

Mechanism of action of phthalate endocrine disruptors in male reproductive development

A thesis submitted to the University of Adelaide in fulfilment of the
requirements for the degree of Doctor of Philosophy

by

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Declaration

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* Ravinder Anand-Ivell, Kee Heng, Bettina Hafen, Brian Setchell, and Richard Ivell. Dynamics of INSL3 peptide expression in the rodent testis. *Biology of Reproduction*, 2009. **81**(3): p. 480-7.

Kee Heng

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Abstract

Phthalates are plasticizers, commonly found in cosmetics, food-wrapping, personal care products and medical devices, and have recently been associated with reduced anogenital distance in male infants, reduced testosterone and altered behavior in boys. In rodents, *in utero* phthalate exposure disrupts the development of the internal and external male reproductive phenotype in the progeny. Testicular Leydig cells may be the primary target of phthalate action, since they often exhibit abnormal aggregation and reduced insulin-like factor 3 (INSL3). Moreover, microarray studies suggest that they are amongst the earliest testicular cell types affected by phthalate treatment, though acute effects on mature cells appear to be minor. The overall aim of this project was to assess the effect of phthalate, with the synthetic non-steroidal estrogen, diethylstilbestrol (DES) as control, on Leydig cell differentiation.

First a time-resolved fluorescent immunoassay (TRFIA) for rodent INSL3, a specific marker of Leydig cell differentiation, was developed, which was highly specific for rat and mouse INSL3 in body fluids or in cell culture medium. Besides showing that INSL3 is a reliable marker for aging Leydig cells, it accurately reflected the differentiation of Leydig cells during puberty.

The second aim of this project assessed whether the kinetics of new Leydig cell differentiation following their ablation by ethane dimethane sulfonate (EDS) was affected by dibutyl phthalate (DBP) treatment at an early stage of Leydig cell regeneration. mRNA expression of Leydig cell markers such as LH receptor (LHR), cytochrome P450 17-alpha-hydroxylase/17,20 lyase (Cyp17a1) and 11-beta-hydroxysteroid dehydrogenase type 1 (Hsd11b1) in the DBP-treated animals were increased, likely due to an increase in cell

proliferation since Leydig cells in treated animals exhibited more clustering and higher numerical density compared to controls.

The long-term effect of DBP on the adult Leydig cell population following *in utero* and lactational exposure was also investigated since most studies have concentrated on acute early effects of phthalates. Maternal DBP treatment appeared to accelerate Leydig cell development, especially when Leydig cells are actively proliferating or differentiating, largely due to an increased proliferation of Leydig cells. The consequences of such treatment do not persist to adulthood, since circulating INSL3 as well as mRNA expression of various Leydig cell markers were comparable in all treatment groups at postnatal day 90 (PND90).

Finally, a long term *in vitro* model of Leydig cell differentiation was established, using undifferentiated adult-type Leydig cells isolated from day 10 rats. Preliminary experiments with DBP and its main metabolite, monobutyl phthalate (MBP), showed that both compounds were probably inhibitory to Leydig cell differentiation in culture in the presence of human chorionic gonadotropin (hCG). The precise mechanism by which these compounds slowed Leydig cell differentiation will be determined in future studies.

Collectively, the findings of this thesis strongly imply that differentiating/developing adult-type Leydig cells are indeed direct targets of endocrine disruption. This thesis has also demonstrated that the EDS model and an *in vitro* Leydig cell differentiation model will be very useful in delineating the underlying mechanism of phthalate action.

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Ravinder Anand-Ivell, Kee Heng, Bettina Hafen, Brian Setchell, and Richard Ivell. Dynamics of INSL3 peptide expression in the rodent testis. *Biology of Reproduction*, 2009. **81**(3): p. 480-7.

Article submitted to scientific journal

Kee Heng, Ravinder Anand-Ivell and Richard Ivell. The endocrine disruptors dibutyl phthalate (DBP) and diethylstilbestrol (DES) influence Leydig cell regeneration following ethane dimethane sulfonate (EDS) treatment of adult male rats. *International Journal of Andrology* (submitted June 2011).

Abstracts published in the proceedings of scientific meetings

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Kee Heng, Bettina Hafen, Malgorzata Kotula-Balak, Richard Ivell and Ravinder Anand-Ivell. Development of a model system to assess the regulation of Leydig stem cell

differentiation. 40th annual conference of the Society for Reproductive Biology (SRB), Adelaide, Australia, August 2009.

Ravinder Anand-Ivell, Kee Heng, Bettina Hafen, Brian Setchell and Richard Ivell. Postnatal development and dynamics of the secreted Leydig cell peptide hormone insulin-like peptide 3 (INSL3) in rodents. 40th annual conference of the Society for Reproductive Biology (SRB), Adelaide, Australia, August 2009.

Abstract arising not directly from the work in this thesis

Richard Ivell, Damien Hunter, Kee Heng, Navdeep Mann, and Ravinder Anand-Ivell. Maternal exposure to phthalate and/or diethylstilbestrol leads to long-term changes in hypothalamic gene expression and adult behavior in male and female offspring. 44th annual meeting of the Society for the Study of Reproduction (SSR), Oregon, USA, accepted for poster presentation in July 2011.

Abbreviations

3 α HSD	3-alpha-hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
AGD	Anogenital distance
AMH	Anti-Mullerian hormone
ALCs	Adult Leydig cells
AR	Androgen receptor
BBP	Benzyl butyl phthalate
BSA	Bovine serum albumin
b.w.	Body weight
cDNA	Complementary DNA
Chrna4	Nicotinic acetylcholine receptor alpha 4
CIS	Carcinoma in situ
Cyp11a1	Cytochrome P450 cholesterol side chain cleavage enzyme
Cyp17a1	Cytochrome P450 17-alpha-hydroxylase/17,20 lyase
CV	Coefficient of variation
Dax1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DBP	Dibutyl phthalate
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DES	Diethylstilbestrol
DHH	Desert hedgehog
DHT	Dihydrotestosterone
DiBP	Diisobutyl phthalate
DINP	Diisononyl phthalate
DMEM	Dulbecco's Modified Eagle Medium
DMP	Dimethyl phthalate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease

dNTP	Deoxyribonucleotide
DOTP	Dioctyl terephthalate
DPP	Dipentyl phthalate
E	Embryonic day
EDS	Ethane dimethane sulfonate
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
FLCs	Fetal Leydig cells
GATA4	GATA binding protein 4
GD	Gestation day
GnRH	Gonadotropin-releasing hormone
GTC	Guanidine Thiocyanate
H&E	Haematoxylin and eosin
hCG	Human chorionic gonadotropin
hpg	Hypogonadal
HPG	Hypothalamic-pituitary-gonadal
HPLC	High-performance liquid chromatography
HRP	Horse radish peroxidase
Hsd3b1	3-beta-hydroxysteroid dehydrogenase
Hsd11b1	11-beta-hydroxysteroid dehydrogenase type 1
Hsd17b3	17-beta-hydroxysteroid dehydrogenase 3
Hsd17b10	17-beta-hydroxysteroid dehydrogenase type 10
IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor-2
IgG	Immunoglobulin G
ILCs	Immature Leydig cells
INSL3	Insulin-like factor 3
i.p.	Intraperitoneal
JAK3	Janus Kinase 3
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MBP	Monobutyl phthalate
MC ₂ R	Melanocortin type 2 receptor
MEHP	Mono(2-ethylhexyl) phthalate

MNG	Multinucleated gonocyte
MQ	MilliQ
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NA	Numerical aperture
NE	Normalized expression
Nr4a1	Nuclear receptor subfamily 4 group A member 1
OD	Optical density
PAS	Periodic Acid Schiff's
PBR	Peripheral-type benzodiazepine receptor
PBS	Phosphate buffered saline
PDGFA	Platelet-derived growth factor A
PDGF-BB	Platelet derived growth factor BB
PDGFR α	Platelet-derived growth factor receptor α
PGCs	Primordial germ cells
PLCs	Progenitor Leydig cells
PND	Postnatal day
PPAR α	Peroxisome proliferator-activated receptor α
PVC	Polyvinyl chloride
RIA	Radioimmunoassay
RNA	Ribonucleic acid
Rps27a	Ribosomal protein S27a
RT-PCR	Reverse transcription polymerase chain reaction
RXFP2	Relaxin/insulin-like family peptide receptor 2
s.c.	Subcutaneous
SCO	Sertoli-cell only
SD	Sprague Dawley
SER	Smooth endoplasmic reticulum
Sf1	Steroidogenic factor 1
SLCs	Stem Leydig cells
SOX9	Sex-determining region Y-box containing gene 9
SRY	Sex-determining region Y
StAR	Steroidogenic acute regulatory protein
TDS	Testicular dysgenesis syndrome

Tfm	Testicular feminization
TMB	3,3',5,5'-tetramethylbenzidine
TRFIA	Time-resolved fluorescent immunoassay
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
Wt1	Wilms tumour 1

Units

°C	Degree Celsius
μl	Microlitre
μg	Microgram
μm	Micron / Micrometre
μsec	Microsecond
bp	Base pairs
cm	Centimetre
cps	Counts per second
g	Gram
×g	Relative centrifugal force
G	Gauge
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
L	Litre
M	Molar
mg	Milligram
min	Minute
mIU	Milli International Unit
ml	Millilitre
mm	Millimetre
mM	Millimolar
n	Number
ng	Nanogram
nm	Nanometer

nM	Nanomolar
pg	Picogram
rpm	Revolutions per minute
sec	Second
U	Unit

Chapter 1

Introduction

1.1 Gonadal development in male fetuses

Mammalian testes and ovaries develop from a common precursor, the undifferentiated, bipotential genital ridge. In mice, the genital ridge appears around embryonic day (E) E10 and remains morphologically undifferentiated until around E12 (Wilhelm & Koopman, 2006). During the indifferent stage, prior to sex determination, the primordial germ cells (PGCs), which arise within the base of the allantois, migrate into the genital ridge region between E10 and E11 in mice (Fig. 1.1) (McLaren, 2000). Once they reach the genital ridge, they continue to proliferate until they are enclosed within the developing testis or ovarian anlage (Tam & Snow, 1981). The decision to make a testis in the fetus depends on the expression of the sex-determining region Y (*Sry*) gene in the pre-Sertoli cells (Albrecht & Eicher, 2001; Koopman et al, 1991; Lovell-Badge, 1992). The activation of the *Sry* gene subsequently drives the differentiation of other testicular cell types such as germ cells and Leydig cells into their mature phenotypes. In the case where *Sry* is absent or not functional, the default pathway leading to ovary development is followed.

In mice, *Sry* expression is first detected on E10.5, reaching a maximum on E11.5 before declining by E12.5 (Bullejos & Koopman, 2001). The role of SRY protein is primarily to activate the protein expression of SRY-box containing gene 9 (SOX9) (Wilhelm & Koopman, 2006). SOX9 is a transcription factor that besides activating other downstream genes also binds to the enhancer upstream of the *Sox9* gene itself, forming a positive feedback loop for more SOX9 protein production during testis development. Since *Sry* is only expressed for a very short time, it is thought that SOX9 might be the pivotal factor for testis development. Indeed, transgenic mice studies showed that *Sox9* expression alone is sufficient to induce testis formation in the absence of *Sry* (Qin & Bishop, 2005; Vidal et al, 2001) and mutation in *Sox9* was found to cause sex reversal in the human (Foster et al,

1994). Besides *Sry* and *Sox9*, other genes such as Wilms tumour 1 (*Wt1*), steroidogenic factor 1 (*Sf1*) and *Dax1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) appear to be important in testis development since mutations in these genes often result in gonadal malformation or sex ambiguity (Englert, 1998; Ludbrook & Harley, 2004; Ozisik et al, 2002).

Following the differentiation of Sertoli cells, development of other testicular cells takes place. The fetal Leydig cells (FLCs) are the steroidogenic cells that reside in the interstitium of the fetal testis. Once they are fully differentiated, they secrete androgens that are essential for the development of the male accessory organs and external genitalia. Besides secreting androgens, the FLCs also secrete a peptide hormone, the insulin-like factor 3 (INSL3) that mediates the first transabdominal phase of testis descent around E14.5 to E18 in mice (Ivell & Bathgate, 2002; Nef & Parada, 1999). The second, so-called inguino-scrotal phase of testes descent is achieved largely under the influence of androgen by postnatal day 20 (PND20) in mice (Hutson, 1986).

The internal genitalia of males and females are mostly derived from the Wolffian duct and the Mullerian duct, respectively (Fig. 1.2). In males, Sertoli cells secrete the anti-Mullerian hormone (AMH) to cause Mullerian duct regression. After the regression of the Mullerian duct, the mature FLCs secrete androgens to develop the Wolffian duct into the epididymides, vasa deferentia and seminal vesicles. In addition, androgens are also essential for the differentiation of the external genitalia of males and brain masculinization (Sato et al, 2004). It is believed that the hypothalamic-pituitary-gonadal (HPG) axis slowly becomes established after the secretion of androgen by FLCs in the testis. However, how the HPG axis becomes established is completely unknown at the present time. The initial

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Figure 1.1: The migratory pathway of primordial germ cells (PGCs). (A) Schematic representation of the localization of PGCs (black dots) at the base of the allantois around the hindgut pocket in an embryonic day 8.5 (E8.5) mouse embryo. (B) Migration of PGCs along the hindgut, dorsal mesentery, and into the genital ridges in an E10.5 mouse embryo [reproduced as originally presented in (Wilhelm et al, 2007)].

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This figure is included on page 21 of the print copy of
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Figure. 1.2. Development and differentiation of the genital tract. At E11.5, a pair of bipotential gonads, fully formed Wolffian ducts and developing Mullerian ducts are present in both XX and XY mice embryos. Testis differentiation is initiated in XY gonads following activation of *Sry* and its downstream signaling pathway while ovarian development takes place in XX gonads in the absence of *Sry*. In males, the Mullerian ducts regress under the influence of AMH secreted by the Sertoli cells, whereas the Wolffian ducts differentiate into epididymides, vasa deferentia, and seminal vesicles under the control of androgens secreted by fetal Leydig cells. INSL3 produced by fetal Leydig cells induces the development of gubernaculum, facilitating the first phase of testicular descent during fetal life. In females, the Wolffian ducts degenerate in the absence of androgens and the Mullerian duct differentiates into oviduct, uterus, and upper vagina [reproduced as originally presented in (Griswold & Behringer, 2009)].

AMH: anti-Mullerian hormone; C: cervix; E: epididymis; Gu: gubernaculum; INSL3: insulin-like factor 3; K: kidney; Mes: mesonephros; MD: Mullerian duct; Od: oviduct; Ov: ovary; SV: seminal vesicle; T: testis; UB: ureteric bud; Ur: ureter; Ut: uterus; V: vagina; VD: vas deferens; WD: Wolffian duct.

androgen production in fetal rat or mouse testis does not appear to require luteinizing hormone (LH) since LH is first detected in plasma 3-4 days following first appearance of FLCs (Aubert et al, 1985; El-Gehani et al, 1998). Moreover, expression of the LH receptor (LHR) was first observed after the initiation of FLC differentiation (Gangnerau & Picon, 1987; Warren et al, 1984). In mice, there appears to be a requirement for another pituitary product, adrenocorticotrophic hormone (ACTH), reacting with the melanocortin type 2 receptor (MC₂R) on FLCs (O'Shaughnessy et al, 2003). This is consistent with a common origin of fetal Leydig and adrenocortical cells (Hatano et al, 1996). Moreover, a novel population of adrenal-like cells was previously identified in fetal mice testis (Val et al, 2006). However, whether ACTH is essential in the FLC development remains debatable since mice lacking ACTH also had normal fetal testosterone level (O'Shaughnessy et al, 2009).

Sexual differentiation is a complicated process that involves signalling pathways that are tightly regulated at different stages of the gonadal development. Due to the bipotential nature of the genital ridge, the active development of the genital ridge into a male gonad at a time when the placenta does not yet form a strongly protective barrier is particularly vulnerable to any insults such as mutations or environmental toxins; the default pathway for ovary development could always be “selected” if the pathway to become a male is compromised.

1.2 Testicular dysgenesis syndrome (TDS) and the possible role of endocrine disruptors in the aetiology of TDS

In recent years, an adverse trend in male reproductive health has been reported. Lower adult testosterone levels and poorer semen quality have been indicated in men from more

recent birth cohorts (Andersson et al, 2007; Auger et al, 1995; Carlsen et al, 1992; Irvine et al, 1996; Jorgensen et al, 2006; Travison et al, 2007). The rate of cryptorchidism and hypospadias in newborn boys appears to be rising in many Western countries (Nassar et al, 2007; Paulozzi, 1999; Toppari et al, 2001; Virtanen & Toppari, 2008). Furthermore, the occurrence of testicular cancer in young men is also increasing (Adami et al, 1994; Bergstrom et al, 1996; Bray et al, 2006; Moller et al, 1995; Richiardi et al, 2004). Cryptorchidism and hypospadias at birth, testicular cancer among young men and reduced sperm quality were proposed to be the symptoms of one underlying entity, the testicular dysgenesis syndrome (TDS) (Fig. 1.3), and are correspondingly thought to originate during fetal life (Skakkebaek et al, 2001). The hypothesis proposed that abnormal testis development could be caused by multiple factors, which subsequently affect the development and/or function of the Sertoli and/or Leydig cells, and in turn cause the manifestations of different reproductive disorders (Skakkebaek et al, 2001).

Cryptorchidism, the failure of testis descent into the scrotum, is the most common congenital birth defect affecting approximately 2-9% of newborn boys (Acerini et al, 2009; Boisen et al, 2004; Toppari et al, 2010; Virtanen & Toppari, 2008). Testicular descent into the scrotum normally occurs in a two-stage process (Hutson, 1985). The transabdominal stage of testicular descent is primarily facilitated by INSL3 acting on the gubernacular ligament connecting the gonad to the ventral body wall (Nef & Parada, 1999; Zimmermann et al, 1999), while androgen (mostly testosterone) is responsible for the inguino-scrotal stage of testicular descent (Foresta et al, 2008; Hutson, 1986; Klonisch et al, 2004). Hypospadias, the second most common congenital anomaly in male newborns, is a genital defect where the urinary meatus is misplaced on the penile shaft. The formation of the urinary meatus is largely androgen-dependent (Manson & Carr, 2003). These congenital abnormalities are often associated with compromised reproductive health and/or fertility

NOTE:

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Figure 1.3: Schematic representation of pathogenic links between the components and clinical manifestations of the testicular dysgenesis syndrome (TDS) [reproduced as originally presented in (Skakkebaek et al, 2001)].

later in life. Testicular cancer is a cancer that affects young men. It originates from the carcinoma in situ (CIS) cells, which are transformed gonocytes that fail to differentiate into spermatogonia (Skakkebaek et al, 1987). Cryptorchidism, hypospadias and testicular cancer have also been associated with low birth weight (Akre et al, 1999; Michos et al, 2007; Weidner et al, 1999). Clinical data showed that men who were born cryptorchid or with hypospadias have a greater risk of testicular cancer, and often have poorer semen quality (Lee & Coughlin, 2001; Moller et al, 1996; Virtanen et al, 2007).

