Effect of fetal or neonatal exposure to monobutyl phthalate (MBP) on testicular development and function in the marmoset

Citation for published version:
McKinnell, C, Mitchell, RT, Walker, M, Morris, K, Kelnar, CJH, Wallace, WH & Sharpe, RM 2009, 'Effect of fetal or neonatal exposure to monobutyl phthalate (MBP) on testicular development and function in the marmoset', Human Reproduction, vol. 24, no. 9, pp. 2244-2254. https://doi.org/10.1093/humrep/dep200

Digital Object Identifier (DOI):
10.1093/humrep/dep200

Link: 
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Human Reproduction

Publisher Rights Statement:
Available under Open Access.

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Effect of fetal or neonatal exposure to monobutyl phthalate (MBP) on testicular development and function in the marmoset

Chris McKinnell¹, Rod T. Mitchell¹, Marion Walker¹, Keith Morris¹, Chris J.H. Kelnar², W. Hamish Wallace², and Richard M. Sharpe¹

¹MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, The Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TF, UK
²Edinburgh Royal Hospital for Sick Children, 9 Sciennes Road, Edinburgh EH9 1LF, UK
³Correspondence address. Tel: +44-131-242-9113; Fax: +44-131-242-6231; E-mail: c.mckinnell@hrsu.mrc.ac.uk

Introduction

Reproductive disorders in newborn boys (cryptorchidism, hypospadias) and young men [low sperm counts, testicular germ cell tumours (TGCT)] are common and/or increasing in incidence (Skakkebaek et al., 2001). These disorders are risk factors for each other and may comprise a testicular dysgenesis syndrome (TDS) with a common origin in fetal life (Skakkebaek et al., 2001; Sharpe and Skakkebaek, 2008). Abnormal testis development leading to altered somatic cell (Sertoli and/or Leydig cell) function, such as altered testosterone production by the fetal testis during the period of the male programming window (Welsh et al., 2008), may underlie TDS disorders (Sharpe and Skakkebaek, 2008).

Evidence suggests that environmental factors/exposures can affect the risk of TDS (Sharpe and Skakkebaek, 2003). Several studies have shown that fetal exposure of male rats to a common environmental chemical, di(n-butyl) phthalate (DBP), or to certain other phthalates, can induce a spectrum of disorders in male offspring similar to TDS (Mylhreest et al., 2000; Parks et al., 2000; Fisher...
et al., 2003). Human exposure to phthalates such as DBP is ubiquitous (Silva et al., 2004; CDC, 2005), and exposure during pregnancy (Marsee et al., 2006) results in fetal exposure (Silva et al., 2004), raising concerns that this could contribute causally to TDS. In rats, fetal testosterone production is inhibited by in utero exposure to DBP (Lehmann et al., 2004; Thompson et al., 2004) or its main metabolite monobutyl phthalate (MBP) (Shono et al., 2000). Similarly, exposure to either DBP (Mylchreest et al., 1998; Fisher et al., 2003) or MBP (Shono and Suito, 2003; Shono et al., 2005) results in a high incidence of cryptorchidism, while MBP exposure in rats also results in reduced fertility in some male offspring (Kai et al., 2005).

While there is indirect evidence to suggest that certain phthalates could impair fetal testosterone production in humans (Swan et al., 2005; Swan, 2008), data from in vitro (Hallmark et al., 2007; Lambrot et al., 2008) and in vivo (Huang et al., 2009) studies suggest the opposite. Furthermore, fetal testosterone production in mice is unaffected by DBP, MBP or monoethylhexyl phthalate (MEHP) (Gaido et al., 2007). In contrast, phthalate effects on germ cell numbers and/or differentiation in the fetal testis have been shown in mice (Gaido et al., 2007), rats (Ferrara et al., 2006) and in vitro in the human (Lambrot et al., 2008). This raises concerns, as impaired fetal germ cell development underlies the origins of TGCT in humans (Rajpert-de Meyts, 2006).

Rodents are not good models for human fetal germ cell development (Mitchell et al., 2008) and, in view of the contrasting effects of phthalates on fetal steroidogenesis in rats and mice described earlier, it is unclear whether the rat is a suitable model for the human. We have therefore used a non-human primate, the marmoset, to investigate if phthalate exposure perinatally can affect testis development. The marmoset is similar to the human in terms of developmental periods in the male (Kelner et al., 2002), perinatal germ cell differentiation (Mitchell et al., 2008) and organisation and (low) efficiency of spermatogenesis (Millar et al., 2000; Sharpe et al., 2000). It may therefore be a better model for the human than rodents. We have already shown (Hallmark et al., 2007) that neonatal administration of MBP can (transiently) inhibit testosterone production similar to the fetal effects of DBP in rats (Thompson et al., 2004). However, there are no reports of testicular effects of fetal exposure to MBP or other phthalates in the marmoset.

The present studies had two aims. First, to determine if TDS-like effects, similar to those found in DBP-exposed rats (Fisher et al., 2003), could be induced in marmosets following fetal or neonatal exposure to MBP. Second, to determine if fetal MBP exposure resulted in adverse testicular effects in adulthood, in particular whether there was evidence of impaired spermatogenesis/fertility or induction of TGCT or its precursor, CIS. Our results show no major, consistent effect of MBP exposure on testis and reproductive development, although some (inconsistent) effects on germ cell development need further investigation.

