the NCI-H295R cells treated for 48 h. Testosterone was expressed as a percentage of the control. Each bar represents the mean  $\pm$  SEM of seven experiments. Values between brackets indicate the percentage decrease compared with control. (B) Time-course of DEHP, MEHP and ketoconazole effects on testosterone production by the NCI-H295R cells. Testosterone was expressed as a percentage of the control. Each bar represents the mean  $\pm$  SEM of five independent experiments. Values between brackets indicate the percentage decrease compared with the control. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  by non-parametric signed rank Mann–Whitney test on unpaired data. NS, not significant.

of human fetal testis explants to study the anti-androgenic action of phthalates is ‘limited’ as stated by Hallmark et al. (2007). It is noteworthy that the administration of dibutyl phthalate (DBP) to neonatal marmosets reduced serum testosterone concentrations (Hallmark et al., 2007).

We investigated whether DEHP and MEHP are metabolized in the human adult testis to (i) elucidate the significance of the anti-androgenic effects of DEHP, (ii) understand why the effects of DEHP and MEHP declined during the course of the cultures and (iii) help interpret the significance of our data to human exposure. We show that DEHP was processed into MEHP by the testis explants, and that the latter was further processed into 5OH-MEHP. Therefore,

the DEHP-induced inhibition of testosterone production by our models most probably results from the activities of MEHP and 5OH-MEHP. The decline in the anti-androgenic effects observed after 48 h of culture most likely results from this biotransformation. Likewise, the time-dependent decrease in the anti-androgenic effects of both DEHP and MEHP in the CELIAS is also likely to be the result of biotransformation processes. In the CELIAS, more than 23% of the DEHP was found to be associated with the cells after 24 h of culture, with these cells being able to metabolize it. Treatment with  $10^{-5}$  M DEHP inhibited testosterone, whereas  $10^{-4}$  M DEHP had no effect after 24 h of culture. Under conditions in which DEHP is not metabolized into MEHP (i.e. in the fetal rat testis ex vivo), it exerts stimulatory effects on testosterone production (pro-androgenic effects; Chauvigne et al., 2009), so possibly when added at  $10^{-4}$  M, the amount of DEHP in the culture before metabolization may be sufficient to counterbalance the anti-androgenic effects of MEHP and 5OH-MEHP.

Using a method exploiting radio-labelled phthalates (Chauvigne et al., 2009), we were able to estimate that, when  $10^{-5}$  M of the phthalates of interest were added for 24 h, about 3–6% of DEHP and MEHP were associated with the testis explants. Taking this into account along with our findings on the testicular DEHP biotransformation, we estimate that phthalate concentrations of the order of 1  $\mu$ g/ml are able to display significant anti-androgenic effects. Interestingly, such concentrations of DEHP or MEHP have been found in the serum and semen of men (Buchta et al., 2005; Pan et al., 2006; Han et al., 2009; Meeker et al., 2009). Consistent with these observations, two epidemiological studies show associations between exposure to MEHP and lower free testosterone concentrations in men (Pan et al., 2006; Meeker et al., 2009).

Studies of the rat *in vivo* have shown that phthalate-induced testosterone suppression is mediated by the inhibition of a number of genes involved in cholesterol transport and metabolism, or of genes encoding steroidogenic enzymes (Shultz et al., 2001; Lehmann et al., 2004; Liu et al., 2005; Borch et al., 2006; Lahousse et al., 2006). The potential involvement of peroxisome proliferator-activated receptors has also been described (Maloney and Waxman, 1999; Bility et al., 2004). In a recent study on the fetal rat testis ex vivo, we showed that the direct inhibitory effects of MEHP are mediated by the inhibition of the *cyp17a1* gene and a decrease in the CYP17A1 protein (Chauvigne et al., 2011).

However, the results presented here show that all testosterone precursors, in both the  $\Delta 4$  and  $\Delta 5$  steroidogenic pathways, were significantly inhibited by exposure of the human testis to MEHP. As this study was not designed to investigate the mechanism of action of phthalates (or of ketoconazole) on human steroidogenesis, the precise mechanisms underlying the suppressive effects of MEHP remain to be elucidated.