The aetiology underlying TDS is not clear. Genetic mutations or polymorphisms appear to be responsible for some cases of TDS (Skakkebaek et al, 2001). The evidence that genetic background might also play a role in the aetiology of TDS came from a study in the USA where it was observed that African-Americans have a significantly lower incidence of testicular cancer than Caucasians living in the same areas of the USA (McGlynn et al, 2003). However, since TDS is increasing at a rapid pace, it suggests that additional factors such as environmental or lifestyle factors could be more important in the aetiology of TDS. Indeed, this was confirmed in epidemiological studies in different countries. Clear geographic difference in the occurrence of TDS has previously been reported, the best-cited example being the different incidences of TDS in the two Nordic countries, Denmark and Finland. It was previously difficult to compare the data reporting TDS rates from different countries since many of these studies used different approaches and selection criteria. Cryptorchidism and hypospadias was shown to be much more frequent in Denmark than in Finland, in a joint Danish-Finnish study where methodology was standardised (Boisen et al, 2005; Boisen et al, 2004; Virtanen et al, 2001). Healthy Danish boys were reported to have slower testis growth and lower serum inhibin B concentration than healthy Finnish boys during infancy (Main et al, 2006b). Slower testis growth during infancy in Danish boys further leads to lower testosterone and serum inhibin B level and

smaller testis size at adulthood in Danish men compared to Finnish men (Jorgensen et al, 2002). The importance of environmental factors was further illustrated by immigrant studies. The testicular cancer risk in first generation immigrants in Europe was similar to that in their countries of origin; while second generation immigrants have a risk that is similar to that in natives of the country to which they migrated (Hemminki & Li, 2002; Myrup et al, 2008).

The effect of environmental factors on reproductive health has been well demonstrated in wild animals. The spill of an estrogenic compound, dichlorodiphenyltrichloroethane (DDT), in Lake Apopka significantly suppressed the testosterone level in juvenile male alligators (Guillette et al, 1994). Estrogenic compounds from the sewage effluent were found to elevate the serum vitellogenin, an egg yolk precursor protein expressed otherwise uniquely in female fish, in male Carp (*Cyprinus carpio*) (Folmar et al, 1996). Serum testosterone was also significantly lower in these male fish (Folmar et al, 1996). At the Experimental Lakes Area in northwestern Ontario, Kidd et al (2007) showed experimentally that chronic exposure to low level of a semi-synthetic steroidal estrogen, 17 α -ethynylestradiol, was able to cause feminization of male fathead minnow (*Pimephales promelas*) and also alteration of oogenesis in the females. This experiment eventually led to a near extinction of this species in the lake after 7 years (Kidd et al, 2007).

Environmental compounds that have the ability to interfere with the endogenous hormonal system are collectively termed “endocrine disruptors” (IPCS, 2002). According to a World Health Organization (WHO) definition, an endocrine disruptor is an exogenous substance or mixture, mainly of man-made origin, that alters function of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations (IPCS, 2002). Endocrine disruptors are thought to exert their effects by

either mimicking an endogenous hormone or blocking the hormone function. Besides affecting fertility in wild animals, endocrine disruptors were also destructive to reproductive health in human population. Spouses of greenhouse workers who were exposed to a mixture of pesticides consisting of atrazine, benomyl-carbendazim, carbaryl and DDT had a higher chance for spontaneous abortion during pregnancy (Petrelli et al, 2003). Moreover, a prolonged time to first conception was reported in male greenhouse workers exposed to various pesticides (Bretveld et al, 2008).

1.3 Disruption of male gonadal development by phthalates

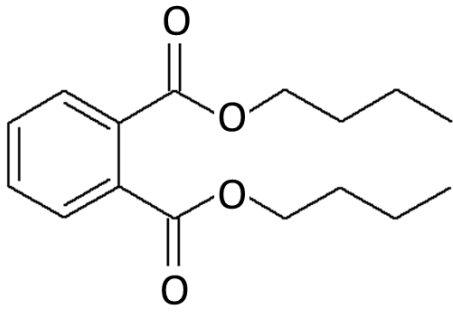
Phthalates have been used extensively as plasticizing agents in polyvinyl chloride (PVC), cosmetics, food-wrapping, personal care products and medical devices (Wormuth et al, 2006). As they are not chemically bound to the polymer and are able to leach into the environment, we are constantly exposed to these compounds in our daily life. Exposure to phthalates can occur via dermal contact, ingestion, inhalation and also intravenous routes. Phthalates are non-persistent chemicals that have short biological half-lives and are rapidly metabolized into monoesters and excreted in urine (Silva et al, 2006; Silva et al, 2007). Various phthalate monoester metabolites have been detected in the human population in urine (Hauser et al, 2007; Weuve et al, 2006), breast milk (Krysiak-Baltyn et al, 2010; Main et al, 2006a), amniotic fluid (Silva et al, 2004) and blood samples (Kato et al, 2004). Infants who received treatment in a neonatal intensive care unit were found to have significantly higher phthalate exposure compared to those who did not (Weuve et al, 2006). Women of child-bearing age (20-40 years of age) have particularly high levels of urinary phthalate metabolites, possibly resulting from the usage of cosmetics in their daily life (Blount et al, 2000). In addition, phthalate metabolites have been detected in cord blood,

suggesting that phthalate exposure in fetuses during pregnancy is directly correlated with exposure of the mothers (Latini et al, 2003).

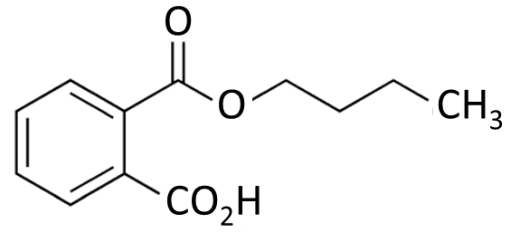
Recently, phthalates are identified as one of the endocrine disruptors, which have the ability to impair the function of the intrinsic endocrine system, as many studies in rodent models were able to show the adverse effect of phthalates on male reproductive development, with phenotypes similar to TDS (except testicular cancer). Whether phthalates can truly affect human reproductive health remains debatable, due to a lack of a direct causal link. Nevertheless, several human studies suggested an important role of phthalates in male reproductive health. Swan et al (2005) reported a significant correlation between shorter anogenital distance (AGD) in newborn boys and high maternal phthalate metabolites in urine (Swan et al, 2005). Data from rodent studies suggested that infants are particularly susceptible to phthalate effects. In fact, a higher level of the dibutyl phthalate (DBP) metabolite, monobutyl phthalate (MBP) (Fig. 1.4), has been associated with lower free serum testosterone levels in infant boys at 3 months of age (Main et al, 2006a). Whether urinary phthalate metabolites are negatively correlated with semen quality and reproductive hormone levels at adulthood remains to be confirmed since findings from different studies have been inconclusive (Duty et al, 2004; Duty et al, 2005; Duty et al, 2003; Hauser et al, 2006; Hauser et al, 2007; Meeker et al, 2009; Murature et al, 1987).

1.3.1 Critical window of exposure

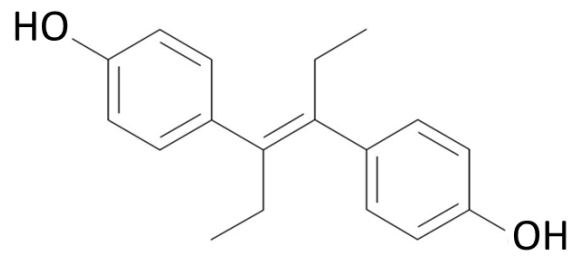
Studies in rats repeatedly showed that the male fetuses or neonates are most susceptible to the detrimental effect of phthalates (Akingbemi et al, 2001; Parks et al, 2000). However, not all phthalate esters are equally toxic to the reproductive system. *In utero* exposure to high doses (~500mg/kg body weight) of di(2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BBP), DBP or diisononyl phthalate (DINP), but not diethyl phthalate (DEP),



Dibutyl phthalate (DBP)



Monobutyl phthalate (MBP)



Diethylstilbestrol (DES)

Figure 1.4: Chemical structures of the endocrine disruptors used in this thesis.

dimethyl phthalate (DMP), or dioctyl terephthalate (DOTP), were shown to alter sexual differentiation and testicular function in the male offspring (Foster et al, 2001; Gray et al, 2000). Prenatal exposure to phthalates in rats during the “male programming window” (E15.5 to E18.5) could cause phenotypes similar to TDS (Welsh et al, 2008). The reason why phthalates are more disrupting to young animals is thought to be because the gonads and the endocrine system are not fully established and have not attained their mature phenotype at that stage of development. Male pups with *in utero* exposure to high doses of phthalates displayed malformations such as cryptorchidism, hypospadias, reduced AGD, reduced testis weight, and retained nipples (Akingbemi et al, 2001; Ema et al, 2003; Foster et al, 2001; Gray et al, 2000; Howdeshell et al, 2007; Parks et al, 2000; Sharpe et al, 1995). On the contrary, exposure of phthalates to adult rats resulted in either limited or no effect on testicular function (Akingbemi et al, 2001). In addition to the timing of administration, dosage of phthalates also determines its effect on sexual differentiation and testicular function. *In utero* exposure to a relatively low dose (10mg/kg body weight) of DEHP significantly advanced the onset of puberty in male offspring while male progeny in a high dose (750mg/kg body weight) group had delayed puberty onset (Ge et al, 2007).

1.3.2 Primary target and mechanism of action of phthalates

Despite the effort put into phthalate research, the main question still remains unresolved. It is still not clear where phthalates are acting primarily. Germ cells, Sertoli cells and the interstitial Leydig cells have been implicated to be targets of phthalate action. *In utero* exposure of DBP was reported to cause the formation of multinucleated gonocytes (MNGs) (Kleymenova et al, 2005; Mylchreest et al, 1999; Mylchreest et al, 2002); poor Sertoli cell-gonocytes contact (Kleymenova et al, 2005) and reduced steroidogenesis by the Leydig cells (Shultz et al, 2001; Thompson et al, 2005). Recently, more studies are suggesting Leydig cells as the primary target of phthalates. It has been shown that *in utero* exposure of

phthalates such as DBP and DEHP reduced testosterone production and the *Insl3* messenger RNA (mRNA) level in fetal rat testes (Akingbemi et al, 2001; Borch et al, 2006; Parks et al, 2000; Wilson et al, 2004). In addition, genes involved in steroidogenesis have also been shown to be down-regulated by phthalates (Barlow et al, 2003; Lehmann et al, 2004; Parks et al, 2000; Plummer et al, 2007; Thompson et al, 2004; Thompson et al, 2005).

In addition to the altered gene expression by Leydig cells, *in utero* exposure to phthalates also caused the formation of abnormal Leydig cell clusters in the fetal testis (Mahood et al, 2005; Mahood et al, 2006). These clusters are thought to be due to abnormal translocation of Leydig cells. In affected animals, the Leydig cell clusters appeared to lead to dysgenetic areas in the testis where Sertoli cells as well as Leydig cells were enclosed within the seminiferous tubules (Mahood et al, 2005). Other groups have also observed Leydig cell aggregation (Akingbemi et al, 2004; Parks et al, 2000). However, in contrast to Mahood et al (2006), the clusters were due to over-proliferation of Leydig cells after phthalate exposure.

In rats, *in utero* exposure to phthalates repeatedly caused agenesis of male organs whose development is androgen-dependent. The phenotypes induced by phthalates include epididymal agenesis, hypospadias, reduced AGD and cryptorchidism (Mylchreest et al, 1999). Although phthalates cause an anti-androgenic phenotype, its action is not androgen-dependent. This conclusion is derived from a number of studies that employed an androgen receptor antagonist, flutamide. Flutamide was found to block the second phase of testes descent which is androgen-dependent but DBP also affected the first phase of testes descent, which is *INSL3*-dependent (Mylchreest et al, 1999). In addition, flutamide did not decrease testosterone production (Mylchreest et al, 2002). Therefore, DBP must be acting

somewhere upstream of androgen signalling. The phenotypes induced were due to an overall reduction in testosterone production. It is unlikely that phthalates act through the classical nuclear receptor pathway as they have been shown to be only very weak activators or without effect in transcriptional activation assays with androgen or estrogen receptors (Harris et al, 1997; Sohoni & Sumpter, 1998; Takeuchi et al, 2005).

In addition to the *in vivo* studies, direct but modest effects of phthalates on hCG-stimulated steroidogenesis have also been demonstrated *in vitro* in primary rat Leydig cell cultures and mouse Leydig tumour cell lines at concentrations in the low micromolar range (Dees et al, 2001; Ge et al, 2007; Jones et al, 1993; Wang et al, 2006; Wang et al, 2007). Alteration to mitochondrial structures and increase in lipid droplets were previously observed in primary rat Leydig cells and MA-10 cells following treatment with the active metabolite of DEHP, mono(2-ethylhexyl) phthalate (MEHP), however only at a high concentration of around 1mM (Dees et al, 2001; Jones et al, 1993).

It has been difficult to delineate the mechanism of action of phthalates because no protein has been found to have a strong interaction with phthalates. Current evidence suggests that the phthalates might act via multiple pathways. Phthalates have been shown to activate peroxisome proliferator-activated receptor α (PPAR α) *in vitro* (Bility et al, 2004; Hurst & Waxman, 2003). However, the PPAR α -mediated pathway alone is not sufficient to elicit the effects of phthalates since testicular toxicity could still be observed in PPAR α -knockout mice (Ward et al, 1998). MEHP was able to cause a modest inhibition on hCG-stimulated steroidogenesis in primary rat Leydig cells and MA-10 cultures, possibly through the suppression of peripheral-type benzodiazepine receptor (PBR), a mitochondrial protein involved in regulation of cholesterol transport across the mitochondrial membranes (Gazouli et al, 2002). Moreover, *in utero* dexamethasone treatment was shown to augment

DBP effects on the reduction of AGD at birth and reproductive parameters such as plasma testosterone, testis weight and penile length at adulthood, suggesting that DBP action might be mediated through a glucocorticoid pathway (Drake et al, 2009).

More recently, researchers have started looking at the effect of endocrine disruptor mixtures on male reproductive development since the human population is more likely to be constantly exposed to a variety of xenobiotics (Kortenkamp, 2008). Low doses of chemicals that singly do not cause adverse reproductive effects, when administered as a mixture with other endocrine disruptors, could result in a cumulative, high incidence of genital anomalies in the progeny (Christiansen et al, 2008; Hass et al, 2007; Hotchkiss et al, 2004). Howdeshell et al (2007) reported that *in utero* exposure to a mixture of DBP and DEHP, two phthalate esters that appeared to act in similar way, could cause genital malformations and reduction in INSL3 and testosterone level in male offspring in a dose-additive manner (Howdeshell et al, 2007). Similar cumulative, dose-additive effects of phthalates were also observed in a study utilising a mixture of five phthalate esters containing DBP, DEHP, BBP, DiBP and dipentyl phthalate (DPP) (Howdeshell et al, 2008).

1.4 Effect of diethylstilbestrol (DES) on male reproductive development

Diethylstilbestrol (DES) (Fig. 1.4) is a synthetic non-steroidal estrogen that was prescribed to pregnant women from the 1940s to the 1970s under the misbelief that it could prevent pregnancy complications and miscarriages (McLachlan, 2006). Its use in pregnant women was later banned following the report of rare occurrence of vaginal carcinoma in young ladies exposed to DES *in utero* (i.e. DES daughters) (Herbst et al, 1971). The *in utero* DES

effect in women has been well studied. In addition to causing abnormalities in the female reproductive system, prenatal DES treatment was also found to cause reproductive tract anomalies in men. A higher incidence of cryptorchidism, hypoplastic testes and epididymal cysts were reported in men exposed to DES prenatally (i.e. DES sons) (Bibbo et al, 1977; Conley et al, 1983; Gill et al, 1977; Gill et al, 1979; Whitehead & Leiter, 1981). The studies on fertility in DES-exposed men have produced mixed results (Bibbo et al, 1977; Gill et al, 1979; Leary et al, 1984; Wilcox et al, 1995). Fertility in these men appeared to depend on the dosage and timing of the DES treatment that their mothers received during pregnancy. In addition, whether prenatal DES increases the risk of testicular cancer remains to be elucidated, since the results from various studies have been inconclusive (Depue et al, 1983; Gershman & Stolley, 1988; Moss et al, 1986; Schottenfeld et al, 1980). A more recent study reported an increase, however non-significant, in the incidence of testicular cancer among men exposed to DES prenatally (Strohsnitter et al, 2001). A possible transgenerational effect of DES was reported as males born to DES daughters appeared to be at a greater risk of having hypospadias (Brouwers et al, 2006; Kalfa et al, 2011; Klip et al, 2002).

Phenotypes similar to TDS can be reproduced in rat or mouse models following gestational or neonatal exposure to DES. Comparable to what has been observed in human DES sons, male rodents exposed to DES during gestation or the neonatal period were found to exhibit cryptorchidism (Emmen et al, 2000), lower testis weight (McKinnell et al, 2001) and reduced fertility (Goyal et al, 2003). Most of the DES effects reported in these studies were short term effects since the animals were sacrificed shortly after the treatment. Several studies showed that the effect of DES treatment during gestation or in the neonatal period in rodents could be permanent since fertility, sperm production and expression of steroidogenic enzymes (e.g. cytochrome P450 cholesterol side chain cleavage enzyme

(Cyp11a1) and steroidogenic acute regulatory protein (StAR)) in these animals was severely compromised also at adulthood, long after the cessation of treatment (Atanassova et al, 2000; Fielden et al, 2002).

The exact mechanism how DES induces male reproductive malformation in rodents is not clear. Several studies suggested an estrogen receptor α (ER α)-dependent mechanism since most of the DES effects on the male reproductive tract could be avoided in ER α knockout mice (Cederroth et al, 2007; Goyal et al, 2007). This was further supported by a study using mice testis culture where DES was able to decrease testosterone production in wild type fetal and neonatal testis but not in the ER α knockout testis (Delbes et al, 2005). Other studies have suggested the importance of androgen-estrogen balance during the development of the male reproductive system, since abnormalities of the male reproductive tract induced by DES in neonatal rats could be prevented by co-administration with testosterone (McKinnell et al, 2001; Rivas et al, 2002; Rivas et al, 2003).