Materials and Methods

Animals, treatments, sample collection and processing

Animals were captive-bred common marmoset monkeys (Callithrix jacchus), maintained in a closed self-sustaining colony since 1973. Two studies were undertaken. In the first, nine pregnant females were administered 500 mg/kg/day MBP (TCI Europe, Zwijndrecht, Belgium) from ~7–15 weeks of gestation. Regular, systematic palpation and/or ultrasound were used to diagnose pregnancy and estimate gestational age. Experience has shown this to be accurate to within one week and was subsequently confirmed by time of birth. A total of 11 male offspring originated from these treated mothers and were killed either around birth (1–5 days; n = 6) or in adulthood (n = 5). The treatment time window was chosen based on comparative analysis of fetal testis development in the marmoset and human using cell-specific markers (Mitchell et al., 2008 and unpublished data) and which indicated that this period will encompass seminiferous cord formation and onset of testosterone production (marmosets have a disproportionately long embryonic period). This period is thought to correspond to a time window in the rat which is critical for androgen-dependent programming of reproductive tract masculinisation (Welsh et al., 2008). Animal welfare considerations precluded the use and treatment of large numbers of animals, so to ensure reasonable numbers of MBP-treated animals, we restricted the number of control newborn males (born to vehicle-gavaged pregnant mothers) to n = 3 and supplemented these with 7 untreated controls to ensure adequate numbers of controls. These supplementary controls were 1–5 day-old animals which had been killed because they originated from triplet births, in which only two animals usually survive. For all parameters investigated in the present studies, values for the supplementary controls were comparable to those for the three offspring from vehicle-treated mothers. For evaluation of testes in adulthood, males exposed in utero to MBP were compared with control animals (n = 5) of comparable age that were derived from other experiments (e.g. Lunn et al., 1994, 1997), but whose mothers had not been treated during pregnancy.

In the second study, 10 newborn marmosets were used, comprising five pairs of co-twin males (one vehicle-treated, one MBP-treated), which enabled pair-wise evaluation using smaller numbers of animals than would otherwise be necessary (Sharpe et al., 2002). Commencing at 4 days of age, marmosets were orally administered vehicle or 500 mg/kg/day MBP for 14 days. The MBP was dissolved in dimethyl sulfoxide then suspended in honey and was taken voluntarily by infants (Hallmark et al., 2007). MBP is considered the active metabolite of DBP and can induce the same fetal testicular effects as DBP in rats (Shono et al., 2000, 2005) and its administration to newborn marmosets (at the presently used dose) has been shown to significantly reduce testosterone levels in blood (Hallmark et al., 2007). MBP was used in the present studies because marmosets are reported to be poor metabolizers of orally administered phthalate diesters to their monoesters (Rhodes et al., 1983, 1986). This means that once the maximum metabolic conversion capacity of a pregnant female marmoset has been reached after DBP dosing, administration of higher doses will not result in any further metabolism, and therefore exposure, to the monoester (MBP). Therefore, to ensure maximum possible exposure in the present studies we administered MBP not DBP. We chose to administer 500 mg/kg/day as this dose of either DBP (Fisher et al., 2003; Mahood et al., 2007) or MBP (Shono et al., 2000, 2005) causes major adverse effects on the fetal testis in rats (and MBP) can suppress testosterone levels in newborn marmosets (Hallmark et al., 2007).

Animals were killed at 1–5 days or 18–21 months of age (fetal exposure) or 4 h after the last (neonatal) treatment, via intraperitoneal injection of an overdose of sodium pentobarbitone (Euthatal; Rhone Meneux Ltd, Harlow, UK). Testes were dissected out, fixed for 6 h in Bouin’s fixative, weighed, then transferred to 70% ethanol and processed into paraffin wax in a Leica automatic processor (Leica Microsystems, Milton Keynes, UK).

For all studies, animals were treated humanely and with regard for alleviation of suffering. Studies were performed according to the Animal (Scientific Procedures) Act 1986 under UK Home Office Project Licence approval, and also approved by the local ethical committee for studies in primates.
**Table I. Antibodies and conditions used for immunohistochemistry**

| Antigen      | Source  | Species | Dilution | Retrieval | Target                      |
|--------------|---------|---------|----------|-----------|-----------------------------|
| 3β-HSD[3]    | Gift    | Rabbit  | 1:800    | N         | Leydig cells                |
| SMA[4]       | Sigma   | Mouse   | 1:1000   | N         | Peritubular myoid cells     |
| Cytokeratin  | Santa Cruz | Mouse | 1:500    | Y         | Sertoli cells               |
| AMH[5]       | Santa Cruz | Goat  | 1:1000   | N         | Sertoli cells               |
| OCT4         | Santa Cruz | Goat  | 1:40     | Y         | Undifferentiated gonocytes   |
| C-KIT        | Dako    | Rabbit  | 1:20     | Y         | Undifferentiated gonocytes   |
| AP-2y        | Santa Cruz | Mouse | 1:40     | Y         | Undifferentiated gonocytes   |
| VASA         | Abcam   | Rabbit  | 1:200    | Y         | Germ cells                  |
| N-Cadherin   | Zymed   | Mouse   | 1:200    | Y         | Cell adhesion molecule       |
| SOX9         | Chemicon | Rabbit | 1:100    | Y         | Sertoli cells               |
| Ki67         | Dako    | Mouse   | 1:40     | Y         | Proliferating cells         |

[3β-Hydroxysteroid dehydrogenase;[4]smooth muscle actin;[5]anti-Müllerian hormone.

---

**Plasma levels of testosterone**

Plasma testosterone was measured using an ELISA adapted from an earlier radioimmunoassay, as described previously (Fisher et al., 2003). The limit of detection was 12 pg/ml.