Ketoconazole has been shown to lower serum testosterone in men (Pont et al., 1982; Schurmeyer and Nieschlag, 1982; Rajfer et al., 1986). Our results demonstrate strong inhibitory effect of ketoconazole on testosterone in the human testis and in the NCI-H295R cell line, as recently described (Hecker et al., 2006). Ketoconazole also increased germ cell apoptosis. It is likely that this ketoconazole-induced germ cell degeneration was at least in part, a consequence of the inhibition of testosterone, as androgen deprivation is known to result in a degeneration of germ cells (Steinberger, 1971).

Consistent with our findings, ketoconazole-treated rats display degeneration of germ cells (Amin, 2008). Interestingly, when the human testis samples were exposed to ketoconazole, pregnenolone was unaffected but progesterone accumulated, which confirms that the Cyp17 hydroxylase enzyme is a target of this drug (Albertson *et al.*, 1988). Thus except for pregnenolone and progesterone, the concentrations of all the precursors of testosterone were found to be decreased by ketoconazole. This may also reflect ketoconazole-induced inhibition of cholesterol synthesis, which has previously been shown *in vivo* in men (Miettinen and Valtonen, 1984).

INSL3 is a Leydig cell product responsible for the first phase of testicular descent during fetal life and is believed to be involved in germ cell survival and bone metabolism (Ivell and Anand-Ivell, 2009; Bay and Andersson, 2011). Neither phthalates nor ketoconazole affected INSL3 production. This contrasts with evidence that prenatal exposure to DBP or MBP inhibits *INSL3* gene expression in the rat fetal testis and may be associated with impaired testicular descent (Liu *et al.*, 2005; Shono *et al.*, 2005). It is also discordant with a report that the intensity of Leydig cell INSL3 immuno-labelling or the *INSL3* gene expression was decreased in the fetal testes of most rats exposed *in utero* to phthalates (Wilson *et al.*, 2004; McKinnell *et al.*, 2005). These discrepancies are likely to reflect differences in the sensitivities of fetal and adult Leydig cells, as discussed above and in the approaches used.

We report that neither DEHP nor MEHP had an effect on inhibin B, consistent with the maintained Sertoli cell integrity. Accordingly, in humans, studies have revealed that environmental exposure to DEHP is not associated with changes in inhibin B serum levels in healthy men (Mendiola *et al.*, 2010) or in men recruited through a study on infertility (Meeker *et al.*, 2009). However, male rats exposed *in utero* and post-natally to DEHP show transitory abnormally low inhibin B serum levels on post-natal Day 22 (Borch *et al.*, 2004). Our observation that ketoconazole significantly increases inhibin B levels is the first evidence that this compound can disrupt human Sertoli cell function. The reason why inhibin B levels were found to be increased when germ cell apoptosis is rising after exposure to ketoconazole, while the reverse would be expected (De Kretser and Robertson, 1989), remains unknown.

The main conclusion of our study is that phthalate esters can alter human steroidogenesis. The active concentrations of phthalates which alter testosterone production appear to be within the range of concentrations found in men in recent epidemiological studies associating exposure to phthalates and impairment of androgenic status.

## Acknowledgements

We thank Jean-Pierre Cravedi for providing us with  $^{14}\text{C}$ -MEHP.

## Authors' roles

B.J. and N.D.-R. designed the research; C.D.-L., O.A., E.P., F.C., and L.L. performed the research; B.L.B. and D.Z. designed the global analysis of the  $\Delta 5$  and  $\Delta 4$  pathways of androgen production and the biotransformation experiments, respectively; and F.G. supervised and performed orchidectomies. All authors contributed to critically reviewing the draft manuscript. All authors saw and approved the final version of the report.