It appears as if both germ cells and somatic cells within the testis are potential targets for DES. In E14.5 mice fetal testis culture, DES treatment was able to decrease the numbers of germ cells, Sertoli cells and Leydig cells and also suppress the testosterone production (Lassurquere et al, 2003). Neonatal DES exposure was previously reported to decrease Sertoli cell number in both rats and mice (Fielden et al, 2002; Sharpe et al, 1998). Reduction in testicular Insl3 mRNA level was observed in fetal mice testis following *in utero* DES exposure (Emmen et al, 2000). A decrease of Leydig cell volume and androgen receptor immunoexpression in rat testis was reported following neonatal DES treatment (McKinnell et al, 2001). In addition to its prenatal and neonatal effects, DES was reported to affect pubertal rats by causing a tubular atrophy, dose-dependent reduction of germ cells, testis weight and testosterone level (Shin et al, 2009). More recently, a reduction of

Cyp11a1 mRNA expression was induced by DES in the Leydig cell line TTE-1 (Warita et al, 2010).

Although we do not understand fully how DES brings about its overt effects in male reproductive development, it is apparent from both human and animal studies that perturbation of hormonal systems during an early developmental stage could have long term detrimental outcomes.

1.5 Leydig cell populations and their maturation

Leydig cells are the steroidogenic cells localised in the interstitium of the testis that produce testosterone and the peptide hormone INSL3 when they become fully differentiated. In most of the mammalian species studied so far, two distinct populations of Leydig cells are present within the testis during prenatal and postnatal life, namely the fetal and adult Leydig cells (ALCs) (Ge & Hardy, 2007; Mendis-Handagama & Ariyaratne, 2001; Yao & Barsoum, 2007). However, in human, there appears to be an additional population of Leydig cells during the neonatal period, the infantile Leydig cells (Prince, 2001). Since Leydig cell differentiation has been extensively studied in mice and rats, differentiation of both FLCs and ALCs described below will concentrate on findings from mouse and rat models.

1.5.1 Fetal Leydig cells (FLCs)

At present, the primary source of FLCs remains unclear. Current evidence suggests that FLC might arise from a mixed population of Sf1-positive progenitor cells that migrate from different origins which include the coelomic epithelium (Karl & Capel, 1998), mesonephros (Merchant-Larios & Moreno-Mendoza, 1998; Nishino et al, 2001) and

possibly the neural crest (Mayerhofer et al, 1996). In addition, it is commonly believed that FLCs and adrenal cortex cells share common precursors that originate from the cranial end of the mesonephros (Hatano et al, 1996).

The exact differentiation process of FLCs is not completely understood, it still remains unclear what triggers the initial differentiation of FLCs. Although recombinant LH was shown to increase FLC number and LH-stimulated testosterone production in fetal rat testis culture (Migrenne et al, 2001), LH is unlikely to be essential in regulating the differentiation of FLCs *in vivo* for the following reasons (Fig. 1.5): (1) LH is only detected in plasma on E17.5, around 3-4 days following the first appearance of FLCs in rats (Aubert et al, 1985; El-Gehani et al, 1998); (2) FLC differentiation precedes the expression of the LHR (Gangnerau & Picon, 1987; Warren et al, 1984); (3) Expression of various FLC markers or circulating testosterone appear to be unaffected in hypogonadal mice and LHR knockout mice (Balvers et al, 1998; O'Shaughnessy et al, 1998; Zhang et al, 2001).

Since 3-beta-hydroxysteroid dehydrogenase (Hsd3b1)-positive FLCs were first observed soon after the differentiation of Sertoli cells at around E13.5 in rats (Gangnerau & Picon, 1987), it is believed that paracrine factors secreted by Sertoli cells might initiate the differentiation of FLCs. Indeed, platelet-derived growth factor A (PDGFA) and desert hedgehog (DHH) secreted by Sertoli cells have been found to be involved in FLC differentiation. A marked reduction in FLC differentiation was observed in platelet-derived growth factor receptor α (PDGFR α) knockout XY gonads at E12.5, indicated by very low or absence of Cyp11a1 expression (Brennan et al, 2003). Similarly, Cyp11a1 expression was also markedly reduced in DHH knockout XY gonads on E14.5 (Yao et al, 2002).

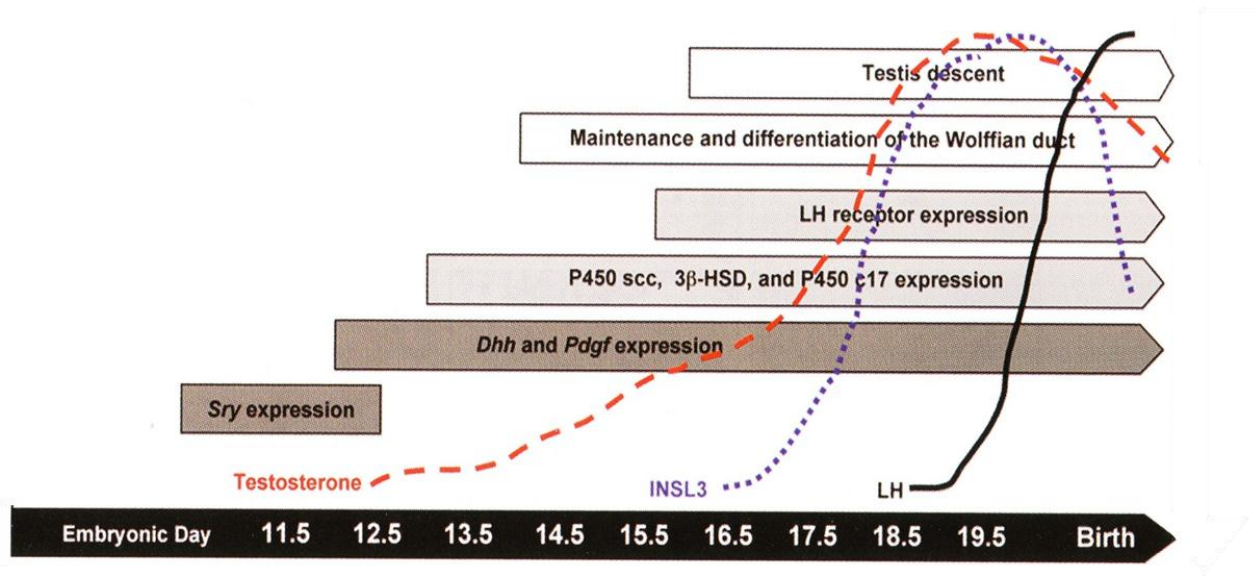


Figure 1.5: Developmental landmarks in fetal testes of rats and mice from E11.5 to birth. Sertoli cell differentiation is initiated following *Sry* activation between E10.5 and E12.5. Differentiated Sertoli cells express DHH and PDGF to regulate fetal Leydig cell (FLC) development. . At E13.5, FLCs begin to express steroidogenic enzymes, including Cyp11a1 (also known as P450scc), Hsd3b1 (or 3β-HSD), and Cyp17a1 (also known as P450c17), and produce androgens. Androgen levels in rats peak just before birth and decline thereafter. The expression of INSL3 starts around E15.5 and declines sharply after birth. LHR is expressed on FLCs from around E15.5 onwards. Since circulating LH only becomes detectable just before birth, it suggests that the initial differentiation of FLCs is independent of LH. The levels of testosterone, INSL3 and LH are arbitrary and should not be used for comparison between the hormones [reproduced as originally presented in (Ge & Hardy, 2007)].

Testosterone production is first detectable at E15.5 in rats and increases thereafter to reach a peak just before birth (Habert & Picon, 1984). The main role of the FLCs is to produce testosterone and INSL3 during fetal life to promote the development of male internal and external genitalia and testes descent, as well as for masculinizing the brain (see section 1.1 for more details).

1.5.2 Adult Leydig cells (ALCs)

The fate of FLCs after birth has been debated for many years. Study in the human testis showed that most FLCs involute after birth (Codesal et al, 1990). In rats, FLCs were shown to persist in the testis for around 2 weeks after birth before their number decreases to around 50% at adulthood (Kerr & Knell, 1988). Although FLCs are observed in the postnatal testis, various studies have conclusively shown that FLCs do not differentiate into ALCs (Haider, 2004; Kerr & Knell, 1988). In addition, FLCs are unlikely to contribute to testosterone production in the postnatal testis (Codesal et al, 1990; Kerr & Knell, 1988; Risbridger et al, 1989). The ALCs are thought to arise from a population of mesenchymal stem cells distinct from those that give rise to the FLC population. The development of the ALC population has been divided into 5 stages (Fig. 1.6): (1) Stem Leydig cells (SLCs); (2) Progenitor Leydig cells (PLCs); (3) Newly-formed Leydig cells; (4) Immature Leydig cells (ILCs); and (5) Mature ALCs (Mendis-Handagama & Ariyaratne, 2001).

Once they are differentiated, the main function of mature ALCs is to produce testosterone postnatally to promote puberty and maintain the male phenotype. INSL3 is also produced by mature ALCs towards puberty. The postnatal function of INSL3 is not clear, however, various studies have suggested a role in germ cell survival and bone metabolism (Ivell & Anand-Ivell, 2009).

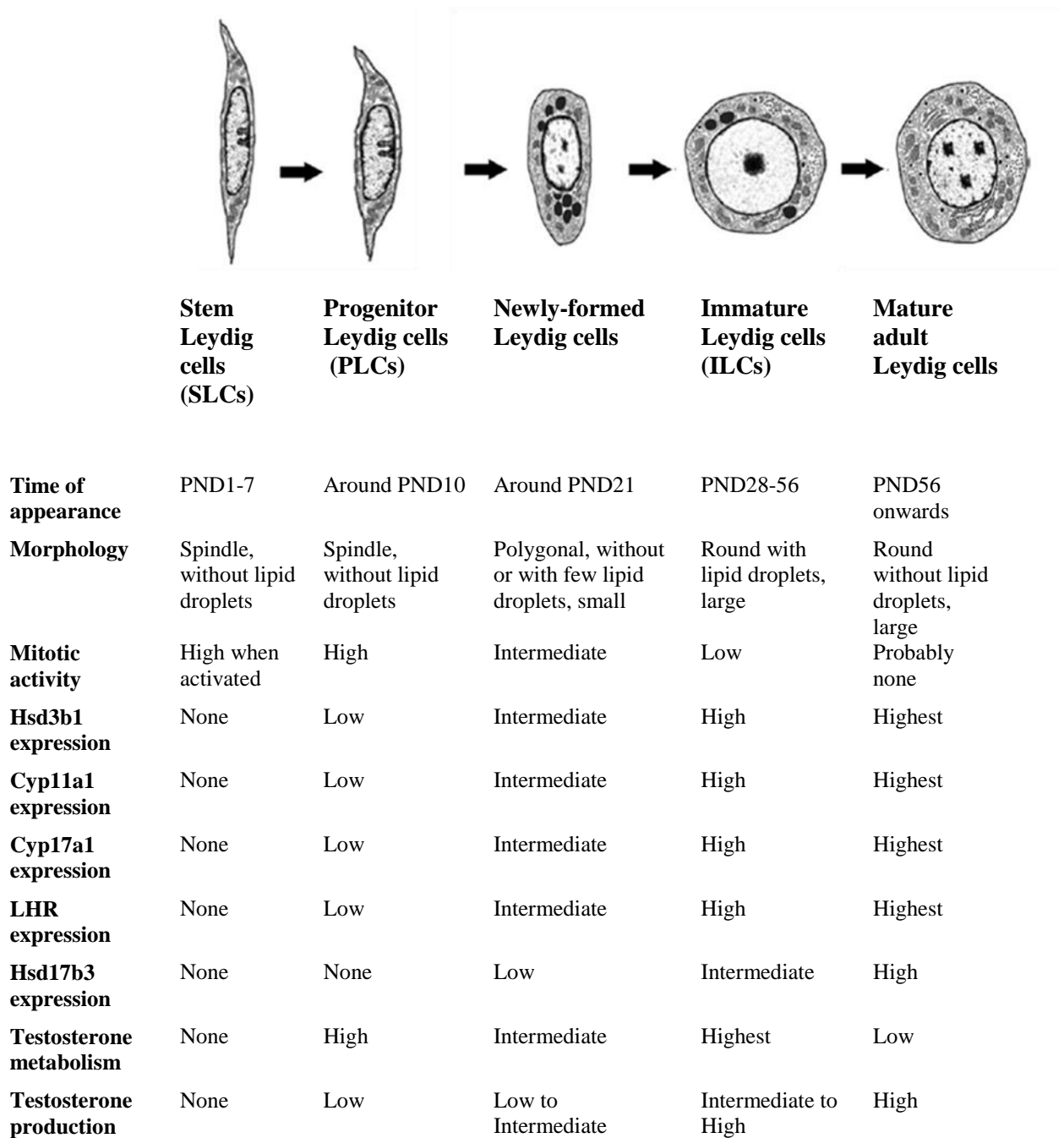


Figure 1.6: Developmental characteristics of adult Leydig cell lineage [modified from (Chen et al, 2010; Ge & Hardy, 2007; Mendis-Handagama & Ariyaratne, 2001)].

1.5.2.1 Stem Leydig cells (SLCs)

The adult population of Leydig cells have always been thought to originate from spindle-shaped mesenchymal stem cells that are either of peritubular (Ariyaratne et al, 2000a) or perivascular origin (Davidoff et al, 2004). Recently, SLCs have been isolated from 7-day-old rat testis (Ge et al, 2006). These cells had the ability for self-renewal, as well as committing into the Leydig cell lineage in the presence of differentiating medium. When these cells were transplanted into the rat testes, they were able to differentiate *in vivo*. The SLCs isolated were spindle-shaped, Hsd3b1-negative, LHR-negative, PDGFR α -positive. They also contained proteins known to be involved in LC development, including GATA binding protein 4 (GATA4), c-kit receptor, and leukemia inhibitory factor receptor (Ge et al, 2006).

1.5.2.2 Progenitor Leydig cells (PLCs)

Following commitment of SLCs into the Leydig cell lineage, PLCs are observed around PND10-11 in the rat testis. PLCs have a spindle shape morphology which is similar to SLCs. As SLCs differentiate into PLCs, the PLCs first gain the expression of Hsd3b1 followed by expression of Cyp11a1 and cytochrome P450 17 α -hydroxylase/17,20 lyase (Cyp17a1) (Ariyaratne et al, 2000a). The presumed functional LHR was also detected by immunohistochemistry in PLCs, although at a later time at around PND12 in rats (Ariyaratne et al, 2000a). As suggested in studies from the EDS rat model where mature ALCs were ablated specifically by a single dose of EDS (Teerds & Rijntjes, 2007), it appears as if the LHR initially expressed in regenerating PLCs are of the truncated form and may not be functional (Tena-Sempere et al, 1994). This is also confirmed since PLCs are insensitive to LH (Shan & Hardy, 1992). Since there is a lack of 17-beta-hydroxysteroid dehydrogenase 3 (Hsd17b3) expression in PLCs, androstenedione

produced is quickly metabolized to androsterone as the major form of androgens produced by these cells.

1.5.2.3 Newly formed adult Leydig cells

The newly formed ALCs arise when the PLCs change their morphology from spindle shape to polygonal. As the newly formed ALCs differentiate, they move away from their peritubular origin towards the centre of the interstitial lacunae. The cytoplasm of newly formed ALCs is sparse and with few lipid droplets. These cells express a high level of steroidogenic enzymes and also LHR. The expression of 11-beta-hydroxysteroid dehydrogenase type 1 (Hsd11b1) is observed in these cells only from PND21 onwards. Testosterone is first expressed in rats when newly formed ALCs are differentiated, although these cells still express higher amounts of another androgen, androstenedione.

1.5.2.4 Immature Leydig cells (ILCs)

ILCs are typically observed in the rat testis around PND28-56 (Ge & Hardy, 2007; Mendis-Handagama & Ariyaratne, 2001). The size of ILCs increases and they become rounder as they become more differentiated. A significant concentration of lipid droplets is observed in the cytoplasm of these cells. They stain strongly for various steroidogenic enzymes such as Hsd3b1, Cyp11a1, and Cyp17a1. With the acquisition of Hsd17b3 expression, ILCs secrete testosterone as well as other androgens (Fig. 1.7). Compared to newly formed LCs, ILCs exhibit a higher level of LHR binding and also express Hsd11b1.

1.5.2.5 Mature adult Leydig cells

Finally, differentiation of adult Leydig cells is completed when mature ALCs are formed around PND56 (Hardy et al, 1989). The mature ALCs are bigger in size and have fewer lipid droplets and contain abundant smooth endoplasmic reticulum (SER) compared to the

NOTE:

This figure is included on page 44 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.7: *De novo* androgen synthesis pathway in Leydig cells [reproduced as originally presented in (Griswold & Behringer, 2009)].

17 α OH P5: 17 α -hydroxy pregnenolone; 17 α OH P4: 17 α -hydroxy progesterone; Hsd17b3: 17-beta-hydroxysteroid dehydrogenase 3; Hsd3b1: 3-beta-hydroxysteroid dehydrogenase; 5 α -red: 5 α -reductase; DHEA: dehydroepiandrosterone; DHT: dihydrotestosterone; Arom: cytochrome P450 aromatase; Cyp17a1: cytochrome P450 17 α -hydroxylase/C17,20 lyase; Cyp11a1: cytochrome P450 cholesterol side chain cleavage enzyme.

ILCs (Zirkin & Ewing, 1987). Since a higher number of LHR are observed in these cells (Shan & Hardy, 1992), it follows that the mature ALCs become more dependent on circulating LH for their functionality and differentiation status. Testosterone is the major form of androgen secreted by the mature ALCs due to the higher level of Hsd17b3 and lower level of testosterone metabolizing enzymes such as 3 alpha-hydroxysteroid dehydrogenase (3 α HSD) compared to the ILCs (Shan et al, 1993). At sexual maturity, there are around 25 million mature ALCs per testis in rats (Ariyaratne & Chamindrani Mendis-Handagama, 2000; Hardy et al, 1989), together producing up to 10 mg testosterone per day (Handelsman, 2001).

1.5.2.6 Regulation of ALC development

The development of ALCs is governed by multiple factors, including circulating hormones and local paracrine factors, at different stages of the differentiation process. Unlike in FLCs, LH is suggested to be one of the most important regulators of ALC maturation and function. The onset of ALC differentiation is most likely to be independent of the action of LH since the circulating LH level is very low during PND10-11 when SLCs first proliferate and differentiate into PLCs (Dohler & Wuttke, 1975; Lee et al, 1975). In addition, the expression of LHR is absent in SLCs (Ge et al, 2006) and LHR expression was only observed around PND12 in rats following the expression of steroidogenic enzymes in the PLCs (Ariyaratne et al, 2000a). However, following the onset of differentiation, the development of ALCs becomes critically dependent on LH (Baker & O'Shaughnessy, 2001). The cell volume and number of Leydig cells were comparable in hypogonadal and control mice from birth until PND5 (Baker & O'Shaughnessy, 2001). Both parameters of Leydig cells were found to be significantly reduced in hypogonadal (hpg) mice, which have a defective HPG axis due to a deletion in the gonadotropin-releasing hormone (GnRH) gene, from PND20 onwards and at adulthood (Baker &

O'Shaughnessy, 2001). These results once again confirm the essential role of LH in the later stages of Leydig cell development.