**Immunohistochemistry**

Specific proteins were detected by immunohistochemistry using methods detailed previously (Fisher et al., 2003; Gaskell et al., 2004; Ferrara et al., 2006; Hutchison et al., 2008; Mitchell et al., 2008). In the case of N-Cadherin and SOX9, protocols were optimised using a range of antibody dilutions and appropriate positive and negative control tissues. The antibodies, dilutions and requirement for antigen retrieval are shown in Table I. Briefly, antigen retrieval used 0.01 M citrate buffer, pH 6.0, except for SOX9, which required retrieval in 0.5 M glycine and 0.01% (w/v) EDTA, pH 3.5. Endogenous peroxidase was blocked by incubating slides in 3% (v/v) H2O2 in methanol and endogenous biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories Inc., Peterborough, UK) according to manufacturer’s instructions. After incubation overnight at 4°C with primary antibody, slides were washed in 0.05 M Tris-buffered saline (TBS), pH 7.4, then incubated with appropriate biotinylated secondary antibody, followed by incubation with streptavidin-conjugated horseradish peroxidase (Dako, Ely, UK) and visualisation of immunostaining using diaminobenzidine (Liquid DAB+, Dako).

**Double immunostaining using Fast Blue visualisation**

VASA/OCT4: after incubation with the primary (VASA) and secondary antibodies as described earlier, slides were washed in TBS, incubated with streptavidin-conjugated alkaline phosphatase (Dako) diluted 1:200 in TBS, and immunostaining visualised using 1 mg/ml Fast Blue (Sigma-Aldrich Ltd, Poole, UK) until staining was optimal. After washing in TBS, slides were immunostained for OCT4 using DAB visualisation as described earlier.

Ki67/VASA: immunostaining for Ki67, using DAB detection, was performed as described earlier. Slides were then washed in TBS and immunostained for VASA using Fast Blue as earlier.

To ensure reproducibility of results and accurate comparison of immunostaining between treatment groups, sections from all groups were run in parallel on at least two occasions. Negative controls were included in each experiment, for which the primary antibody was replaced with either peptide-preabsorbed antibody (OCT4) or the appropriate normal serum (all other antibodies), and in all such cases immunostaining was absent. Representative sections were photographed using a Provis AX70 microscope (Olympus Optical, London, UK) fitted with a Canon DS6031 digital camera (Canon Europe, Amsterdam, The Netherlands). Images were compiled using Photoshop CS2 (Adobe Systems Inc., Mountain View, CA, USA).

**Determination of germ cell and Sertoli cell numbers**

Sections were analysed using a Zeiss Axio-Imager microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) fitted with a Hitachi HV-C20 camera (Hitachi Denshi Europe, Leeds, UK) and a Prior automatic stage (Prior Scientific Instruments Ltd, Cambridge, UK). Image-Pro 6.2 with Stereologger plug-in software (MagWorldwide, Wokingham, UK) was used to select random fields for counting and to place a grid over the tissue. Germ cell counting used sections double-immunostained for VASA and OCT4 (1–5 days of age) to label all subpopulations, VASA alone (17–20 days of age) or sections stained with hematoxylin (adults); in adults, spermatogonia, spermatocytes and round spermatids were counted separately, while elongate spermatids and spermatozoa were combined. Sertoli cell counting used sections immunostained for SOX9, a Sertoli cell-specific nuclear marker (Swain and Lovell-Badge, 1997). Relative cell volume per testis was first determined (Sharpe et al., 2003). The number of fields counted per animal (~15–75 fields) was dependent on obtaining a percentage standard error value of <5%. Data were converted to absolute volume per testis by multiplying by testis weight (equivalent to volume), then converted to cell number per testis after determination of mean cell nuclear diameter and volume (average of 70–100 nucleoli) using the Stereologger software nucleator function. For elongate spermatids and spermatozoa, only cell volume per testis was calculated due to extreme variation in shape of the nucleus.

**Determination of the germ cell PI**

PI was determined stereologically on sections double-immunostained for Ki67/VASA, which enabled identification of both proliferating and nonproliferating germ cells. A total of ~150 germ cells, from two sections per animal, were counted. The proliferation index (PI) was calculated as
the number of Ki67+/VASA+ cells divided by the number of Ki67+/VASA+ plus Ki67-/VASA+ cells × 100.

Statistical analysis
All data were analysed using the Student’s t-test (two-tailed). Paired t-test was used for the co-twin study.

Results

Effect of in utero exposure to MBP on normality of reproductive tract development

Our findings are summarised in Table II. We compared these with the incidence of abnormalities observed in our studies in rats (Fisher et al., 2003; Mahood et al., 2007), and calculated the number of animals expected to exhibit each abnormality based on a total of n = 11 MBP-exposed male offspring studied and assuming the same percentage incidence as found in our rat studies. All males exposed in utero to MBP were normally masculinised at birth and showed no evidence of hypospadias; this was confirmed when males were killed either at age 1–5 days (termed ‘at birth’, n = 6) or in adulthood (18–21 months; n = 5). At birth, testes could be visualised in transit through the inguinal canal and were similarly placed in MBP-exposed animals and controls. Plasma testosterone levels in MBP-exposed animals at birth (2.32 ± 0.76 ng/ml; mean ± SEM; n = 6) were comparable to controls (2.41 ± 0.72 ng/ml; n = 5). When killed, no gross abnormalities of the epididymis, vas deferens, prostate or seminal vesicles were apparent in MBP-exposed males or in controls (data not shown).