## Funding

This work was funded by Inserm (Institut national de la santé et de la recherche médicale), Ministère de l'Enseignement Supérieur et de la Recherche, and grants 212844 [DEER (Developmental Effects of Environment on Reproductive Health)] from the European Union, by grants from the European Council for Plasticisers and Intermediates, by ANR-06-PNRA-008-05 (PLAST-IMPACT), by ANR-07-SEST-019 (PHTALATESTIS) and by the French Ministry of Environment in a national program 'Grenelle de Environnement programme LOLF 189' funding (Specific Tools and Methods for Reprotoxicity program).

## Conflict of interest

The authors declare they have no competing interests be it financial, personal or professional.

## References

- Albertson BD, Frederick KL, Maronian NC, Feuillan P, Schorer S, Dunn JF, Loriaux DL. The effect of ketoconazole on steroidogenesis: I. Leydig cell enzyme activity in vitro. *Res Commun Chem Pathol Pharmacol* 1988; **61**:17–26.
- Amin A. Ketoconazole-induced testicular damage in rats reduced by Gentiana extract. *Exp Toxicol Pathol* 2008; **59**:377–384.
- Barlow NJ, McIntyre BS, Foster PM. Male reproductive tract lesions at 6, 12, and 18 months of age following in utero exposure to di(n-butyl) phthalate. *Toxicol Pathol* 2004; **32**:79–90.
- Bay K, Andersson AM. Human testicular insulin-like factor 3: in relation to development, reproductive hormones and andrological disorders. *Int J Androl* 2011; **34**:97–109.
- Bility MT, Thompson JT, McKee RH, David RM, Butala JH, Vanden Heuvel JP, Peters JM. Activation of mouse and human peroxisome proliferator-activated receptors (PPARs) by phthalate monoesters. *Toxicol Sci* 2004; **82**:170–182.
- Borch J, Ladefoged O, Hass U, Vinggaard AM. Steroidogenesis in fetal male rats is reduced by DEHP and DINP, but endocrine effects of DEHP are not modulated by DEHA in fetal, prepubertal and adult male rats. *Reprod Toxicol* 2004; **18**:53–61.
- Borch J, Metzdorff SB, Vinggaard AM, Brokken L, Dalgaard M. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. *Toxicology* 2006; **223**:144–155.
- Buchta C, Bittner C, Heinzl H, Hocker P, Macher M, Mayerhofer M, Schmid R, Seger C, Dettke M. Transfusion-related exposure to the plasticizer di(2-ethylhexyl)phthalate in patients receiving plateletpheresis concentrates. *Transfusion* 2005; **45**:798–802.
- Chauvigne F, Menuet A, Lesne L, Chagnon MC, Chevrier C, Regnier JF, Angerer J, Jegou B. Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis in vitro. *Environ Health Perspect* 2009; **117**:515–521.
- Chauvigne F, Plummer S, Lesne L, Cravedi JP, Dejucq-Rainsford N, Fostier A, Jegou B. Mono-(2-ethylhexyl) phthalate directly alters the expression of Leydig cell genes and CYP17 lyase activity in cultured rat fetal testis. *PLoS One* 2011; **6**:e27172.
- Courant F, Antignac JP, Laille J, Monteau F, Andre F, Le Bizec B. Exposure assessment of prepubertal children to steroid endocrine disruptors. 2. Determination of steroid hormones in milk, egg, and meat samples. *J Agric Food Chem* 2008; **56**:3176–3184.