With the possible exception of SLCs, all the other stages of ALCs express the androgen receptor (AR), with highest and lowest expression observed in ILCs and mature ALCs, respectively (Bremner et al, 1994; Shan & Hardy, 1992; Shan et al, 1995). The presence of AR in ALCs suggests that androgen might play a role in the regulation of Leydig cell maturation. The *in vitro* differentiation of rat ALCs requires both dihydrotestosterone (DHT) and LH (Hardy et al, 1990). In the testicular feminization (Tfm) mice where AR is absent, there was a considerably lower number of ALCs at adulthood (O'Shaughnessy et al, 2002). In addition, these Leydig cells failed to express markers of mature ALCs compared to both normal and cryptorchid controls (Murphy et al, 1994; Murphy & O'Shaughnessy, 1991; O'Shaughnessy et al, 2002), suggesting an incomplete maturation of ALCs in the absence of androgen action.

Although it has been well documented that various xenoestrogens may have inhibitory or adverse effects on testicular function, the effect of estrogen on Leydig cell development is not completely understood. A single injection of estrogen in immature rats on PND5 prevented the maturation of Leydig cells in immature rats of 60 days of age (Dhar & Setty, 1976). The effect of estrogen on the regenerating Leydig cells in the EDS rat model appears to depend on the timing of estrogen treatment. Administration of estrogen in the EDS-treated rats during days 0-5 and 5-30 following EDS treatment had no effect on the regeneration process of the ALC population in the testis (Abney & Myers, 1991). However, ALC regeneration was blocked when daily estrogen treatment was given from days 5 to 30 post EDS (Abney & Myers, 1991), suggesting that development of SLCs into PLCs might be most sensitive to estrogen action. Another supporting evidence to this

notion is that the mRNA expression of estrogen receptor ER α is highest in PLCs compared to mature ALCs (Zhai et al, 1996). ER α is the predominant subtype of estrogen receptor expressed in ALCs (Fisher et al, 1997). Whether another subtype of estrogen receptor, estrogen receptor β (ER β), is expressed in ALCs is still not clear. Studies in adult rat and human testes showed that ER β is only expressed in germ cells and Sertoli cells (Saunders et al, 2001). Its expression in Leydig cells was mostly limited to the fetal testis (Saunders et al, 1998; Saunders et al, 1997). Hence, estrogen most probably exerts its effect through ER α in ALCs.

As observed in both the EDS model and in prepubertal rats, the differentiation of ALCs is inhibited by experimental hypothyroidism (Ariyaratne et al, 2000b; Ariyaratne et al, 2000c; Teerds et al, 1998). On the other hand, higher number of mature ALCs was often observed in hyperthyroid animals, indicating the positive regulatory effect of thyroid hormone on ALC differentiation (Ariyaratne et al, 2000c; Teerds et al, 1998). Various studies suggested that the differentiation of ALC is negatively regulated by AMH (Lyet et al, 1995; Racine et al, 1998). Overexpression of AMH in male mice decreased the mRNA expression of Cyp17a1 while disruption of AMH increased the gene expression of Cyp17a1 (Racine et al, 1998).

In addition to the aforementioned hormones, growth factors such as DHH and PDGFA appear to be essential in the development of ALCs since Leydig cell loss or failure of ALC development was observed in knockout mice for either factor (Clark et al, 2000; Gnessi et al, 2000). The effect of insulin-like growth factor-1 (IGF-1) on ALC development appears to be two-fold: to increase proliferation of Leydig cell precursors/progenitors and promote maturation of ILCs into mature ALCs (Khan et al, 1992; Moore & Morris, 1993). Furthermore, IGF-1 appears to be able to work synergistically with LH since it was able to

enhance the LH-stimulated androgen production in ILCs isolated from 30-day-old rats (Gelber et al, 1992). Furthermore, factors secreted by testicular macrophages seem to be essential in ALC differentiation since the ALC population failed to develop in immature rats where testicular macrophages had been depleted (Gaytan et al, 1994a; Gaytan et al, 1994b; Gaytan et al, 1994c).

1.6 Objectives of this thesis

Overall, current evidence suggests that the development of the male reproductive tract during fetal life is sensitive and vulnerable to any early hormonal perturbations. Many adverse effects on male reproductive tract development induced by phthalates are most likely to be the secondary effect of lower testosterone production by Leydig cells. To date, *in utero* maternal exposure studies have mainly looked at phthalate effects in the fetal testis at the time when FLCs are fully differentiated. No information is known as to whether FLCs are affected at an earlier stage by these xenobiotics.

In this study, we propose that xenobiotic treatments, such as DBP or DES, are likely to influence the differentiation process of Leydig cells. DES was used in this study as a positive control as similar phenotypic effects were observed in rodents following gestational or neonatal exposure to DBP or DES (Emmen et al, 2000). Since the differentiation process in the fetal testis still remains largely unclear, and FLCs themselves as an experimental model are relatively inaccessible, I decided to employ a well-established model, the EDS adult rat model, to study the effect of phthalates on adult-type Leydig cell differentiation. As most of the studies have only looked at the acute effects of phthalate treatment, we were also interested to see if the reproductive malformation that manifests during fetal life could affect testicular function later at adulthood. INSL3 is a

marker of the mature Leydig cell phenotype (Ivell & Bathgate, 2002). Since INSL3 is an important end-point in this project and there are currently no good commercial kits available for measuring rodent INSL3, the first aim of the project was to develop and an immunoassay for measuring rat INSL3 in both plasma and culture medium.

The specific aims of the thesis were:

- (1) To develop a time-resolved fluorescent immunoassay (TRFIA) for rat INSL3 for measurement in plasma and culture medium.
- (2) To investigate the effect of DBP on the regeneration process of Leydig cells in the EDS adult rat model, thereby determining the stage of differentiation that is most sensitive to phthalate action.
- (3) To investigate whether the adverse effect of *in utero* DBP exposure could persist through to adulthood in male progeny.
- (4) To develop an *in vitro* Leydig cell differentiation model using primary rat Leydig cells isolated from 10-day-old animals, and to look at the effects of DBP and MBP on the differentiation process in order to understand the mechanism of action of these endocrine disruptors.

Chapter 2

Materials and Methods

2.1 - Materials

The suppliers of all reagents used throughout this thesis are indicated as following:

Reagent	Reagent supplier
Absolute ethanol	Merck (Kilsyth, Victoria, Australia)
Affinity-purified goat anti-rabbit IgG	Rockland (Gilbertsville, PA, USA)
Affinity-purified testosterone antiserum	Ivell laboratory
Agarose (Type I)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Antibiotic-antimycotic (100×)	Invitrogen (Mulgrave, VIC, Australia)
Bovine Serum Albumin (BSA), Fraction V	Merck (Kilsyth, Victoria, Australia)
Bovine/sheep INSL3 peptide	Dr. John Wade (Howard Florey Institute, Melbourne, Victoria, Australia)
Cell Proliferation Reagent WST-1	Roche Applied Science (Castle Hill, NSW, Australia)
Chloroform	APS Specialty Chemicals (Greenfields, SA, Australia)
Collagenase (type IA)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Corn oil	Sigma-Aldrich (Castle Hill, NSW, Australia)
Density marker beads	GE Healthcare Australia Pty. Ltd (Rydalmere, NSW, Australia)
Deoxynucleotide Mix (10mM)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Dibutyl phthalate (DBP)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Diethylstilbestrol (DES)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen (Mulgrave, VIC, Australia)
EDTA, Disodium Salt, Dihydrate	Merck (Kilsyth, Victoria, Australia)
Enhancement solution	Perkin Elmer (Waltham, MA, USA)

Entellan® mounting medium	Merck (Kilsyth, Victoria, Australia)
Eosin 1% alcoholic stain	Amber Scientific Pty Ltd (Midvale, WA, Australia)
EDS	Gift from A/Prof. Katja Teerds (Wageningen, Netherlands)
Ethidium bromide solution (10mg/ml)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Europium-labelled rat INSL3	Ivell laboratory
Europium-labelled testosterone	Ivell laboratory
Fetal calf serum	Invitrogen (Mulgrave, VIC, Australia)
Formaldehyde RNA dye	Ambion (Austin, TX, USA)
Formaldehyde solution, 40% (w/v)	Merck (Kilsyth, Victoria, Australia)
Gill's #2 Haematoxylin	Amber Scientific Pty Ltd (Midvale, WA, Australia)
Glacial acetic acid	BDH Chemicals/VWR International (North Shore, Auckland, New Zealand)
Guanidine Thiocyanate (GTC)	MP Biomedicals (Seven Hills, NSW, Australia)
Heparin	Lyppard Australia Limited (Adelaide, Australia)
HistoBond® adhesive microscope slides	Paul Marienfeld GmbH & Co. KG (Lauda-Koenigshofen, Germany)
Histolene	Fronine/Lomb Scientific (Kensington, VIC, Australia)
Horse serum	Sigma-Aldrich (Castle Hill, NSW, Australia)
Human chorionic gonadotropin (hCG)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Human insulin (recombinant)	Invitrogen (Mulgrave, VIC, Australia)
Human insulin-like growth factor 1 (IGF-1)	Gift from Dr. Briony Forbes (Adelaide, SA, Australia)
Human insulin-like growth factor 1 (IGF-2)	Gift from Dr. Briony Forbes (Adelaide, SA, Australia)

Human INSL3 peptide	Dr. John Wade (Howard Florey Institute, Melbourne, Victoria, Australia)
Hydrochloric acid (HCl), 36.5-38.0%	Sigma-Aldrich (Castle Hill, NSW, Australia)
Isopropanol	Merck (Kilsyth, Victoria, Australia)
L-Glutamine	Invitrogen (Mulgrave, VIC, Australia)
Minisart® sterile syringe filter (0.2µm)	Sartorius Stedim Australia Pty Ltd (Dandenong South, VIC, Australia)
Monobutyl phthalate (MBP)	TCI (Chuo-ku, Tokyo, Japan)
Non-essential amino acids solution 10mM (100×)	Invitrogen (Mulgrave, VIC, Australia)
Nuclease-free water	Ambion (Austin, TX, USA)
Nutrient mixture F-12 HAM	Invitrogen (Mulgrave, VIC, Australia)
Oligo(dT) ₁₂₋₁₈ Primer	Invitrogen (Mulgrave, VIC, Australia)
Oligonucleotide primers	Geneworks (Hindmarsh, SA, Australia)
Paraplast® Plus paraffin wax	Ted Pella Inc. (Redding, CA, USA)
Penicillin-Streptomycin	Invitrogen (Mulgrave, VIC, Australia)
Percoll™	GE Healthcare Australia Pty. Ltd (Rydalmere, NSW, Australia)
Periodic acid 1% solution	Amber Scientific Pty Ltd (Midvale, WA, Australia)
Phosphate buffered saline (PBS) (1×, pH7.4)	Invitrogen (Mulgrave, VIC, Australia)
Picric acid solution (~1.3% in H ₂ O) (saturated aqueous)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Polyclonal rabbit anti-rat INSL3 antibody	IMVS (Gilles Plain, SA, Australia)
Porcine relaxin	Gift from Prof. David Sherwood (Urbana, IL, USA)
Rat INSL3 peptide	Dr. John Wade (Howard Florey Institute, Melbourne, Victoria, Australia)
Rat LH ELISA kit	USCN Life (Wuhan, Hubei, China)

Recombinant human H2 relaxin	Dr. John Wade (Howard Florey Institute, Melbourne, Victoria, Australia)
Schiff's reagent (for analysis of aldehydes)	BDH Prolabo/VWR International (North Shore, Auckland, New Zealand)
Sodium chloride (NaCl)	Merck (Kilsyth, Victoria, Australia)
Sodium hydroxide (NaOH)	Merck (Kilsyth, Victoria, Australia)
Sodium Phosphate, Dibasic (Na ₂ HPO ₄)	Merck (Kilsyth, Victoria, Australia)
Sodium Phosphate, Monobasic, Monohydrate (NaH ₂ PO ₄ • H ₂ O)	Merck (Kilsyth, Victoria, Australia)
Sodium pyruvate solution 100mM (100×)	Invitrogen (Mulgrave, VIC, Australia)
Soy-free Semi-Pure Growth Diet (AIN-93G)	Specialty feeds (Glenforrest, WA, Australia)
Standard rat chow (rat and mouse cubes)	Specialty feeds (Glenforrest, WA, Australia)
SuperScript® II Reverse Transcriptase	Invitrogen (Mulgrave, VIC, Australia)
SYBR® Premix Ex Taq™ (Perfect Real Time)	Takara Bio (Otsu, Shiga, Japan)
Testosterone (T)	Sigma-Aldrich (Castle Hill, NSW, Australia)
Thimerosal	Merck (Kilsyth, Victoria, Australia)
TrackIt™ 100bp DNA ladder	Invitrogen (Mulgrave, VIC, Australia)
TrackIt™ Cyan/Orange Loading Buffer	Invitrogen (Mulgrave, VIC, Australia)
Tris	Merck (Kilsyth, Victoria, Australia)
TRIzol® reagent	Invitrogen (Mulgrave, VIC, Australia)
TURBO DNA-free™ kit	Ambion (Austin, TX, USA)
Tween 20	Bio-Rad Laboratories (Gladesville, NSW, Australia)
Whatman filter paper	Whatman International Ltd (Maidstone, Kent UK)
Xylene	Chem-Supply (Gillman, SA, Australia)

2.2 – Buffers and solutions

The composition of buffers and solutions used throughout this thesis are described in detail below. Unless specified otherwise, all solutions were made with autoclaved milli-Q (MQ) water and stored at room temperature. Wherever necessary, pH was adjusted with either 10M sodium hydroxide (NaOH) or concentrated hydrochloric acid (HCl, 36.5-38.0%) using an UltraBasic (UB-10) Benchtop pH Meter (Denver Instrument, Bohemia, NY, USA).

Assay buffer (for testosterone TRFIA), pH7.0

0.2g of BSA

2.76g of NaH₂PO₄•H₂O

0.528g of NaOH

400µl of 0.5M EDTA, pH 8.0

0.02g of Thimerosal

40µl of 10% Tween 20 (w/v)

pH to 7.0

Made up to 400ml with autoclaved MQ water.

Bouin's fixative

300ml of picric acid solution (~1.3% in H₂O) , filtered with Whatman filter paper

100ml of 40% (w/v) formaldehyde solution

20ml of glacial acetic acid

Stored at 4°C.

EDTA, 0.5M, pH8.0

18.61g EDTA

pH to 8.0.

Made up to 100ml with autoclaved MQ water.

Ethanol, 50%

500ml of absolute ethanol

500ml of MQ water

Ethanol, 70%

700ml of absolute ethanol

300ml of MQ water

Ethanol, 95%

950ml of absolute ethanol

50ml of MQ water

Ethanol, 80%

40ml of absolute ethanol

10ml of nuclease free water

Na₂HPO₄, 10mM

0.142g of Na₂HPO₄

Made up to 100ml with autoclaved MQ water.

NaH₂PO₄, 10mM

0.138g of NaH₂PO₄ • H₂O

Made up to 100ml with autoclaved MQ water.

NaOH, 10M

40g of NaOH

Made up to 100ml with autoclaved MQ water.

Periodic acid, 0.5%

125ml of Periodic acid (1% solution)

125ml of MQ water

Made fresh before use.

Sodium phosphate buffer, 10mM, pH7.0

61ml of 10mM Na₂HPO₄

39ml of 10mM NaH₂PO₄

pH to 7.0 with 10mM Na₂HPO₄

Filter sterilized with 0.2µm filter.

TAE buffer, 50×

242g of Tris base

57.1ml of glacial acetic acid

100ml of 0.5M EDTA (pH8.0)

Made up to 1L with autoclaved MQ water.

TAE buffer, 1×

100ml of 50× TAE buffer

Made up to 5L with MQ water.

Tween 20 (10% w/v)

10g of Tween 20

Made up to 100ml with autoclaved MQ water.

Stored at 4°C.

Wash buffer (for TRFIA), 1×

0.5g of NaCl

20µl of Tween 20

Made up to 100ml with autoclaved MQ water.

Stored at 4°C.

2.3 – General Methods

General experimental procedures and methods that are used in more than one chapter are described in detail below. Methods that are used in specific chapters are described in detail in appropriate chapters.

2.3.1 Animal experiments

All animal experiments were approved by the Adelaide University Animal Ethics Committee (ethics number S19/07). Sprague-Dawley (SD) rats were used throughout in this thesis (except where specified in Chapter 3) and they were either purchased from the Animal Resources Centre (ARC, Perth, WA, Australia) or University of Adelaide

Laboratory Animal Services (LAS, Adelaide, SA, Australia). The rats were housed under a 12 hour light/ 12 hour dark cycle with *ad libitum* access to standard rat chow and water, unless otherwise specified.

2.3.1.1 Collection of testes and blood

The rats were euthanized via CO₂ asphyxiation. One testis from each animal was snap-frozen in liquid nitrogen for reverse transcription polymerase chain reaction (RT-PCR) analysis while the other testis was fixed in Bouin's fixative. Blood was collected from the posterior vena cava using heparinised 19G needles. Following collection, the blood was kept on ice before being centrifuged at 1000 ×g at 4°C for 20 minutes. Plasma was collected from the top clear layer after centrifugation and stored at -20°C until measurement for INSL3, testosterone and LH.

2.3.1.2 Fixation and processing of testes

Upon collection, the testes were immediately fixed in Bouin's fixative for 2 hours at room temperature. Testes were then cut into half cross-sectionally using a sharp scalpel and fixed further for 4 hours at room temperature. After 6 hours fixation at room temperature, the fixative was changed to 70% ethanol. Specimens were kept at room temperature and ethanol was changed several times before being processed and automatically paraffin-embedded at the University of Adelaide Histology Services using a Leica Semi-enclosed Benchtop Tissue Processor Leica TP1020 (Leica, Wetzlar, Germany). The processing protocol and paraffin wax embedding protocol are shown in Table 2.1.

Table 2.1: Processing and paraffin wax embedding protocol for fixed tissues:

Steps	Reagents	Duration
1.	70% Ethanol	1 hour
2.	80% Ethanol	1 hour
3.	80% Ethanol	1 hour
4.	95% Ethanol	30 minutes
5.	95% Ethanol	1 hour
6.	100% Ethanol	90 minutes
7.	100% Ethanol	90 minutes
8.	Histolene	1 hour
9.	Histolene	2 hours
10.	Histolene	2 hours
11.	Paraffin wax (under vacuum)	90 minutes
12.	Paraffin wax (under vacuum)	90 minutes
13.	Paraffin wax (under vacuum)	1 hour

2.3.2 Immunoassays

2.3.2.1 Rat INSL3 time-resolved fluorescent immunoassays (TRFIA)

Please refer to section 3.2.1 in Chapter 3 for detailed procedure.