Effect of in utero exposure to MBP on testis morphology and size at birth

Immunohistochemistry for cell-specific markers was used to evaluate normality of testis formation/morphology; immunostaining for smooth muscle actin (SMA) (Fig. 1A and B) was used to label peritubular myoid cells, cytokeratin (Fig. 1C and D) and anti-Müllerian hormone (AMH) (Fig. 1E and F) in 1–5-day-old marmosets after fetal exposure to 500 mg/kg/day MBP or in controls. Immunostaining of all three proteins in MBP-exposed animals was comparable to controls. Seminiferous cord structure and size appeared normal, with no evidence of focal dysgenesis. Sertoli cell differentiation and function was similarly unaffected. Bar = 50 μm.

Table II Expected number of MBP-exposed marmosets exhibiting testicular or reproductive tract abnormalities, based on the incidence induced in rat studies using the same dose of the parent compound DBP

| Abnormality                              | Incidence in rat studiesb | Number of affected marmosetsc | Expected | Observed |
|------------------------------------------|---------------------------|-------------------------------|----------|----------|
|                                          |                           |                               |          |          |
| Hypospadias                              | 17%                       | 2                             | 0        |
| Cryptorchidism                           | ≥70%                      | ≥7                            | 0        |
| Small testes/impaired spermatogenesis    | ≥70%                      | ≥7                            | 0        |
| Focal testicular dysgenesis              | ≥50%                      | ≥5                            | 0        |

*aAs MBP is considered to be the active moiety, exposure of the marmosets to MBP should have been at least equivalent (and probably more) to that in rats administered the same dose of DBP; bIncidence observed in previous studies (Fisher et al., 2003; Mahood et al., 2007); cExpected number based on 11 MBP-exposed offspring studied at birth or in adulthood and assuming the same % incidence as found in our rat studies.

Figure 1 Representative testicular histology and immunostaining for smooth muscle actin (SMA) (A and B), cytokeratin (C and D) and anti-Müllerian hormone (AMH) (E and F) in 1–5-day-old marmosets after fetal exposure to 500 mg/kg/day MBP or in controls.
Germ cell aggregation/clusters at birth in MBP-exposed animals

Immunostaining of serial testis sections with AMH (Fig. 2A), OCT4 (Fig. 2B), AP2γ and C-KIT (not shown) confirmed the presence of unusual clusters of germ cells in the two aforementioned MBP-exposed animals. Almost all of the germ cells in these clusters were immunopositive for OCT4 (Fig. 2B), AP2γ and C-KIT (not shown), indicating that they were undifferentiated gonocytes. In one animal, several clusters were observed in every section examined from different regions of both testes; in the second animal, clusters were less frequent but distributed in different parts of the testis. Germ cell clusters were also observed in sections from 2 of 10 control animals, but were much fewer in number, sporadic and much smaller in size. In the two MBP-exposed animals, the clusters typically contained 10–20 germ cells, but clusters of up to 27 germ cells were found. In controls, clusters rarely contained more than 10 germ cells.

Abnormal germ cell aggregation occurs in the centre of seminiferous cords in fetal testes of DBP-exposed rats, due to withdrawal of Sertoli cell cytoplasm from around the germ cells (Kleymenova et al., 2005). We investigated this using immunohistochemistry for the adhesion protein N-Cadherin (Johnson and Boekelheide, 2002). This showed N-Cadherin immunoreactivity in MBP-exposed animals with germ cell clusters comparable to controls (Fig. 2C), suggesting that cell–cell adhesion in clusters was normal. Immunostaining of serial sections with OCT4 and Ki67 revealed no clusters of Ki67þ cells intermediate (OCT4þ/VASAþ) cells expressing both (Mitchell et al., 2008). This enables distinction of the least differentiated gonocytes (OCT4þ/VASA−) from the most differentiated spermatogonia (OCT4−/VASA−) with some germ cells intermediate (OCT4þ/VASA−). Double immunostaining for OCT4 and VASA was therefore used to identify all germ cells for counting and to discriminate the relative proportions of undifferentiated versus differentiated germ cells.

Total germ cell number per testis varied considerably between animals, ranging from 0.12 to 0.56 × 10⁶ in controls and from 0.29 to 0.84 × 10⁶ in MBP-exposed animals. The majority of germ cells in both groups were VASA− (Fig. 3A), while OCT4−/VASA− cells comprised only 1–2% of the total (not shown). There was no significant difference in numbers of VASA+ cells, OCT4+ cells or in total germ cell number per testis in MBP-exposed animals compared with controls (Fig. 3A).

Germ cell differentiation, quantified by expressing OCT4+ germ cells as a percentage of the total germ cell population, varied widely (0.4–28.9%) between control animals. There was no significant effect of MBP exposure on germ cell proliferation (Fig. 4), although in one MBP-exposed animal the PI was unexpectedly high and more than double the mean control value. This was not one of the animals described earlier in which we found extensive germ cell clustering.

Effect of in utero exposure to MBP on germ cell numbers and differentiation at birth

The neonatal marmoset testis contains a mixed complement of germ cells, some expressing the pluripotency marker OCT4, others expressing the differentiation marker VASA, and a small population expressing both (Mitchell et al., 2008). This enables distinction of the least differentiated gonocytes (OCT4þ/VASA−) from the most differentiated spermatogonia (OCT4−/VASA−) with some germ cells intermediate (OCT4þ/VASA−). Double immunostaining for OCT4 and VASA was therefore used to identify all germ cells for counting and to discriminate the relative proportions of undifferentiated versus differentiated germ cells.