- De Kretser DM, Kerr JB. The cytology of the testis. In: Knobil EN, Neill JD, Ewing LL, Greenwald GS, Markert CL, Pfaff DW (ed). *The Physiology of Reproduction*. New York: Raven Press, 1988.
- De Kretser DM, Robertson DM. The isolation and physiology of inhibin and related proteins. *Biol Reprod* 1989;**40**:33–47.
- Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 1986;**89**:271–277.
- Gracia T, Hilscherova K, Jones PD, Newsted JL, Zhang X, Hecker M, Higley EB, Sanderson JT, Yu RM, Wu RS et al. The H295R system for evaluation of endocrine-disrupting effects. *Ecotoxicol Environ Saf* 2006;**65**:293–305.
- Gray TJ, Gangoli SD. Aspects of the testicular toxicity of phthalate esters. *Environ Health Perspect* 1986;**65**:229–235.
- Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L. Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicol Sci* 2000;**58**:350–365.
- Hallmark N, Walker M, McKinnell C, Mahood IK, Scott H, Bayne R, Coutts S, Anderson RA, Greig I, Morris K et al. Effects of monobutyl and di(n-butyl) phthalate in vitro on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects in vivo in the fetal rat and neonatal marmoset and in vitro in the human. *Environ Health Perspect* 2007;**115**:390–396.
- Han SW, Lee H, Han SY, Lim DS, Jung KK, Kwack SJ, Kim KB, Lee BM. An exposure assessment of di-(2-ethylhexyl) phthalate (DEHP) and di-n-butyl phthalate (DBP) in human semen. *J Toxicol Environ Health A* 2009;**72**:1463–1469.
- Hecker M, Newsted JL, Murphy MB, Higley EB, Jones PD, Wu R, Giesy JP. Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: hormone production. *Toxicol Appl Pharmacol* 2006;**217**:114–124.
- Huang PC, Kuo PL, Chou YY, Lin SJ, Lee CC. Association between prenatal exposure to phthalates and the health of newborns. *Environ Int* 2009;**35**:14–20.
- Imajima T, Shono T, Zakaria O, Suita S. Prenatal phthalate causes cryptorchidism postnatally by inducing transabdominal ascent of the testis in fetal rats. *J Pediatr Surg* 1997;**32**:18–21.
- Ivell R, Anand-Ivell R. Biology of insulin-like factor 3 in human reproduction. *Hum Reprod Update* 2009;**15**:463–476.
- Jackson AE, O'Leary PC, Ayers MM, de Kretser DM. The effects of ethylene dimethane sulphonate (EDS) on rat Leydig cells: evidence to support a connective tissue origin of Leydig cells. *Biol Reprod* 1986;**35**:425–437.
- Jonsson BA, Richthoff J, Rylander L, Giwercman A, Hagmar L. Urinary phthalate metabolites and biomarkers of reproductive function in young men. *Epidemiology* 2005;**16**:487–493.
- Koch HM, Bolt HM, Preuss R, Angerer J. New metabolites of di(2-ethylhexyl)phthalate (DEHP) in human urine and serum after single oral doses of deuterium-labelled DEHP. *Arch Toxicol* 2005;**79**:367–376.
- Lahousse SA, Wallace DG, Liu D, Gaido KW, Johnson KJ. Testicular gene expression profiling following prepubertal rat mono-(2-ethylhexyl) phthalate exposure suggests a common initial genetic response at fetal and prepubertal ages. *Toxicol Sci* 2006;**93**:369–381.
- Lambrot R, Muczynski V, Lecureuil C, Angenard G, Coffigny H, Pairault C, Moison D, Frydman R, Habert R, Rouiller-Fabre V. Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. *Environ Health Perspect* 2009;**117**:32–37.
- Lehmann KP, Phillips S, Sar M, Foster PM, Gaido KW. Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di (n-butyl) phthalate. *Toxicol Sci* 2004;**81**:60–68.
- Lin H, Ge RS, Chen GR, Hu GX, Dong L, Lian QQ, Hardy DO, Sottas CM, Li XK, Hardy MP. Involvement of testicular growth factors in fetal Leydig cell aggregation after exposure to phthalate in utero. *Proc Natl Acad Sci USA* 2008;**105**:7218–7222.
- Liu K, Lehmann KP, Sar M, Young SS, Gaido KW. Gene expression profiling following in utero exposure to phthalate esters reveals new gene targets in the etiology of testicular dysgenesis. *Biol Reprod* 2005;**73**:180–192.
- Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, Schmidt IM, Suomi AM, Virtanen HE, Petersen DV et al. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. *Environ Health Perspect* 2006;**114**:270–276.
- Maloney EK, Waxman DJ. trans-Activation of PPARalpha and PPARgamma by structurally diverse environmental chemicals. *Toxicol Appl Pharmacol* 1999;**161**:209–218.
- McKinnell C, Sharpe RM, Mahood K, Hallmark N, Scott H, Ivell R, Staub C, Jegou B, Haag F, Koch-Nolte F et al. Expression of insulin-like factor 3 protein in the rat testis during fetal and postnatal development and in relation to cryptorchidism induced by in utero exposure to di (n-Butyl) phthalate. *Endocrinology* 2005;**146**:4536–4544.
- Meeker JD, Calafat AM, Hauser R. Urinary metabolites of di(2-ethylhexyl) phthalate are associated with decreased steroid hormone levels in adult men. *J Androl* 2009;**30**:287–297.
- Mendiola J, Jorgensen N, Andersson AM, Calafat AM, Silva MJ, Redmon JB, Sparks A, Drobnis EZ, Wang C, Liu F et al. Associations between urinary metabolites of di(2-ethylhexyl) phthalate and reproductive hormones in fertile men. *Int J Androl* 2010;**20**:128–138.
- Miettinen TA, Valtonen VV. Ketoconazole and cholesterol synthesis. *Lancet* 1984;**2**:1271.
- Pan G, Hanaoka T, Yoshimura M, Zhang S, Wang P, Tsukino H, Inoue K, Nakazawa H, Tsugane S, Takahashi K. Decreased serum free testosterone in workers exposed to high levels of di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP): a cross-sectional study in China. *Environ Health Perspect* 2006;**114**:1643–1648.
- Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray LE Jr. The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat. *Toxicol Sci* 2000;**58**:339–349.
- Pont A, Williams PL, Azhar S, Reitz RE, Bochra C, Smith ER, Stevens DA. Ketoconazole blocks testosterone synthesis. *Arch Intern Med* 1982;**142**:2137–2140.
- Prevost S, Buisson C, Monteau F, André F, Le Bizec B (2004) Is GC-C-IRMS a possible analytical approach to clear up misuse situations for forbidden natural substances in edible tissues? *Proceedings of the Euroresidue V Conference*, Noordwijkerhout, the Netherlands [insert City of Publication].
- Rajfer J, Sikka SC, Rivera F, Handelsman DJ. Mechanism of inhibition of human testicular steroidogenesis by oral ketoconazole. *J Clin Endocrinol Metab* 1986;**63**:1193–1198.
- Roulet V, Denis H, Staub C, Le Tortorec A, Delaleu B, Satie AP, Patard JJ, Jegou B, Dejuq-Rainsford N. Human testis in organotypic culture: application for basic or clinical research. *Hum Reprod* 2006;**21**:1564–1575.
- Sanderson JT, Seinen W, Giesy JP, van den Berg M. 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? *Toxicol Sci* 2000;**54**:121–127.
- Schurmeyer T, Nieschlag E. Ketoconazole-induced drop in serum and saliva testosterone. *Lancet* 1982;**2**:1098.

- Shono T, Shima Y, Kondo T, Suita S. In utero exposure to mono-*n*-butyl phthalate impairs insulin-like factor 3 gene expression and the transabdominal phase of testicular descent in fetal rats. *J Pediatr Surg* 2005;**40**:1861–1864.
- Shultz VD, Phillips S, Sar M, Foster PM, Gaido KW. Altered gene profiles in fetal rat testes after in utero exposure to di(*n*-butyl) phthalate. *Toxicol Sci* 2001;**64**:233–242.
- Steinberger E. Hormonal control of mammalian spermatogenesis. *Physiol Rev* 1971;**51**:1–22.
- Svechnikov K, Landreh L, Weisser J, Izzo G, Colon E, Svechnikova I, Soder O. Origin, development and regulation of human Leydig cells. *Horm Res Paediatr* 2010;**73**:93–101.
- Swan SH. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. *Environ Res* 2008;**108**:177–184.
- Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S et al. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect* 2005;**113**:1056–1061.
- Wilson VS, Lambright C, Furr J, Ostby J, Wood C, Held G, Gray LE Jr. Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis. *Toxicol Lett* 2004;**146**:207–215.