2.3.2.2 Testosterone time-resolved fluorescent immunoassays (TRFIA)

Testosterone in plasma was extracted using the ethanol extraction method as previously described (Anand-Ivell et al, 2008; Paust et al, 2002). To extract testosterone from plasma, 150µl of plasma was incubated with 1050µl of cold absolute ethanol (i.e. final concentration of ethanol = 87.5%) for 1 hour at 4°C in BD Falcon™ 5ml round-bottom polystyrene tubes (BD Biosciences, San Jose, CA, USA). The tubes were centrifuged at 1700 ×g in a Heraeus® Megafuge® 2.0 (Thermo Scientific, Langenselbold, Germany) for 45 minutes at room temperature. Following centrifugation, supernatants were decanted into glass tubes and the contents were dried using the RapidVap Vacuum Evaporation System (Labconco, Kansas City, MO, USA) at 60°C for 90 minutes. The residue in the tubes was reconstituted in 150µl of assay buffer and kept at 4°C until measurement by the testosterone TRFIA on the same day.

The total testosterone level in plasma was measured using a semi-competitive TRFIA as described previously (Anand-Ivell et al, 2008). The range of detection for this assay was 0.06–14.58 ng/ml testosterone. A sample volume of 50µl was used throughout. Affinity-purified testosterone antiserum was applied at a final dilution of 1 in 1300. After addition of samples or standards, antiserum and Europium-labeled tracer (100,000 cps/well), the plates were incubated for 20 hours in a dark, humid chamber, without shaking at 4°C. Plates were subsequently washed four times before addition of 200µl of enhancement solution and further incubation at room temperature on an orbital mixer (Ratek Instruments, Boronia, VIC, Australia) at speed of 4.5. Plates were read after 60 minutes in

a Victor³ 1420 multilabel counter (Perkin Elmer, Boston, MA, USA), by pulsing samples with 1000/sec excitation light of 340nm band detecting emitted light at 615nm, with a delay of 400µsec after each flash to minimize non-specific background fluorescence. The intra-assay and inter-assay coefficients of variation (CV) were <7 and <16%, respectively (Anand-Ivell et al, 2008). Cross-reactivity of the assay was previously reported to be 42% for dihydrotestosterone, 1.6% for androstenedione, 1.7% for 17β-dihydroandrosterone and <0.01% for a wide range of other steroids tested (Anand-Ivell et al, 2008).

2.3.2.3 Rat luteinizing hormone (LH) enzyme-linked immunosorbent assay (ELISA)

Luteinizing hormone in rat plasma was measured using a commercial rat LH ELISA kit (USCN Life, Wuhan, Hubei, China), according to the manufacturer's protocol. Briefly, 100µl of plasma, standards or matrix(=blank) was added to a 96-well microtiter plate pre-coated with antibody which was specific for rat LH, and incubated at 37°C for 2 hours. The content in wells was aspirated followed by 1 hour incubation with biotin-conjugated polyclonal anti-rat LH antibody at 37°C. Subsequently, the plate was washed thrice, followed by 1 hour incubation with horse radish peroxidase (HRP)-conjugated avidin at 37°C. Finally, the plate was washed thrice before the addition of the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution. The colorimetric reaction was stopped after 40 minutes by the addition of sulphuric acid. The plates were read in a Victor³ 1420 multilabel counter (Perkin Elmer, Boston, MA, USA) at 450nm. The range of detection for this assay was 0.78mIU/ml to 50mIU/ml. The intra-assay and inter-assay CV were <4.5% and <7.5%, respectively. The assay was specific for natural or recombinant rat LH and had no reported cross-reactivity with other related proteins.

2.3.3 Ribonucleic acid (RNA) extraction and RT-PCR analysis

2.3.3.1 RNA extraction from frozen testes

Upon collection, rat testes were snap-frozen in liquid nitrogen and subsequently stored at -80°C. Frozen rat testes were powdered using a mortar and pestle which were pre-cooled with liquid nitrogen. Total RNA was prepared from frozen testes powder using TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Mulgrave, VIC, Australia). Briefly, testes powder was resuspended in TRIzol® reagent for 10 minutes at room temperature. 0.2 volumes of chloroform were added to separate the organic and aqueous phase of the samples. After mixing and centrifugation at 2000rpm for 10 minutes in a microcentrifuge at room temperature, the upper aqueous phase, which contains the RNA, was transferred to new Eppendorf tubes. RNA was then precipitated with 0.5 volumes of isopropanol. The RNA was pelleted by centrifuging at 13,000 ×g for 10 minutes at 4°C. To remove contaminants, the RNA pellet was then washed twice with 1 volume of 80% ethanol. The RNA pellet was subsequently air-dried on ice, re-dissolved in nuclease-free water and kept at -80°C until further use.

2.3.3.2 RNA extraction from cells

At the end of the experiments, cells in culture dishes were washed once with cold PBS, and subsequently lysed with TRIzol® reagent for 10 minutes at room temperature. Total RNA was extracted from the cells according to manufacturer's instructions (Invitrogen, Mulgrave, VIC, Australia), as in section 2.3.3.1.

2.3.3.3 Deoxyribonuclease (DNase) treatment

To avoid deoxyribonucleic acid (DNA) contamination, which might lead to erroneous results in the subsequent PCR, purified total RNA was subjected to DNase treatment using TURBO DNA-free™ kit according to the manufacturer's instructions (Ambion, Austin,

TX, USA). Briefly, the RNA samples were incubated with 0.1 volumes of 10× TURBO DNase buffer and 4 units of TURBO DNase at 37°C for 20 minutes. The reaction was inactivated by 0.2 volumes of DNase inactivation reagent at room temperature for 2 minutes. Finally, the samples were centrifuged at 10,000 ×g for 5 minutes at 4°C. Supernatants which contained RNA were then transferred to fresh Eppendorf tubes and kept at -20°C until further use.

2.3.3.4 Estimation of RNA concentration

The concentration of RNA was determined using the Eppendorf® BioPhotometer (Eppendorf, North Ryde, NSW, Australia). RNA samples were diluted 1:50 in 10mM sodium phosphate buffer and optical density (OD) was measured at 230nm, 260nm, 280nm and 320nm. The ratio of OD₂₆₀/OD₂₈₀ was used to estimate the quality of the RNA, with ratios of 1.8~2.0 indicating good quality of the RNA preparation.

2.3.3.5 Checking the quality of RNA

The quality of RNA was additionally checked by agarose gel electrophoresis using 1% agarose gels containing 0.25% guanidine thiocyanate and 1% ethidium bromide in 1× TAE buffer. A 2:1 ratio of sharp and clear ethidium bromide stained 28S:18S ribosomal RNA bands were observed for all samples, indicating that the RNA samples were of consistently high quality.

2.3.3.6 Synthesis of complementary DNA (cDNA)

Single-stranded cDNA was synthesized from 3µg of total RNA (for testes) or 1µg of total RNA (for cells) using SuperScript® II Reverse Transcriptase, primed with oligo(dT)₁₂₋₁₈ primer, according to the manufacturer's instructions (Invitrogen, Mulgrave, VIC, Australia). Briefly, the RNA samples were primed with oligo(dT)₁₂₋₁₈ primer at 70°C for 5

minutes. The reaction mixture was then made up to a total volume of 20µl with the addition of 0.2 volumes of 5× first strand buffer, 0.1 volume of 0.1M DTT, 0.5mM of deoxyribonucleotide (dNTP) mix and 200 units of Superscript™ II Reverse Transcriptase. The reaction mixture was incubated at 42°C for 90 minutes in a Shake 'n' Stack hybridisation oven (Thermo Scientific, Langenselbold, Germany). The synthesized cDNA was cooled on ice and diluted 1:5 with nuclease-free water and stored at -20°C until further use.

2.3.3.7 Quantitative real-time RT-PCR analysis

Quantitative real-time RT-PCR was carried out to quantify the mRNA expression of various Leydig cell markers following different treatments. All quantitative real-time RT-PCR was performed using the Rotor-Gene RG-3000 (Corbett Research/QIAGEN, VIC, Australia). The sequence of oligonucleotide primers and the amplicon sizes are listed in Table 2.2.

The cycling programs for each primer set are listed in Table 2.3. Briefly, after an initial denaturation at 95°C for 3 minutes, cycling (denaturation 95°C, annealing, elongation 72°C) progressed for a total of 40 cycles (except for the housekeeping gene ribosomal protein S27a (Rps27a), used as control, with 20 cycles) with data capture at the end of each elongation cycle. After a 2 minute final incubation at 40°C, melt curves were obtained to determine reaction efficiency and effectiveness, with samples being heated at 1°C increments from 55°C to 99°C. All PCR products were subsequently checked by agarose gel electrophoresis to confirm that the amplicon was a single band of the required size.

The real-time RT-PCR results were processed using Q-gene 96, a Microsoft Excel-based software application program (Muller et al, 2002). The normalized expression (NE) of genes was calculated using the equation:

$$NE = \frac{(E_{Reference})^{CT_{Reference}}}{(E_{Target})^{CT_{Target}}}$$

where:

E_{Target} and $E_{Reference}$ are the PCR amplification efficiencies of the target and housekeeping gene, respectively; and

CT_{Target} and $CT_{Reference}$ are the threshold cycles (where the fluorescence curve intersects threshold) of the PCR amplification of the target gene and housekeeping gene, respectively.

2.3.3.8 Agarose gel electrophoresis for PCR products

As stated above, to verify that the required product had indeed been amplified, the PCR products were checked using agarose gel electrophoresis. 8µl of PCR products were mixed with 2µl of 1× TrackIt™ Cyan/Orange Loading Buffer and run on 1.5% agarose gels containing 1% ethidium bromide in 1× TAE buffer. 2µl of TrackIt™ 100 bp DNA ladder was loaded on all gels for determination of band sizes. All gels were run at 80 volts for approximately 2 hours. The gels were photographed under UV illumination using a Bio-rad Gel Documentation System (Bio-Rad Laboratories, Gladesville, NSW, Australia).

Table 2.2: Oligonucleotide primers used in quantitative real-time PCR.

Gene	Gene accession	Orientation	Sequence (5'-3')	Amplicon size
Chrna4	NM_024354.1	Forward	cattgacgtggacgagaagaac	176bp
Chrna4	NM_024354.1	Reverse	ctccatccgcattgttagag	176bp
Cyp11a1	NM_017286.2	Forward	gtggcactcgtggggacag	430bp
Cyp11a1	NM_017286.2	Reverse	acccaatgggcctctggta	430bp
Cyp17a1	NM_012753.1	Forward	gcagaggtttgacttgatgtg	180bp
Cyp17a1	NM_012753.1	Reverse	gaggtatggatcggggatgta	180bp
Hsd11b1	NM_017080.2	Forward	ggtcaacgtgtccatcactctc	214bp
Hsd11b1	NM_017080.2	Reverse	catgatcctccttctggattc	214bp
Hsd17b3	NM_054007.1	Forward	tggaaaagctacaggtcatctcag	455bp
Hsd17b3	NM_054007.1	Reverse	ggttgaaacagaataaggggtcag	455bp
Insl3 (rat)	NM_053680.1	Forward	ctgctactgctgctcctggctcta	372bp
Insl3 (rat)	NM_053680.1	Reverse	gtggggacacagacccaaaa	372bp
Insl3 (mouse)	NM_013564.5	Forward	gcagtggctagagcagagacatc	306bp
Insl3 (mouse)	NM_013564.5	Reverse	gtccttgcttactgcatctatga	306bp
LHR	NM_012978.1	Forward	ctcatgcctgtcttctact	481bp
LHR	NM_012978.1	Reverse	tgcaaaagtctgcaaaggagag	481bp
Nr4a1	NM_024388.1	Forward	gcttcttcaagcgacagtaca	375bp
Nr4a1	NM_024388.1	Reverse	gggagccagagagcaagtcata	375bp
Rps27a	NM_031113.1	Forward	ccaggataaggaaggaattcctcctg	297bp
Rps27a	NM_031113.1	Reverse	ccagcaccacattcatcagaagg	297bp
StAR	NM_031558.2	Forward	ggaccttgaaaggctctgga	592bp
StAR	NM_031558.2	Reverse	tacagcgtacgcttacgaag	592bp
Sult1e1	NM_012883.1	Forward	cttctctcacaggagcatctggac	409bp
Sult1e1	NM_012883.1	Reverse	tgcaattctttcccaaatgatg	409bp

Table 2.3: Cycling program for genes analyzed in quantitative real-time PCR.

Gene	Denaturation	Annealing	Elongation
Chrna4	95°C for 30 secs	67°C for 30 secs	72°C for 45 secs
Cyp11a1	95°C for 30 secs	65°C for 30 secs	72°C for 45 secs
Cyp17a1	95°C for 30 secs	64°C for 30 secs	72°C for 45 secs
Hsd11b1	95°C for 30 secs	62°C for 30 secs	72°C for 45 secs
Hsd17b3	95°C for 30 secs	60°C for 30 secs	72°C for 45 secs
Insl3 (rat)	95°C for 30 secs	67°C for 30 secs	72°C for 45 secs
Insl3 (mouse)	95°C for 30 secs	60°C for 30 secs	72°C for 45 secs
LHR	95°C for 30 secs	62°C for 30 secs	72°C for 45 secs
Nr4a1	95°C for 30 secs	62°C for 30 secs	72°C for 45 secs
Rps27a	95°C for 30 secs	64°C for 30 secs	72°C for 30 secs
StAR	95°C for 30 secs	60°C for 30 secs	72°C for 45 secs
Sult1e1	95°C for 30 secs	63°C for 30 secs	72°C for 45 secs

2.3.4 Periodic Acid Schiff's (PAS) and haematoxylin staining

In order to quantify the number of Leydig cells in a testis, testis sections were stained with PAS and haematoxylin as described previously (Gould et al, 2007; Leblond & Clermont, 1952), with slight modification. Testes sections of 5µm (for the physical disector method) and 30µm (for the optical disector method) were mounted on HistoBond® adhesive microscope slides. Briefly, testes sections were de-paraffinised in xylene twice, 100% ethanol, 95% ethanol and distilled water, each step for 2 minutes (5µm sections) or 10 minutes (30µm sections). The sections were then incubated with 0.5% periodic acid for 10 minutes to oxidize the glucose residues. The slides were washed 3 times with distilled water, each time for 5 minutes before incubation with Schiff's reagent for 20 minutes at room temperature. The stain was developed by incubating the sections under running tap water for 30 minutes. The sections were counterstained with Gill's #2 Haematoxylin for 2 minutes. Finally, the sections were de-hydrated in 95% ethanol, 100% ethanol, xylene twice, each step for 2 minutes (for 5µm sections) or 10 minutes (for 30µm sections) and mounted in the Entellan® mounting medium.

2.3.5 Haematoxylin and Eosin (H&E) staining

Testes sections of 5µm were mounted on HistoBond® adhesive microscope slides and de-paraffinised in histolene twice for 5 minutes each time, and subsequently rehydrated in 100% ethanol twice, 70% ethanol, 50% ethanol and distilled water, each step for 3 minutes. Nuclear staining was achieved by incubation in Gill's #2 Haematoxylin for 2 minutes. The slides were washed in running tap water followed by incubation in 0.5% ammonia for 1 minute. The slides were given one wash in running tap water before the cytoplasm was stained with 1% eosin alcoholic stain for 1 minute. The slides were then washed once in running tap water. Finally, the sections were de-hydrated in 100% ethanol

thrice for 3 minutes each time and histolene twice, each step for 5 minutes. The sections were subsequently mounted in the Entellan® mounting medium.

2.3.6 Statistical analysis

Graphpad Prism 5.0 software was used to analyze all data. Statistical differences between treatment groups were first analyzed by ANOVA followed by post hoc student t-test, unless otherwise specified. A *p*-value of <0.05 was considered statistically significant.

Chapter 3

Development and validation of an immunoassay for rat insulin-like factor 3 (INSL3)

The contents of this chapter were included as part of the following publication in *Biology of Reproduction*.

Ravinder Anand-Ivell, Kee Heng, Bettina Hafen, Brian Setchell, and Richard Ivell. *Dynamics of INSL3 peptide expression in the rodent testis*. *Biol Reprod*, 2009. **81**(3): p. 480-7.

STATEMENT OF AUTHORSHIP

DYNAMICS OF INSL3 PEPTIDE EXPRESSION IN THE RODENT TESTIS.

Biology of Reproduction 2009; 81(3): p.480-487.

Anand-Ivell, Ravinder

(Lead author, responsible for conception of study and collection of testicular fluids. Designed and led establishment of INSL3 TRFIA. Contributed to the writing of the resulting article.)

I give consent for Kee Heng to present this paper for examination towards the Doctor of Philosophy.

Heng, Kee (Candidate)

(Carried out a significant part of the validation and establishment of the TRFIA assay protocol, was responsible for blood sample collection, design and carrying out of the EDS animal study. Contributed to writing of the resulting article)

Signed.....Date.....

Hafen, Bettina

(Assisted in the development and testing of the TRFIA assay)

I give consent for Kee Heng to present this paper for examination towards the Doctor of Philosophy.

Setchell, Brian

(Advised and assisted in collection of testicular fluids)

I give consent for Kee Heng to present this paper for examination towards the Doctor of Philosophy

Ivell, Richard

(Overall responsibility for conception, design and carrying out of the project, and the conception and writing of the resulting article)

I give consent for Kee Heng to present this paper for examination towards the Doctor of Philosophy

3.1 – Introduction

The insulin-like factor 3 (INSL3) is a small peptide hormone of 6.3kDa that belongs to the insulin/relaxin superfamily. It was first discovered as a novel gene transcript sequence in 1993 to be specifically expressed in Leydig cells in the boar testis both prenatally and postnatally (Adham et al, 1993). It is now considered to represent one of the ‘neohormones’ that has recently evolved in order to cope with the mammalian reproductive needs such as having a scrotal testes (Ivell & Bathgate, 2006). The INSL3 peptide is encoded by a small gene of <1kb that contains 2 exons and 1 intron (Ivell & Anand-Ivell, 2009) and is closely linked to the Janus Kinase 3 (JAK3) gene locus (Spiess et al, 1999). Although the *Insl3* cDNA sequence has been known for some time, the precise protein structure is still relatively unknown. The mature INSL3 protein is predicted to resemble the structure of relaxin, with separately cleaved A and B chains connected by inter- and intra-chain disulphide bonds (Ivell & Anand-Ivell, 2009; Smith et al, 2001). Rat INSL3 which had been chemically synthesized based on the cDNA sequence was shown to be fully bioactive in inducing the growth of the rat fetal gubernaculum in organ culture (Smith et al, 2001).

The expression of INSL3 in the male is primarily confined to the Leydig cells within the testis (Adham et al, 1993; Sadeghian et al, 2005). INSL3 is also expressed in the female, however at a relatively much lower level compared to the male, in the cells of the follicular theca interna and in the corpus luteum within the ovary (Bathgate et al, 1996), in the placenta (Hombach-Klonisch et al, 2001) and in breast tissue (Hombach-Klonisch et al, 2000a).