Table III Testis and body weights (means ± SEM) in the marmosets used for the present studies, and in other (unquantified) adults for comparison

| Treatment | Age at evaluation | No. of animals | Testis wt (mg) | Body wt (g) |
|-----------|------------------|----------------|---------------|-------------|
| Controls (vehicle-treated) | 1–5 days | 3 | 5.5 ± 0.8 | 29.7 ± 1.0 |
| Controls (non-treated) | 7 | 4.7 ± 0.4 | 29.0 ± 1.9 |
| Controls (combined) | 10 | 4.9 ± 0.6 | 29.2 ± 1.3 |
| MBP 500 mg/kg/day | 6 | 4.8 ± 0.6 | 29.3 ± 1.9 |
| Controls (vehicle-treated)a | 17–20 days | 5 | 11.5 ± 0.9 | 56.8 ± 4.1 |
| MBP 500 mg/kg/daya | 5 | 11.0 ± 0.8 | 51.8 ± 3.8 |
| Controls (quantified)b | Adult | 5 | 522 ± 85 | 366 ± 15 |
| Controls (not quantified)c | 12 | 516 ± 9 | 388 ± 17 |
| Controls (combined) | 17 | 518 ± 24 | 381 ± 13 |
| MBP 500 mg/kg/day | 5 | 605 ± 34 | 411 ± 21 |

a Co-twin males; b Untreated control adults most closely age-matched to MBP-exposed animals; c Data from other untreated adults, showing that quantified adults are representative.

Effect of in utero exposure to MBP on Sertoli cell number and the germ cell:Sertoli cell ratio at birth

Sertoli cell number in controls was ~4 × 10⁶ per testis and was unaffected by MBP exposure (Fig. 5A). There was a non-significant trend...
Effect of neonatal treatment with MBP on germ cell numbers and differentiation

Because there was ~12 weeks between cessation of fetal treatment and evaluation (around birth), during which MBP effects might disappear, we also studied male co-twin marmosets treated with vehicle or 500 mg/kg/day MBP for ~14 days. Testis weights in MBP-treated co-twins were comparable to controls (Table III). In control co-twins, OCT4 expression fell dramatically compared with animals at birth, with OCT4+ cells either absent or occurring only sporadically in control testes. MBP-treated co-twins were comparable to controls (not shown), suggesting no effect on germ cell differentiation neonatally. Total germ cell number was determined by quantifying VASA+ cells, and in controls this gave a mean (± SEM; n = 5) value of 1.63 ± 0.20 × 10^6 (Fig. 6), indicating a 2.5-fold increase in germ cell number compared with values at birth (Fig. 3A). Germ cell number was reduced by 45 and 35% in two out of five co-twins exposed neonatally to MBP, when compared with their control co-twins, but was increased by 25% in another MBP-exposed co-twin (Fig. 6). Overall, there was no significant difference between control and MBP-treated co-twins (Fig. 6). There was no straightforward relationship between testis weight and germ cell number in individual animals.

Effect of in utero exposure to MBP on the adult marmoset testis and fertility

To determine if in utero exposure to MBP had any long-term effects on the marmoset testis or on fertility, some animals were evaluated in adulthood by comparing them with (control) animals previously used in other experiments. All testes were scrotal in MBP-exposed animals (Table II) and testicular morphology was completely normal (Fig. 7); testis weights were also comparable to controls (Table III). Examination of sections from different parts of both testes from each animal revealed no abnormal seminiferous tubules or foci of dysgenesis (Table II). Quantification of germ cells confirmed normal spermatogenesis in MBP-exposed animals (Table II), with no effect on numbers of different germ cell types (Fig. 8). Immunohistochemistry for OCT4 was used to look for presence of fetal-like germ cells, but none were detected in testes from control or MBP-exposed animals. Fertility was assessed in three MBP-exposed animals and was normal, but was not investigated in others.

Discussion

The aim of this study was to determine if fetal or neonatal exposure of male marmosets to MBP resulted in effects on testicular (focal dysgenesis, effects on Sertoli and germ cells) and reproductive tract (epididymal or penile abnormalities, testis maldescent) development similar to those found in rats exposed to phthalates, when studied either at birth or in adulthood. As male marmosets exhibit similar phases of testis development (McKinnell et al., 2001; Kelnar et al., 2002) and germ cell differentiation (Mitchell et al., 2008) to the human, whereas rodents show pronounced differences, the marmoset may be a better model than rodents for study of MBP effects. In particular, differences in germ cell differentiation between humans and marmosets on the one hand and rodents on the other hand, suggest that marmosets could be susceptible to induction of CIS (the precursor of TGCT in humans) unlike the rat (Mitchell et al., 2008). Therefore,
one focus of our studies was on perinatal germ cell differentiation. Treatment of pregnant marmosets for 7 weeks with a high dose of MBP did not induce any of the testicular or reproductive tract abnormalities in male offspring seen in similar experiments in rats (after DBP treatment), although there was limited, and inconclusive, evidence for effects on germ cells perinatally. No CIS or abnormalities in germ cell numbers or fertility were found in adult marmosets exposed fetally to MBP.

Several studies have shown that administration of DBP, or certain other phthalates, to pregnant rats induces TDS-like effects in male offspring (Parks et al., 2000; Mylchreest et al., 2000; Fisher et al., 2003). Defects found neonatally include focal areas of seminiferous tubule dysgenesis (Fisher et al., 2003), abnormal Leydig cell aggregates (Mahood et al., 2005), occurrence of multinucleated gonocytes and abnormal gonocyte aggregation (Kleymenova et al., 2005) and reduced germ cell numbers and delayed gonocyte differentiation (Ferrara et al., 2006). Using similar immunohistochemical techniques to these studies in rats, we found no evidence for similar effects in MBP-exposed marmosets at birth or in adulthood.