The expression of INSL3 during fetal development is sexually dimorphic. In mice, INSL3 expression was first detectable on E13.5 in the fetal testis, and remained highly expressed until the end of gestation, while no detectable INSL3 expression was observed in the female embryo (Zimmermann et al, 1997). The role of INSL3 in the first, transabdominal phase of testicular descent in male fetuses was first demonstrated with the generation of INSL3 knockout mice (Nef & Parada, 1999; Zimmermann et al, 1999). Male mice that were homozygous for the *Insl3* gene deletion exhibited bilateral cryptorchidism with both testes located high in the abdomen, caused by the failure of gubernaculum development (Nef & Parada, 1999; Zimmermann et al, 1999). In INSL3 knockout male mice, the gubernacula appear thin and undeveloped, and resemble those that are found in female fetuses (Kubota et al, 2001; Nef & Parada, 1999; Zimmermann et al, 1999). On the other hand, overexpression of the *Insl3* gene in female mouse embryos resulted in the ‘descent’ of the ovaries into the inguinal region due to the inappropriate overdevelopment of the gubernacula (Adham et al, 2002). While, several polymorphisms and mutations have been identified in the *INSL3* gene in some cryptorchid patients, however these are unlikely to be the main cause for cryptorchidism since most of the polymorphisms and mutations appeared to be present in both normal and cryptorchid populations (Baker et al, 2002; Lim et al, 2001; Marin et al, 2001; Takahashi et al, 2001).

After birth, testicular *Insl3* gene expression remains low until PND25 in mice, during the time of the first wave of spermatogenesis (Zimmermann et al, 1997). While INSL3 function in male fetal development has been known for some time, the function of INSL3 in the adult male has remained unclear. Accumulating evidence suggests that INSL3 might play a role in germ cell survival. Germ cell apoptosis induced by a GnRH antagonist in immature rats could be prevented by co-treatment of INSL3 (Kawamura et al, 2004). Furthermore, men who are experimentally receiving a pharmacological contraceptive

regime to suppress pituitary function and hence spermatogenesis responded differently to the treatment. Circulating INSL3 levels in the poor responders (with higher residual sperm) were found to be significantly higher than for the good responders (Amory et al, 2007). Finally, testis weight in rats was significantly reduced following injection of INSL3 B-chain (an INSL3 antagonist) into the testis (Del Borgo et al, 2006). Consistent with these findings, the INSL3 receptor, relaxin/insulin-like family peptide receptor 2 (RXFP2), was previously reported to be expressed specifically in post-meiotic germ cells (Anand-Ivell et al, 2006b; Feng et al, 2007). In addition to its effect on germ cells, it was recently reported that young men with T222P mutation in the RXFP2 gene had significantly reduced bone density despite having normal level of plasma testosterone, suggesting that INSL3 might also play a role in bone metabolism (Ferlin et al, 2008).

INSL3 appears to mediate its effect through binding to its specific receptor, RXFP2 (Bathgate et al, 2006; Kumagai et al, 2002). RXFP2 belongs to a family of G-protein coupled receptors containing multiple leucine-rich repeats in their large extracellular domains. RXFP2 was shown to be expressed in rat gubernaculum (Kubota et al, 2002; Kumagai et al, 2002), germ cells (Anand-Ivell et al, 2006b; Feng et al, 2007), and ovary (Kawamura et al, 2004). Similar to INSL3 knockout mice, deletion of RXFP2 also caused cryptorchidism in male mice (Gorlov et al, 2002). To date, only one mutation identified in RXFP2 (i.e. T222P) has been found to be associated with cryptorchidism in the human (Bogatcheva et al, 2007). Whether the mutation in RXFP2 plays a general role in cryptorchidism remains debatable since the same mutation in RXFP2 was not linked to cryptorchidism in other studies (El Houate et al, 2008; Nuti et al, 2008).

The function and regulation of INSL3 has been made clearer with the recent development of various radioimmunoassay (RIA) and TRFIA for the measurement of human INSL3.

Using a commercial RIA kit (Phoenix Pharmaceuticals, Belmont, CA, USA), Foresta et al (2004) showed that INSL3 is circulating in adult men while only low levels of INSL3 were detected in adult women and orchidectomized men, showing that in men this peptide hormone is exclusively secreted by the testis (Foresta et al, 2004). Previously, Insl3 mRNA levels as well as immunocytochemical staining intensity were shown to decrease dramatically in the Leydig cells of old rats (Paust et al, 2002). Using the human INSL3 TRFIA developed in our laboratory (Bay et al, 2005), we previously reported that the serum INSL3 level similarly declined with age in a large population of Australian men (Anand-Ivell et al, 2006a).

A number of studies are supporting the view that INSL3 secretion is independent of the acute regulation of the HPG axis. Patients who received hCG stimulation test showed that their INSL3 serum concentration remained unchanged 72 and 96 hours after treatment, despite a substantial rise in serum testosterone, suggesting that INSL3 secretion was not acutely regulated by hCG (Bay et al, 2005). This result was in agreement with the results reported in rodent models, where INSL3 was shown to be constitutively expressed (Sadeghian et al, 2005). Furthermore, INSL3 in unilaterally orchidectomised patients was lower than normal adult men although the serum testosterone level in these patients was normal (Anand-Ivell et al, 2006a). On the other hand, serum INSL3 was markedly increased after a long-term (3 months) hCG treatment in hypogonadotropic patients (Bay et al, 2005). These results strongly suggest that INSL3 secretion is acutely independent of LH and could only be regulated by the long-term differentiating effect of LH on Leydig cells. Therefore, INSL3 should serve as a better marker than testosterone as a measure of Leydig cell capacity (numbers and differentiation status).

Because of cross-species variation in the INSL3 peptide sequence, it has not been possible until now to make use of rodents as physiological models to test INSL3 secretion and function. The only piece of available information on INSL3 level in rodent models came from a study in 2001. Boockfor and colleagues measured serum INSL3 in male rats by making use of an anti-mouse INSL3 antibody and ¹²⁵I-labelled human INSL3 as radioligand on a RIA platform (Boockfor et al, 2001). In the current study, we describe the development of a new rodent-specific immunoassay, which allows us for the first time to study the physiology of INSL3 in experimental rodent models, its importance as a puberty marker, and in aging research.

3.2 – Methods

3.2.1 Rodent INSL3 time-resolved fluorescent immunoassays (TRFIA)

Heterodimeric rat INSL3 was chemically synthesized by Dr. John Wade at the Howard Florey Institute, Melbourne, Australia (Smith et al, 2001). The peptide was shown by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) to be >99% pure, and circular dichroism analysis showed that it conformed to the expected molecular structure. The rat INSL3 synthesized was found to be biologically active in inducing the growth of fetal rat gubernacula in whole organ cultures (Smith et al, 2001). Polyclonal antibodies were raised in New Zealand white rabbits, as previously described (Bay et al, 2005). The serum indicating the highest titer against the immunizing antigen was then used to develop a specific immunoassay on a TRFIA platform.

A new TRFIA specific for rat INSL3 was recently developed in our laboratory. Europium-labeled tracer was produced exactly as described for human INSL3 (Anand-Ivell et al, 2006a; Bay et al, 2005), except using the pure rat INSL3 peptide (Fig. 3.1). The TRFIA

assay was designed essentially as previously described (Bartsch et al, 2004; Bay et al, 2005), except that microtiter fluoroNunc strips (Nunc, Roskilde, Denmark) were coated with 1µg/well affinity-purified goat anti-rabbit immunoglobulin G (IgG) with long-term storage at -20°C. A sample volume of 50µl was used throughout. Standards represented concentrations of 20pg to 5ng rat INSL3 per ml, and covered the full range of measurement. Rabbit anti-rat INSL3 antiserum was applied without further purification at a final dilution of 1 in 5000. After addition of samples or standards and antibody, plates were incubated for 20 hours in a dark, humid chamber, with gentle shaking at 4°C. Plates were then washed once, and tracer added (500,000 cps/well) with further incubation for 2 hours in the dark at 4°C without shaking. Plates were then washed four times before addition of 200µl of enhancement solution and further incubation at room temperature. Plates were read after 60 minutes in a Victor³ 1420 multilabel counter (Perkin Elmer, Boston, MA, USA), by pulsing samples with 1000/sec excitation light of 340nm band detecting emitted light at 615nm, with a delay of 400µsec after each flash to minimize non-specific background fluorescence.

3.2.2 Specificity of rodent INSL3 TRFIA

Related peptides that belong to the insulin/relaxin family of hormones were checked for cross-reactivity in this newly developed rat INSL3 TRFIA. Human INSL3, bovine/sheep INSL3, human insulin, human IGF-1, human insulin-like growth factor 2 (IGF-2), porcine relaxin and recombinant human H2 relaxin of various concentrations (0.02ng/ml to 50ng/ml) were used in the specificity test. The procedure of the rat INSL3 TRFIA was followed as described in section 3.2.1.

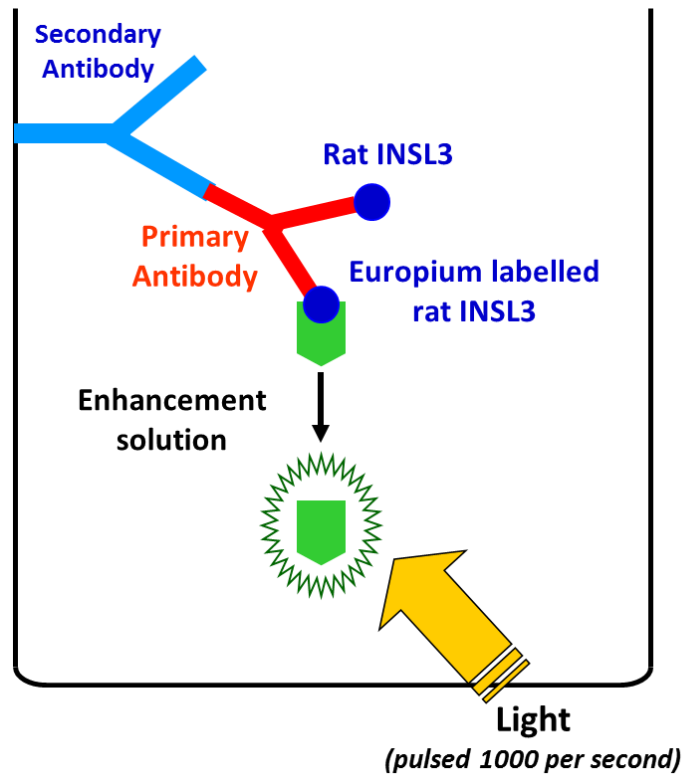


Figure 3.1: Principle of rat INSL3 TRFIA. The microtiter fluoroNunc strips are coated with secondary affinity-purified goat anti-rabbit IgG which has high affinity for the primary antibody. During sample incubation, rat INSL3 present in the sample will bind to the primary polyclonal rabbit anti-rat INSL3 antiserum. Any vacant capacity left on the primary antibody will be taken by the Europium labelled-rat INSL3. The acidic enhancement solution then dissociates Eu^{3+} from the Europium labelled-rat INSL3 to form a homogenous and highly fluorescent $\text{Eu}-(2\text{-NTA})_3(\text{TOPO})_{2-3}$ micellar chelate solution. Fluorescence could then be measured using a Victor³ 1420 multilabel counter, by pulsing samples with 1000/sec excitation light of 340nm band detecting emitted light at 615nm, with a delay of 400 μsec after each flash to minimize non-specific background fluorescence. The number of counts obtained will be inversely proportional to the concentration of rat INSL3 within the sample.

3.2.3 Postnatal time course of peripheral INSL3 concentration

In order to obtain blood from rats of precise age postnatally through puberty, pregnant SD rats were followed, and the exact date of delivery determined. Male pups were weaned on PND21 and thereafter fed *ad libitum* with standard rat chow and water. On PNDs 5, 10, 15, 20, 25, 30, 35, 40, 60 and 90, the rats were sacrificed between 1400 and 1500 h, and trunk blood and other tissues collected, as described in section 2.3.1.1 and stored at -20°C until analysis for INSL3 and testosterone.

3.2.4 Peripheral INSL3 profile following Leydig cell ablation

Adult male SD rats of around 90 days of age were given a single bolus intraperitoneal (i.p.) injection (Day 1) of EDS (75mg/kg body weight, dissolved in DMSO/water, 1:3) to ablate all mature adult Leydig cells within the testes. The animals were sacrificed on Days 10, 14, 19, 27 and 37. Blood was collected from the posterior vena cava, as described in section 2.3.1.1 and stored at -20°C until analysis for INSL3 and testosterone.

3.2.5 Culture of MA-10 cells

The MA-10 mouse Leydig tumour cell line was a kind gift from Dr. Mario Ascoli (University of Iowa, IA, USA). The cells were cultured as previously described (Sadeghian et al, 2005). Briefly, MA-10 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's media (containing 1g/L d-glucose, 4mM L-glutamine, 25mM HEPES buffer, 4mg/L pyroxene HCl and 110mg/L sodium pyruvate) and nutrient mixture F-12 HAM (with NaHCO₃, without L-glutamine) supplemented with 7.5% horse serum, 2.5% fetal calf serum, 100 units Penicillin(sodium salt) and 100µg Streptomycin sulfate in 0.85% saline. Initial experiments looked at the accumulation over time of INSL3 in culture media in unstimulated cells. For subsequent experiments, 200,000 cells were seeded per well in 12-well plates the day before experiment. Media in all wells were changed the next day

and cells were stimulated, as indicated, with testosterone, and/or monobutyl phthalate (MBP; the chief natural metabolite of DBP in rodents). Each experiment was performed in triplicate and repeated 3 times. Supernatant was collected from each well after 8 or 32 hours and stored at -20°C until analysis for INSL3. After 32 hours, the cells were harvested for RNA extraction (see sections 2.3.3.1 to 2.3.3.5). cDNA was synthesized as described in section 2.3.3.6.

Messenger RNA (mRNA) was measured using quantitative RT-PCR (qRT-PCR), as previously described (Anand-Ivell et al, 2006b), with mouse INSL3- and Rps27a-specific primers (Table 2.2).

3.2.6 Isolation of primary Leydig cells from adult rats

Experiments were also carried out with primary cultures of Leydig cells prepared from adult (90-day-old) Wistar rats, as previously described (Anand et al, 2003; Paust et al, 2002). Briefly, the rats were euthanized via CO₂ asphyxiation. Testes were removed from the animal and decapsulated. Decapsulated testicular mass were treated with 0.25mg/ml collagenase for 20 minutes at 37°C in a shaking water bath. The homogeneous cell suspension was subsequently filtered through a nylon mesh to yield a crude Leydig cell suspension. The crude Leydig cell suspension was centrifuged at 300 ×g for 10 minutes. The cell pellet was resuspended in culture media and 2ml of cell suspension was layered on top of a continuous Percoll gradient. A similar Percoll gradient was prepared and contained only the density marker beads and no cells. Both gradients were centrifuged at 800 ×g for 25 minutes. Highly purified Leydig cells were aspirated between 1.033g/ml and 1.062g/ml. Purified Leydig cells were washed twice in culture medium using centrifugation at 300 ×g for 10 minutes. Finally, the cells were suspended in a known volume of culture medium and the cell number was determined using a Neubauer chamber.

Purified Leydig cells were maintained in culture medium containing 1:1 mixture of Dulbecco's modified Eagle's media and nutrient mixture F-12 HAM supplemented with 0.1% bovine serum albumin (BSA), 100 units Penicillin(sodium salt) and 100µg Streptomycin sulfate in 0.85% saline. For experiments, 100,000 cells were seeded per well in 48-well plates. Supernatant was collected from each well after 14, 22, 38 and 46 hours and stored at -20°C until analysis for INSL3.

3.2.7 Statistical analysis

All experiments were completely repeated at least once. Significant differences (minimum $P < 0.05$) were determined either by two-tailed ANOVA followed by a post hoc Neuman-Keuls test, or by t statistics.

3.3 – Results

3.3.1 Establishment of a sensitive TRFIA measuring rodent INSL3 in peripheral sera and other body fluids

A sensitive sandwich TRFIA was developed, similar to the one we had produced to measure human INSL3 (Anand-Ivell et al, 2006a; Bay et al, 2005). The new assay uses a rabbit polyclonal antiserum raised against the mature A-B heterodimeric rat INSL3 in its native conformation.

As tracer, rat heterodimeric INSL3 was labeled with Europium chelate. The upper and lower limits of detection of the new assay are 5.0 and 0.02ng/ml, respectively, (Fig. 3.2A) for sera, plasma, or cell culture media, with within-plate coefficients of variation in the upper, middle, and lower detection range of 5.10%, 0.22%, and 11.21%, respectively. Between-plate coefficient of variation was 2.8% in the midrange. There was no detectable

cross-reactivity in this assay with porcine relaxin, human recombinant relaxin, human IGF1, human IGF2, human insulin, human INSL3, or bovine/sheep INSL3 (Fig. 3.2B), although this assay appears to detect mouse INSL3 as effectively as rat INSL3.

3.3.2 Peripheral concentrations of INSL3 during postnatal rat development

For SD rats, INSL3 is first detectable in peripheral serum (trunk blood) at Day 20 and increases to a maximum of 4–10ng/ml by Day 40 (Fig. 3.3). Importantly, this maximum is substantially higher than the final established value in adulthood at 3 months of age. Before Day 20, there appears to be no circulating INSL3, even though there are still remnants of the fetal Leydig cell population (Ivell et al, 2003). Samples collected in aged rats (>23 month old) have markedly lower levels of circulating INSL3, irrespective of strain (Table 3.1), and confirm previous reports of an age-dependent loss in testicular INSL3 immunoreactivity as well as mRNA expression (Paust et al, 2002). It should be noted that female rodents consistently have very low peripheral INSL3 concentrations, although generally above the detection limits of the assay.

3.3.3 Circulating concentration of INSL3 following EDS treatment of adult male rats

The alkylating agent, EDS, specifically destroys the differentiated population of fetal and adult-type Leydig cells (Teerds & Rijntjes, 2007). In the absence of supplementary androgens, there is a compensatory increase in LH, which promotes the differentiation of new Leydig cells from peritubular stem cells (Teerds & Rijntjes, 2007). Here (Fig. 3.4), we show first that EDS treatment effectively reduced circulating INSL3 levels by Day 10 to below the level of detection for the TRFIA assay. Significant levels of INSL3 were again detectable in the circulation at Day 27 after EDS treatment, increasing to almost normal levels by Day 37.

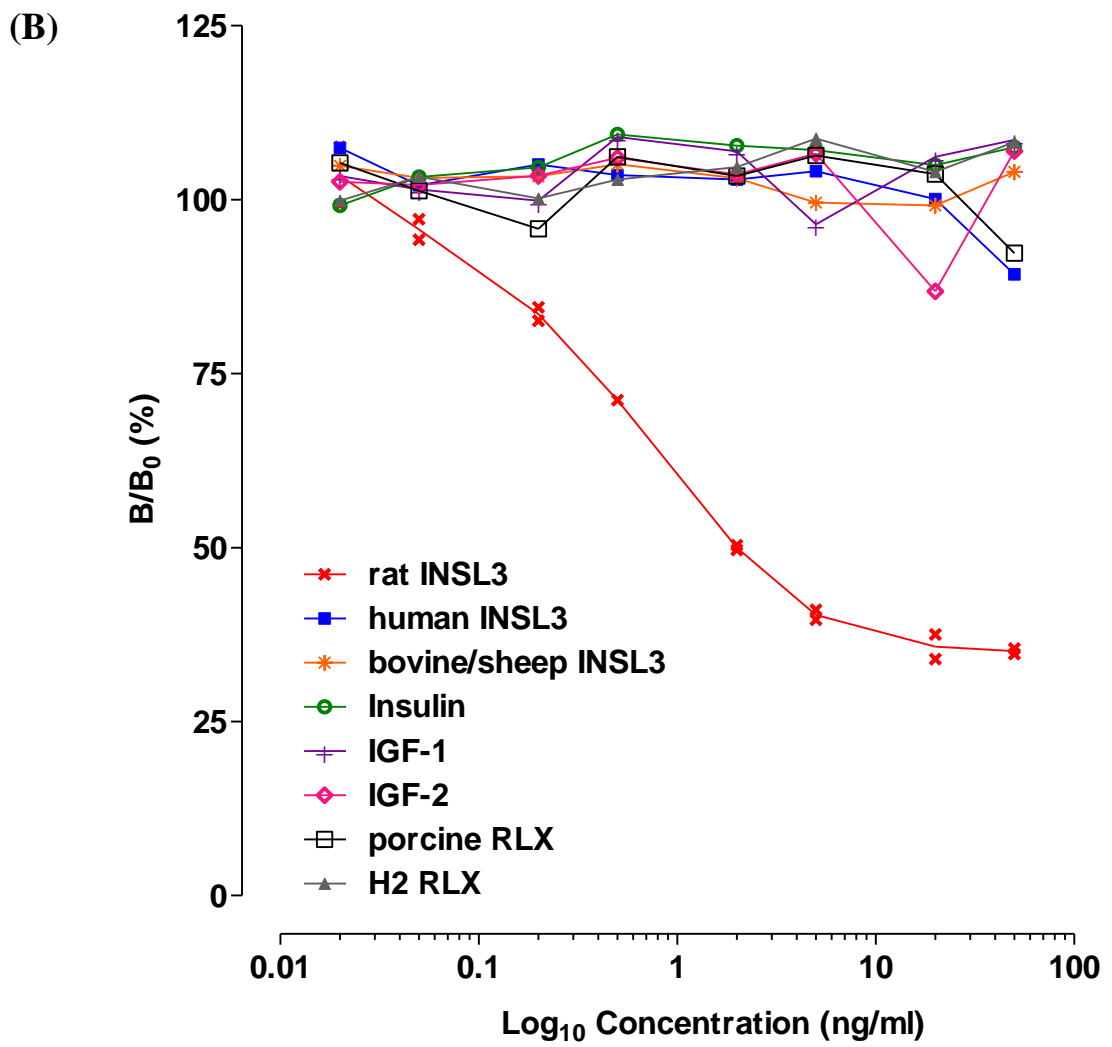
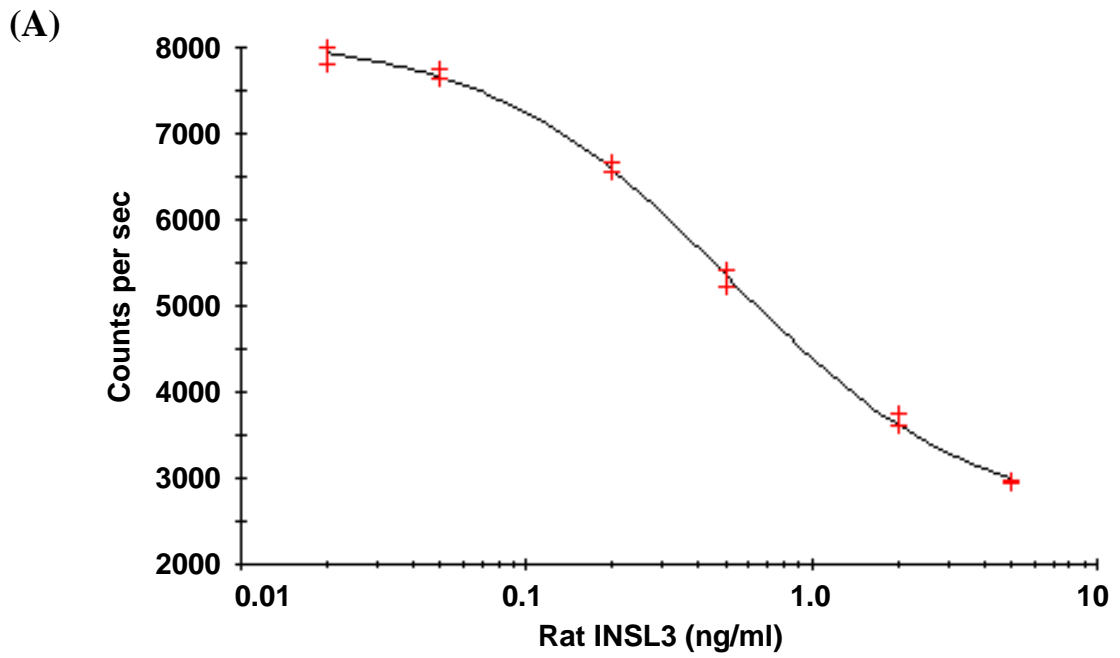


Figure 3.2 (A) Standard curve for rodent INSL3 TRFIA. The assay makes use of 50 μ l samples of cell culture supernatant, plasma, serum or any other body fluid without extraction, and has a standard range of sensitivity of 0.02ng/ml to 5ng/ml. **(B) Sensitivity of rat INSL3 TRFIA.** Rat INSL3 and several structurally-related peptides were tested for binding to the antiserum in rat INSL3 TRFIA. None of the peptides tested showed cross-reactivity to this assay. The results are expressed as $\%B/B_0 = B / B_0 \times 100\%$; where B = absorbance (A_{615nm}) at 'x'ng/ml protein concentration; $B_0 = A_{615nm}$ for blank matrix (i.e. 0ng/ml).

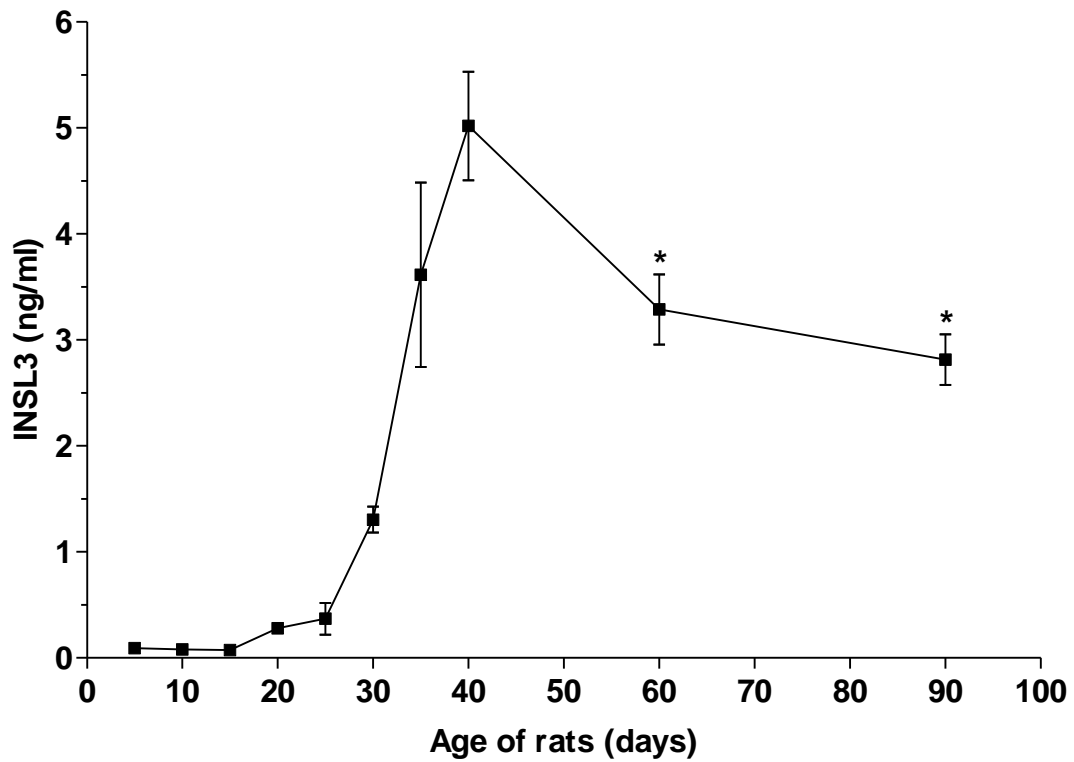


Figure 3.3: Concentration (mean \pm SEM; n = 6) of INSL3 in peripheral blood plasma from SD rats at various postnatal ages. Circulating INSL3 is first detectable around PND20 and reaches its peak value on PND40. It is important to note that this maximum INSL3 level is significantly higher than the final established value on PND60 and PND90 (*Significant difference ($P < 0.01$) from concentration at PND40).

Table 3.1: INSL3 concentration (ng/ml; mean \pm SEM) in peripheral blood plasma from rats and mice of different strains and age, as indicated.

Strain	Gender	Age	INSL3 concentration in peripheral blood plasma	N
Rat				
Sprague Dawley	Male	3 months	2.814 \pm 0.168	3
Sprague Dawley	Female	3 months	0.080 \pm 0.027	6
Wistar	Male	3 months	1.510 \pm 0.092	10
Sprague Dawley	Male	> 22 months	0.937 \pm 0.027	4
Wistar	Male	> 22 months	0.764 \pm 0.209	6
Wistar	Female	> 22 months	0.077 \pm 0.019	3
Mouse				
CBA	Male	2 months	0.775 \pm 0.029	8
CBA	Female	2 months	0.053 \pm 0.002	4

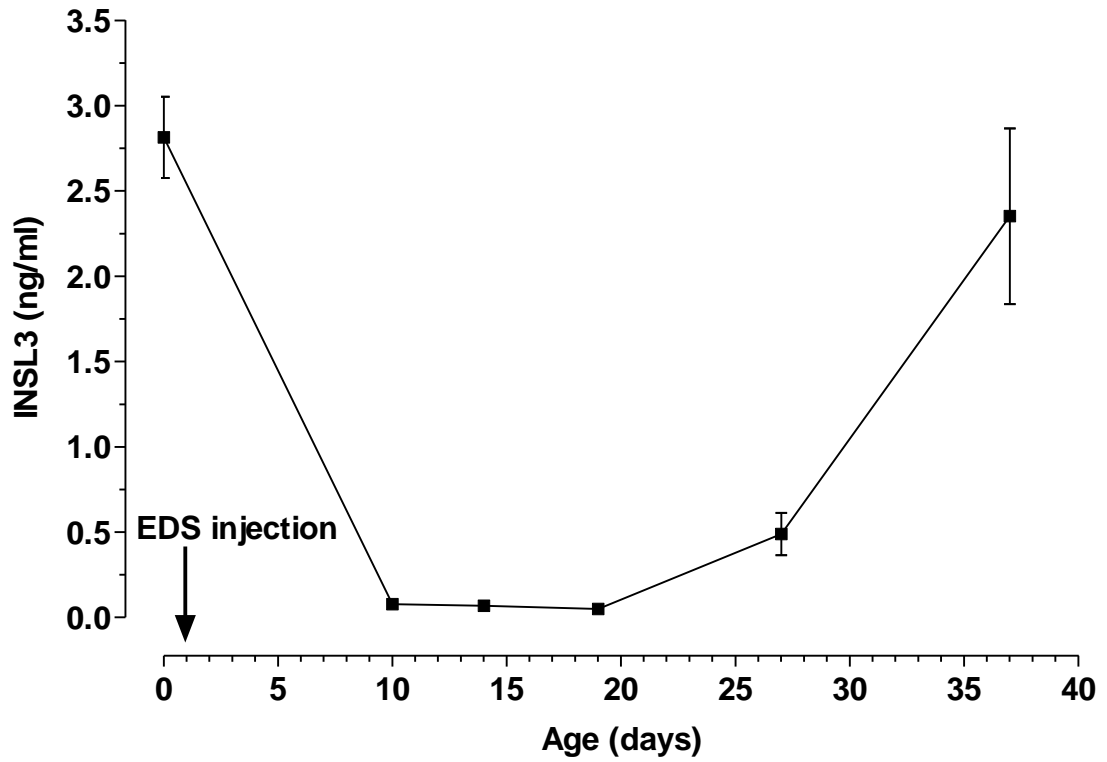


Figure 3.4: Concentration (mean \pm SEM; n = 6) of INSL3 in peripheral blood plasma from adult (90 days of age) SD rats treated with a single bolus injection of EDS on Day 1.

3.3.4 Expression of INSL3 peptide by Leydig cells in culture

Many studies have looked at *Insl3* gene expression by assessing the specific mRNA inside cells. It is assumed that this represents a useful physiological parameter. However, this is only true if it can be shown that mRNA is translated and that the protein product is secreted in a biologically appropriate form and in an amount that corresponds to the mRNA measured. Because the TRFIA is designed to recognize heterodimeric rat INSL3 in native conformation, we tested whether Leydig cells in culture indeed secrete potentially functional INSL3 into the medium, and whether this can be regulated. We show here, for the first time, that INSL3 is indeed secreted by both MA-10 mouse tumor Leydig cells and by primary adult rat Leydig cells in culture (Fig. 3.5). Recently, it has been suggested (Lague & Tremblay, 2008) that androgens and phthalates at high concentration may modulate *Insl3* gene expression in cultured Leydig cells. However, we failed to detect any change with testosterone or MBP at several concentrations, either at the level of mRNA expression (Fig. 3.6, inset), or at the level of INSL3 peptide secretion at both 8 and 32 hours (Fig. 3.6).

3.4 – Discussion

INSL3 is a major Leydig cell product in every eutherian mammal so far investigated. In humans and now rodents, it has been shown also to be a major secreted hormone. Studies in humans show that, unlike testicular steroids, which are acutely governed by the components of the HPG axis, INSL3 appears to be constitutively expressed, and thus representative of the quality (functionality and number) of the Leydig cells present in the

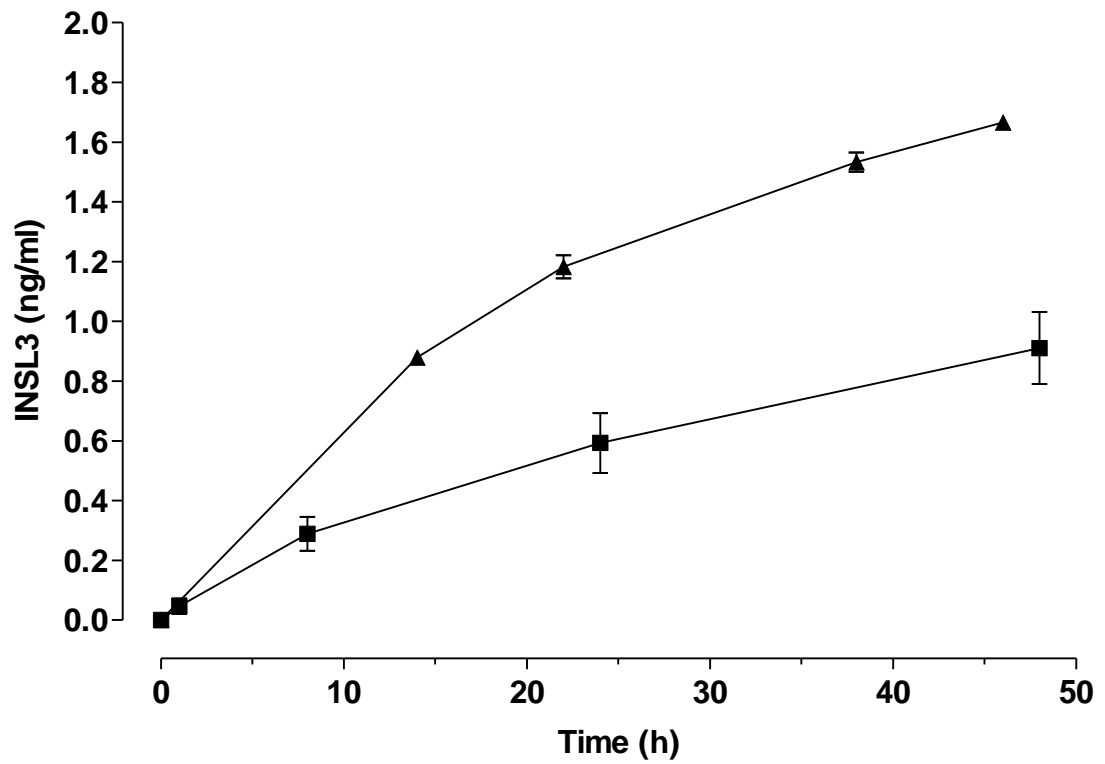


Figure 3.5: Time course of INSL3 production (mean \pm SD; n = 3 independent experiments) into culture media from MA-10 mouse Leydig tumor cells (filled squares) and primary adult (3 month old) rat Leydig cells (filled triangles).