One of the major effects of DBP/MBP exposure in rats is inhibition of testosterone production by the fetal testis (Shono et al., 2000; Lehmann et al., 2004) which leads to downstream effects such as reduced anogenital distance, cryptorchidism and hypospadias in occasional animals (Foster et al., 2001; Fisher et al., 2003) and reduced Sertoli cell number at birth (Scott et al., 2007, 2008). We found no evidence for penile abnormalities or cryptorchidism in MBP-exposed marmosets either at birth or in adulthood, and Sertoli cell number was comparable to controls at birth. Although these findings do not rule out the possibility that MBP exposure reduced testosterone production by the fetal testis during treatment, they show that if this occurred, it was not sufficient to affect penile development. Based on rat studies, the critical phase for penile effects due to androgen suppression is during the male programming window, estimated to be within the MBP treatment period (~7–15 weeks gestation) when extrapolated from rat and human (Welsh et al., 2008) and based on fetal testis development in the marmoset (Mitchell et al., 2008).

A key focus of the present study was the potential effects of MBP exposure on germ cell development. Two main aspects were investigated, germ cell number and differentiation. We investigated the effect of fetal and neonatal MBP exposure in separate studies, as with fetal exposure there was ~12 weeks between cessation of treatment and evaluation (around birth), in which effects of MBP might have been recovered from, whereas this was not the case in neonatally-treated animals. Previous studies in vivo in rats (Ferrara et al., 2006) and in vitro in rats and humans (Lambrot et al., 2008) have shown that certain phthalates (DBP/MBP, MEHP) can reduce fetal germ cell number. We found that neither fetal nor neonatal exposure to MBP had any significant effect on germ cell number. However, in the neonatally treated animals, two of the five MBP-treated co-twins showed a marked reduction in germ cell number compared with their control co-twins. If this was due to MBP treatment, it is unclear why the remaining three MBP-exposed animals did not show evidence for a similar effect, so the difference could be unrelated to treatment.
In terms of relevance to the human, the most important aspect of germ cell development that we evaluated is their differentiation, because incomplete fetal germ cell differentiation is thought to underlie formation of CIS from which TGCT develops in adulthood (Rajpert-de Meyts, 2006). A previous study in rats showed that fetal DBP exposure delays (but does not prevent) germ cell differentiation (Ferrara et al., 2006). The marmoset appears an excellent model for the human as in both species perinatal germ cell differentiation is asynchronous (in contrast to the rat), and the testes at birth contain a mixed population of differentiated (VASA⁺) and undifferentiated (OCT4⁺) germ cells (Mitchell et al., 2008). The marmoset appears an excellent model for the human as in both species perinatal germ cell differentiation is asynchronous (in contrast to the rat), and the testes at birth contain a mixed population of differentiated (VASA⁺) and undifferentiated (OCT4⁺) germ cells (Mitchell et al., 2008). In marmosets (Mitchell et al., 2008) and humans (Gaskell et al., 2004), the proportion of OCT4⁺ germ cells declines progressively during fetal and neonatal life, as gonocytes differentiate. In the present study, in newborn control marmosets OCT4⁺ cells comprised ~12% and VASA⁺ cells ~87% of total germ cells. Although these proportions varied considerably between animals, in control animals (n = 10) OCT4⁺ cells never comprised >29% of the total. If differentiation was delayed by MBP exposure, the proportion of OCT4⁺ cells would be increased, but we found no evidence for this in either fetally or neonatally MBP-exposed animals. However, in one marmoset exposed fetally to MBP, the % OCT4⁺ cells was outside the control range (~5-fold...
higher than the mean control value). This could indicate that OCT4 expression had been abnormally prolonged in some germ cells in this animal, but we cannot exclude the possibility that it was simply at the extreme of the normal range (based on 10 controls in this time period).

Another unusual finding in two marmosets after fetal MBP exposure, was the occurrence of large germ cell clusters. These mainly comprised cells immunopositive for OCT4, AP2γ and C-KIT, and therefore classified as undifferentiated gonocytes. Neither of these animals was the one referred to above with an unusually high proportion of OCT4⁺ germ cells. Aggregation of germ cells has previously been observed in the testes of fetal rats exposed to DBP, due to withdrawal of Sertoli cell cytoplasm from around the germ cells (Kleymenova et al., 2005). In the present study, however, germ cell clusters were more localised within the cords and generally bordered the basal lamina. Additionally, N-cadherin immunoexpression in Sertoli cell cytoplasm around the clusters indicated normal Sertoli cell-germ cell contact, unlike in the rat lesions. Exactly how these germ cell clusters arose is unclear, and we found no previous reference to such clusters in the literature. One possibility is that they are foci of clonal expansion, although no unusual pattern of Ki67 expression was observed within these areas.

Likewise it is not possible to conclude whether germ cells within these clusters are abnormal. Clusters contained a much higher proportion of OCT4⁺ cells than in the germ cells outside of the clusters, perhaps suggesting that differentiation in these areas is occurring more slowly than in the testes as a whole. If the clusters represent foci of delayed development, it could be significant in relation to origins of CIS in humans (Rajpert-de Meyts, 2006), which is characterised by expression of the pluripotency marker OCT4 and other markers of undifferentiated or fetal germ cells such as AP2γ and C-KIT, which we also found in these germ cell clusters. However, since such clusters were found in 2 out of 10 control animals, albeit smaller in size and with much lower frequency, it is possible that the clusters in MBP-exposed animals are merely at the extreme end of the normal range. For certain, we found no such clusters or CIS-like cells in adulthood, but as this is based on only five animals exposed in utero to MBP, animal numbers may have been too low to detect this phenomenon or its consequences (if any). In view of its importance, this needs clarifying.

Based on the present studies, we conclude that administration of high doses of MBP to pregnant marmosets does not affect steroidogenesis by the fetal testis during the critical period of the male programming window, sufficient to cause any detectable downstream effects; nor is there any evidence for focal or wider testicular dysgenesis. This conclusion is based on a sufficient number of animals (n = 11 offspring in total) to provide a measure of confidence. While these are relatively small numbers, they should be considered in the context of the high incidence of abnormalities found in rat studies using DBP. Assuming that the high dose of MBP used in our study resulted in a similar incidence, we would have expected to see several animals exhibiting one or more abnormality.

However, this conclusion rests on the assumption that all effects of DBP are mediated via MBP in the marmoset, and we cannot exclude the possibility that the lack of effects of MBP in the present study is due to differences in metabolism of MBP by pregnant/fetal marmosets as compared with rodents.

While the findings also do not demonstrate any overt abnormality in germ cell numbers or development in MBP-exposed animals, some changes were detected at birth in a minority of animals that could indicate an effect, especially as only six animals were evaluated at birth for these parameters; this requires clarification. This is particularly the case in view of recent evidence indicating that MEHP has effects on germ cells but not on steroidogenesis during exposure of the human fetal testis in vitro (Lambrot et al., 2008).

**Funding**

These studies were funded entirely by the UK Medical Research Council (WBS U.1276.00.003.00003.01).

**References**

CDC (Centers for Disease Control and Prevention). Third national report on human exposure to environmental chemicals. 2005; http://www.cdc.gov/exposurereport/report.htm.
Ferrara D, Hallmark N, Scott HM, Brown R, McKinnell C, Mahood IK, Sharpe RM. Acute and long-term effects of in utero exposure of rats

---

**Figure 8** Numbers (A) of spermatogonia (SG), spermatocytes (PS) and round spermatids (RS), and absolute volume (abs. vol) (B) of elongating spermatids plus spermatozoa (ES + SPZ) in testes of adult marmosets after fetal exposure to 500 mg/kg/day MBP or in controls. MBP had no significant effect on numbers of any germ cell type.
to di(n-butyl) phthalate on testicular germ cell development and proliferation. *Endocrinology* 2006;14:5352–5362.

Fisher JS, Macpherson S, Marchetti N, Sharpe RM. Human 'testicular dysgenesis syndrome': a possible model based on in utero exposure of the rat to dibutyl phthalate. *Hum Reprod* 2003;18:1383–1394.

Foster PM, Mylchreest E, Gaido KW, Sar M. Effects of phthalate esters on the developing reproductive tract of male rats. *Hum Reprod Update* 2001;7:231–235.

Gaido KW, Hensley JB, Liu D, Wallace DG, Borghoff S, Johnson KJ, Hall SJ, Boekelheide K. Fetal mouse phal TH I exposure shows that gonocyte multinucleation is not associated with decreased testicular testosterone. *Toxicol Sci* 2007;97:491–503.

Gaskell TL, Ensal A, Robinson LL, Anderson RA, Saunders PT. Immunohistochemical profiling of germ cells within the fetal human testis: identification of three subpopulations. *Biol Reprod* 2004;71:2012–2021.

Hallmark NJ, Walker M, McKinnell C, Mahood IK, Scott HS, 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.

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.

Hutchinson GR, Scott HM, Walker M, McKinnell C, Ferrara D, Mahood IK, Sharpe RM. Sertoli cell development and function in an animal model of testicular dysgenesis syndrome. *Biol Reprod* 2008;78:352–360.

Johnson KJ, Boekelheide K. Dynamic testicular adhesion junctions are immunologically unique. II. Localization of classic cadherins in rat testis. *Biol Reprod* 2002;66:992–1000.

Kai H, Shono T, Tajiri T, Suits S. Long-term effects of intruterine exposure to mono-n-butyl phthalate on the reproductive function of postnatal rats. *J Pediatr Surg* 2005;40:429–433.

Kelnar CJ, McKinnell C, Walker M, Morris KD, Wallace WH, Saunders PT, Fraser HM, Sharpe RM. Testicular changes during infancy ‘quiescence’ in the marmoset and their gonadotrophin dependence: a model for investigating susceptibility of the prepubertal human testis to cancer therapy? *Hum Reprod* 2002;17:1367–1378.

Kleyumenova E, Swanson C, Boekelheide K, Gaido KW. Exposure in utero to di(n-butyl) phthalate alters the vimentin cytoskeleton of fetal rat Sertoli cells and disrupts Sertoli cell-gonocyte contact. *Biol Reprod* 2005;73:482–490.

Lambrot R, Muczyński V, Lécureuil C, Angenard G, Coffigny H, Parault C, Moison D, Tedman R, Habert R, Roulleau-Fabre V. Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production. *Environ Health Perspect* 2008;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.

Lunn SF, Recio R, Morris K, Fraser HM. Blockade of the neonatal rise in testosterone by a gonadotrophin-releasing hormone antagonist: effects on timing of puberty and sexual behaviour in the male marmoset monkey. *J Endocrinol* 1994;141:439–447.

Lunn SF, Cowen GM, Fraser HM. Blockade of the neonatal increase in testosterone by a GnRH antagonist: the free androgen index, reproductive capacity and postmortem findings in the male marmoset monkey. *J Endocrinol* 1997;154:125–131.

Mahood IK, Hallmark N, McKinnell C, Walker M, Fisher JS, Sharpe RM. Abnormal Leydig cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. *Endocrinology* 2005;146:613–623.

Mahood IK, Scott HM, Brown R, Hallmark N, Walker M, Sharpe RM. In utero exposure to di(n-butyl) phthalate and testicular dysgenesis: comparison of fetal and adult end points and their dose sensitivity. *Environ Health Perspect* 2007;115(Suppl 1):55–61.

Marsee K, Woodruff TJ, Axelrad DA, Calafat AM, Swan SH. Estimated daily phthalate exposures in a population of mothers of male infants exhibiting reduced anogenital distance. *Environ Health Perspect* 2006;114:805–809.

McKinnell C, Collins PT, Fraser HM, Kelnar CJ, Kivlin C, Morris KD, Sharpe RM. Comparison of androgen receptor and oestrogen receptor beta immunoneoexpression in the testes of the common marmoset (Callithrix jacchus) from birth to adulthood: low androgen receptor immunoneoexpression in Sertoli cells during the neonatal increase in testosterone concentrations. *Reproduction* 2001;12:419–429.

Milar MR, Sharpe RM, Weinbauer GF, Fraser HM, Saunders PT. Marmoset spermatogenesis: organizational similarities to the human. *Int J Androl* 2000;23:266–277.

Mitchell R, Cowan G, Morris KD, Anderson RA, Fraser HM, Mckenzie KJ, Wallace WHB, Kelnar CJH, Saunders PTK, Sharpe RM. Germ cell differentiation in the marmoset (Callithrix jacchus) during fetal and neonatal life closely parallels that in the human. *Hum Reprod* 2008;23:2755–2765.

Mylchreest E, Cattley RC, Foster PM. Male reproductive tract malformations in rats following gestational and lactational exposure to Di(n-butyl) phthalate: an antandrogenic mechanism? *Toxicol Sci* 1998;43:47–60.

Mylchreest E, Wallace DG, Cattley RC, Foster PM. Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to Di(n-butyl) phthalate during late gestation. *Toxicol Sci* 2000;55:143–151.

Parks LG, Osbey 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.

Rajpert-de Meyts E. Developmental model for the pathogenesis of testicular carcinoma in situ: genetic and environmental aspects. *Hum Reprod Update* 2006;12:303–323.

Rhodes C, Elcombe CR, Batten PL, Bratt H, Jackson SJ, Pratt IS, Orton TC. The disposition of 14C-di-2-ethylhexylphthalate (DEHP) in the marmoset. *Dev Toxicol Environ Sci* 1983;1:579–581.

Rhodes C, Orton TC, Pratt IS, Batten PL, Bratt H, Jackson SJ, Elcombe CR. Comparative pharmacokinetics and subacute toxicity of di(2-ethylhexyl) phthalate (DEHP) in rats and marmosets: extrapolation of effects in rodents to man. *Environ Health Perspect* 1986;65:299–307.

Scott HM, Hutchinson GR, Mahood IK, Hallmark N, Welsh M, De Gendt K, Verhoeven G, O’Shaughnessy P, Sharpe RM. Role of androgens in fetal testis development and dysgenesis. *Endocrinology* 2007;148:2027–2036.

Scott HM, Hutchinson GR, Jobling MS, McKinnell C, Drake AJ, Sharpe RM. Relationship between androgen action in the ‘male programming window’, fetal Sertoli cell number, and adult testis size in the rat. *Endocrinology* 2008;149:5280–5287.

Sharpe RM, Skakkebaek NE. Male reproductive disorders and the role of endocrine disruption: advances in understanding and identification of areas for future research. *Pure Appl Chem* 2003;75:2023–2038.

Sharpe RM, Skakkebaek NE. Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. *Fertil Steril* 2008;89(Suppl 1):e33–e38.

Sharpe RM, Walker M, Millar MR, Morris K, McKinnell C, Saunders PTK, Fraser HM. Effect of neonatal GnRH antagonist administration on Sertoli cell multinucleation in the marmoset. *J Endocrinol* 2000;166:77–86.
cell number and testicular development in the marmoset: comparison with the rat. Biol Reprod 2000;62:1685–1693.
Sharpe RM, Martin B, Morris K, Greig I, McKinnell C, McNeilly AS, Walker M. Infant feeding with soy formula milk: effects on the testis and on blood testosterone levels in marmoset monkeys during the period of neonatal testicular activity. Hum Reprod 2002;17:1692–1703.
Sharpe RM, Fraser HM, Brougham MF, McKinnell C, Morris KD, Kelnar CJ, Wallace WH, Walker M. Role of the neonatal period of pituitary-testicular activity in germ cell proliferation and differentiation in the primate testis. Hum Reprod 2003;18:2110–2117.
Shono T, Suta S. Dose-dependent effect of phthalate ester on testicular descent in pre- and postnatal rats. Urol Res 2003;31:293–296.
Shono T, Kai H, Suta S, Nawata H. Time-specific effects of mono-n-butyl phthalate on the transabdominal descent of the testis in rat fetuses. BJU Int 2000;86:121–125.
Shono T, Shima Y, Kondo T, Suta 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.
Silva MJ, Reidy JA, Herbert AR, Preau JL Jr, Needham LL, Calafat AM. Detection of phthalate metabolites in human amniotic fluid. Bull Environ Contam Toxicol 2004;72:1226–1231.
Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 2001;16:972–978.
Swain A, Lovell-Badge R. A molecular approach to sex determination in mammals. Acta Paediatr Suppl 1997;423:46–49.
Swan SH. Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. Environ Res 2008;108:107–184.
Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon B, Ternand CL, Sullivan S et al., and the Study for Future Families Research Team. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect 2005;113:1056–1061.
Thompson CJ, Ross SM, Gaido K. Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism. Endocrinology 2004;145:1227–1237.
Welsh M, Saunders PTK, Fisken M, Scott HM, Hutchison GR, Smith LB, Sharpe RM. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. J Clin Invest 2008;118:1479–1490.

Submitted on March 5, 2009; resubmitted on April 27, 2009; accepted on May 6, 2009.

McKinnell et al.