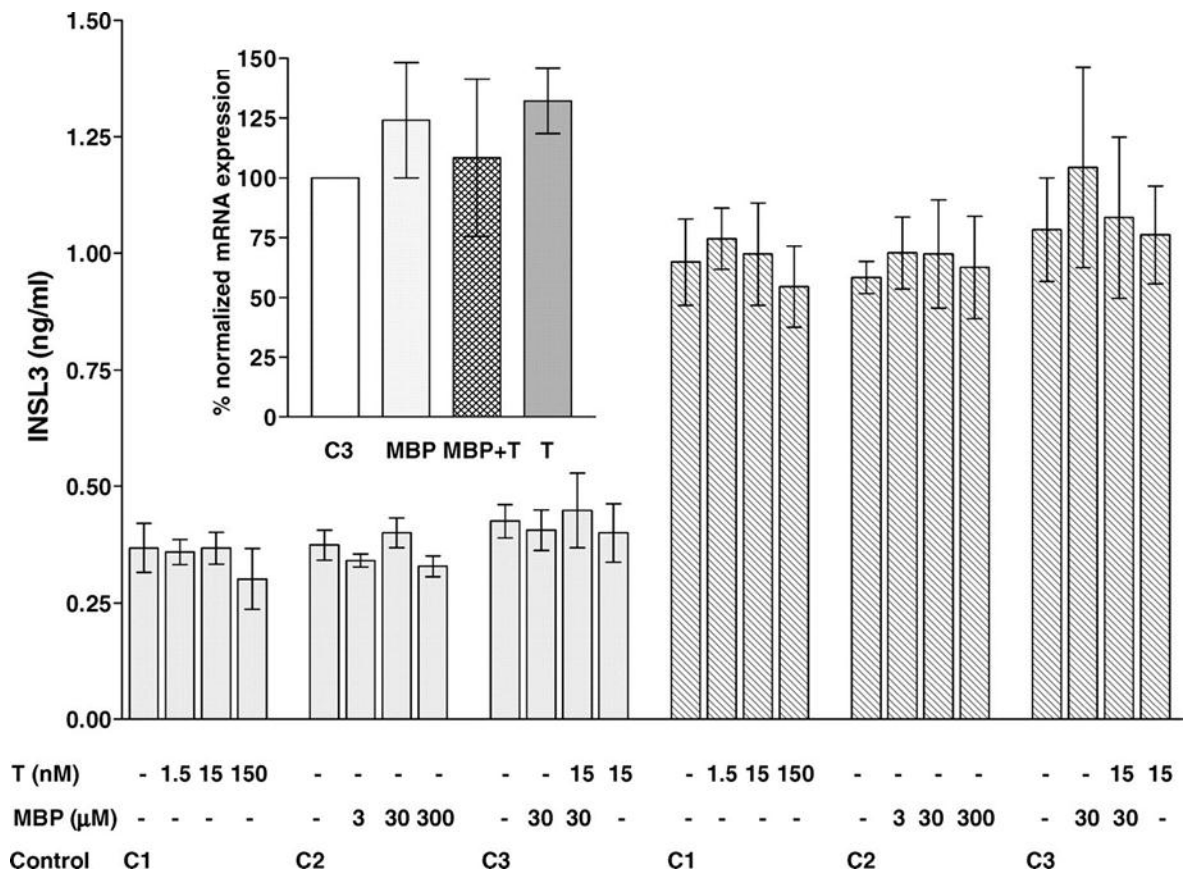


Figure 3.6: Lack of effect of testosterone (T) and/or MBP on INSL3 production after 8h (open bars) and 32h (hatched bars) by MA-10 mouse tumour Leydig cells at various concentrations and combinations of reagent. Inset displays the results of three independent qRT-PCR measurements of the specific Insl3 mRNA in the same MA-10 cells following stimulation with 30μM MBP, 15nM T, or both together, and incubated for 32 h, normalized against Rps27a mRNA. C1 to C3 are appropriate controls using the corresponding vehicle for T and MBP: C1, 0.001% ethanol; C2, 0.05% DMSO; C3, 0.001% ethanol plus 0.05% DMSO, final concentrations. All results are mean ± SEM of 3 independent experiments.

testes (Anand-Ivell et al, 2006a; Atlantis et al, 2009; Ivell & Anand-Ivell, 2009). Nevertheless, as Leydig cells alter their differentiation state {e.g., in aging (Paust et al, 2002), seasonal breeders (Hombach-Klonisch et al, 2000b; Hombach-Klonisch et al, 2004; Ivell et al, 2003), mouse mutants (Balvers et al, 1998), Leydig cell tumors (Klonisch et al, 1999), androgen-treated men (Anand-Ivell et al, 2006a), or in those subject to a male hormonal contraceptive regimen [(Amory et al, 2007) and (Anand-Ivell and Ivell, unpublished results)]}, INSL3 expression is also correspondingly altered. It can be argued that INSL3 offers a more accurate parameter to determine true hypogonadism than those that are influenced by factors perturbing the HPG axis. This acute independence from the HPG axis is best illustrated by a study of men who have undergone unilateral orchidectomy for testicular cancer (Anand-Ivell et al, 2006a). Testosterone levels in these men are not significantly different from those in normal intact men due to a compensatory upregulation of LH expression by the HPG axis. In contrast, not only are INSL3 serum concentrations significantly reduced in men with one testis, but there is a highly significant inverse correlation to LH levels, which would not be the case if INSL3 was being acutely controlled by the HPG axis (Anand-Ivell et al, 2006a). Given the potential that an independent measure of Leydig cell functionality would have in assessing and treating hypogonadism, for example, in the aging male, it is important to have good model systems in which to explore the appropriate physiology. This study was carried out to extend our understanding of INSL3 physiology in the rodent from the mRNA level to that of the expressed phenotype.

We have developed a sensitive and robust immunoassay using a TRFIA platform, which is able to detect INSL3 in both rats and mice without extraction or concentration. Employing this assay, we have mapped the development of INSL3 expression (secretion) in the postnatal SD rat. Apart from reflecting the pubertal increase in Leydig cell functionality,

the serum INSL3 profile indicates that there is an “overshoot” in Leydig cell functioning on the attainment of adulthood at 40 days (4–10 ng/ml INSL3). This is not evident for testosterone production (data not shown), which, in agreement with numerous other studies, appears to rise consistently from about Day 25 to attain steady adult levels at around Day 50–60 [e.g., (Huhtaniemi et al, 1986; Ketelslegers et al, 1978; Lee et al, 1975)]. In addition to showing high diurnal variability (Leal & Moreira, 1997), testosterone is presumably regulated by the acute feedback mechanisms of the HPG axis, which act to maintain a relatively constant circulating androgen concentration. Only after the completion of several full spermatogenic cycles at ~3 month old do we see a stabilized, lower adult norm level for INSL3 of ~3 ng/ml in this strain. As expected based on earlier mRNA and immunohistochemical studies (Paust et al, 2002), circulating INSL3 is considerably reduced in aging male rats, similar to what is observed in men (Anand-Ivell et al, 2006a). Our results differ somewhat from those reported previously for Holtzman rats (Boockfor et al, 2001), which showed average INSL3 concentrations at Days 40–50 of about 0.6 ng/ml. These were measured using a heterologous assay with anti-mouse INSL3 antibodies and human INSL3 as tracer, which might have underestimated the rat INSL3, and also indicated high variance for the older age groups. Our own data (Table 3.1) also suggest that there may be a marked strain-specific component to the expression of rodent INSL3.

Postpubertal female rats all have circulating INSL3 levels just above the assay detection limit (Table 3.1). The source of this INSL3 is presumably the growing follicles of the ovary, which are recognized in humans and rodents as being able to generate INSL3 (Balvers et al, 1998; Ivell & Anand-Ivell, 2009). Treatment of rats with a single bolus injection of EDS kills all mature Leydig cells (Teerds & Rijntjes, 2007). In the absence of any further treatment, the HPG axis is consequently up-regulated through the loss of

inhibitory androgen, with an increased LH output driving the differentiation of a new population of Leydig cells from resident peritubular stem cells in the testes (Teerds & Rijntjes, 2007). We can follow the differentiation of the new Leydig cells via measurement of INSL3 in circulating blood, and show that, by Day 27, an INSL3 concentration can be determined, equivalent to that observed at about the same time postnatally in untreated rats. This experiment also confirms that the Leydig cells are the only significant source of circulating INSL3 in male rats.

All our studies suggest that INSL3 secretion from cultured Leydig cells is also constitutive. We have failed to find any effector able to influence *Insl3* gene expression at the mRNA level (Balvers et al, 1998; Sadeghian et al, 2005) and now at the level of peptide secretion, in spite of addressing several key second messenger systems. This is interesting, because of a recent report (Lague & Tremblay, 2008) in which it was suggested that high concentrations of androgens, and also phthalates, could act on Leydig cells to modulate *Insl3* gene transcription. We have repeated this study, and fail to see a significant effect at the mRNA level or at the level of the secreted peptide. We do not understand this discrepancy. Together with our earlier results, we strongly support the view that, at the physiological level of the secreted hormone, and also at the level of mRNA expression, INSL3 is unregulated and constitutive, and reflects the functional capacity and/or number of Leydig cells.

In summary, we have now developed a sensitive immunoassay specific for rodent INSL3. We believe that this assay will prove to be very useful as a tool for understanding INSL3 and Leydig cell function and physiology in rodent models in the future.

Chapter 4

**Effect of DBP and DES on Leydig
cell regeneration following EDS
treatment of adult male rats**

4.1 – Introduction

As mentioned in Chapter 1, fetal Leydig cells (FLCs) appear to be one of the early targets of phthalate action. As it is technically difficult to access FLCs, we made use of the EDS-treated adult rat model to look at the effect of phthalates on the differentiation process of adult Leydig cells (ALCs). With the principal endocrine systems of the body already established in the adult rat, and thus unlike the situation in the fetal or pubertal animal, this model provides a tightly regulated and synchronized Leydig cell differentiation process ideal for studies of phthalate action.

EDS is an alkylating agent that selectively ablates the mature, differentiated adult type Leydig cells, as well as differentiated FLCs, in adult rat testis (Molenaar et al, 1985; Morris et al, 1986; Risbridger et al, 1989; Teerds, 1996; Teerds et al, 1990). In addition to its effect in testis, EDS is also able to affect the epididymis, although this effect is generally neglected (Klinefelter et al, 1990; Liu et al, 1993). A single intraperitoneal injection of EDS leads to ablation of both mature adult and fetal Leydig cells via apoptosis within 48 hours following the treatment (Molenaar et al, 1985; Teerds et al, 1990; Yan et al, 2000). The absence of mature Leydig cells in the testis causes a dramatic drop in the circulating level of testosterone, which by negative feedback causes an increase in pituitary LH production (O'Leary et al, 1987), and this in turn stimulates the differentiation of new Leydig cells from presumably resident stem cells. Testosterone withdrawal also eventually disrupts spermatogenesis, with germ cell sloughing occurring around 7 days after EDS treatment (Bakalska et al, 2001; Henriksen et al, 1995; O'Leary et al, 1987). Following EDS treatment, new Leydig cells proliferate and differentiate from resident mesenchymal stem cells to restore the population in the testis in the absence of exogenous testosterone. Regenerated morphologically identifiable Leydig cells are first seen around 14 days after

EDS treatment and the whole population appears to be fully regenerated by 56 days after the treatment (Teerds, 1996; Teerds et al, 1999; Teerds et al, 1990). This regeneration process is temporally comparable to Leydig cell development during puberty and therefore has been widely used to study aspects of Leydig cell differentiation.

The present studies aimed to investigate if the endocrine disruptors DBP and DES, when given at an early stage of Leydig cell regeneration (differentiation), are able to influence the differentiation status and functionality of those cells.

4.2 – Methods

4.2.1 Treatment of rats – 1st study

Adult male SD rats of around 90 days of age were given a single bolus intraperitoneal (i.p.) injection (Day 1) of EDS (75mg/kg body weight, dissolved in DMSO/water, 1:3) to ablate all mature adult Leydig cells within the testes (Fig. 4.1). Following EDS treatment, the rats were divided into 3 groups (EDS only; EDS+DBP; EDS+ DES, n = 6 per group) according to body weight randomization. From Days 5 to 7 after EDS administration, the rats either received vehicle (by oral gavage and subcutaneous (s.c.) injection), DBP (500mg/kg body weight/day in corn oil by oral gavage and vehicle via s.c. injection) or DES (125µg/kg body weight/day in corn oil containing 0.5% ethanol via s.c. injection and vehicle by oral gavage). Animals were killed on Days 10, 14, 19, 27 and 37. Testes and blood were collected, as described in section 2.3.1.1.

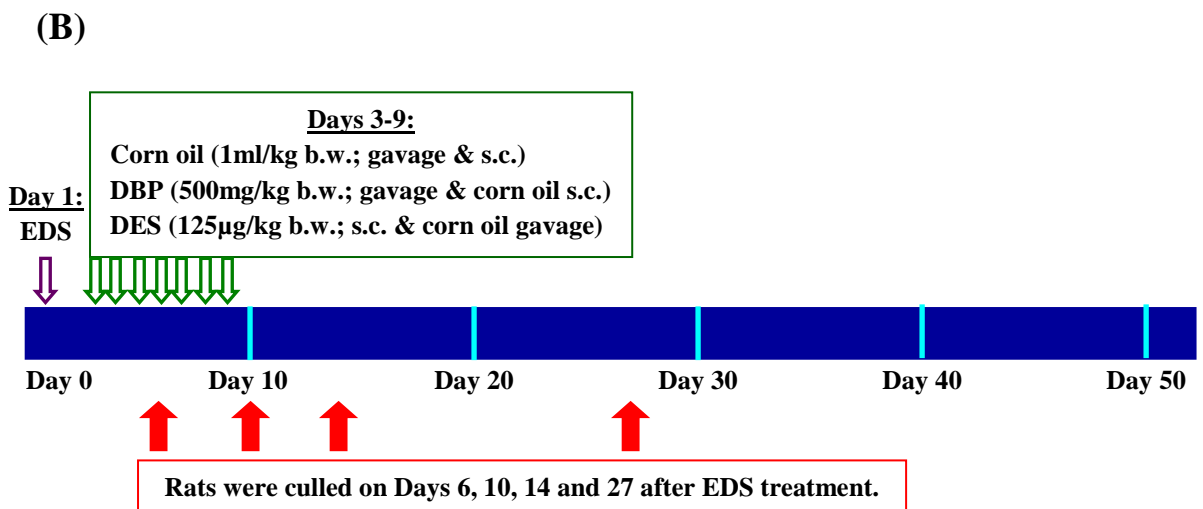
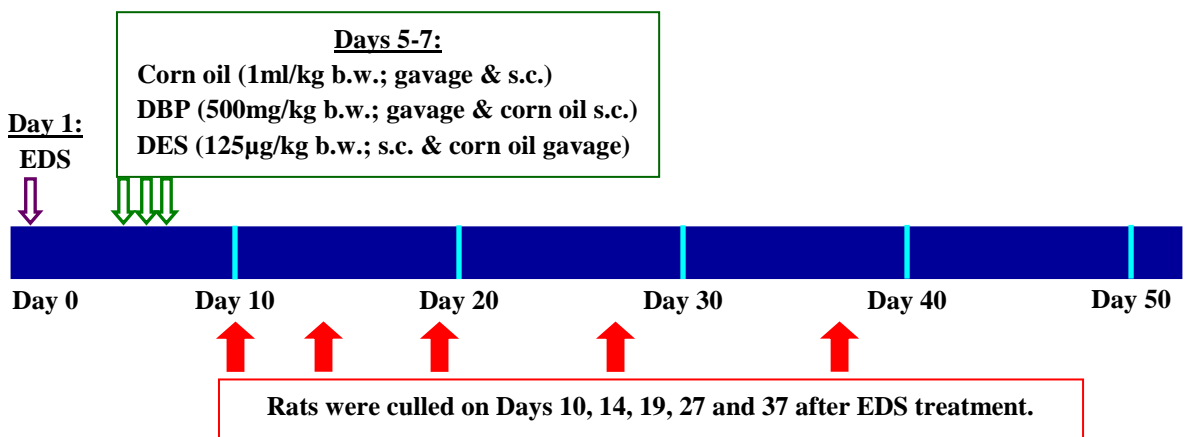
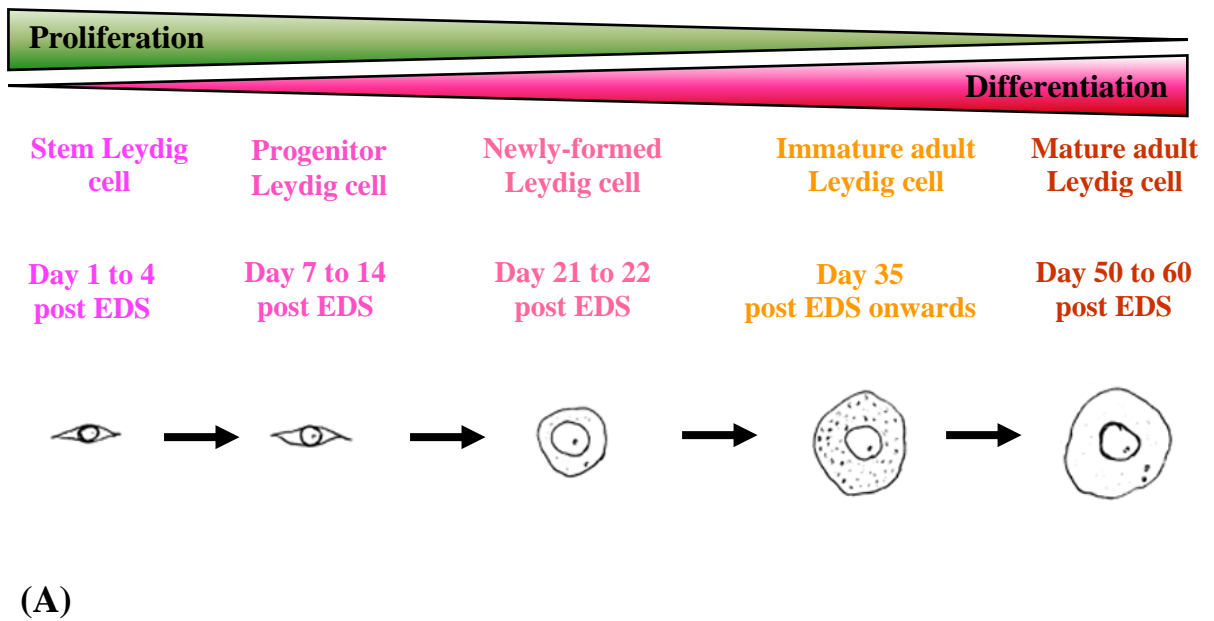


Figure 4.1: Animal treatment periods and sampling time-points for the first(A) and second(B) studies.

4.2.2 Treatment of rats – 2nd study

The 1st study was repeated with modification of the dosing regimen and culling time-points (Fig. 4.1). The animals were dosed exactly as described in section 4.2.1, except that dosing occurred from Days 3 to 9 following EDS treatment, and rats were sacrificed on Days 6, 10, 14 and 27. Only 4 rats were used per group in this experiment. The pre-treatment control rats (n = 4) were killed on Day 0, prior to EDS treatment.

4.2.3 Estimated numerical density of Leydig cells using the ‘physical disector’ method

The numerical density of Leydig cells was estimated using the disector method (referred as ‘physical disector’ in this thesis) (Sterio, 1984). Paraffin-embedded testes were sectioned into 5µm sections using a Leica RM2025 rotary microtome (Leica, Wetzlar, Germany). To ensure that sections selected represent the whole testis, sections were taken from different parts of testis and the sets of random serial sections selected were at least 15 sections apart. Random serial sections were mounted on superfrost slides, stained with PAS and Gill’s #2 Haematoxylin (see section 2.3.4). Microscope pictures were captured on the Cell^F program using a 40× objective on an Olympus BX-51 microscope attached with an Olympus DP72 digital camera. First, a random area on the reference section was viewed by the microscope and the image was captured and saved. The same area on the adjacent section (i.e. look-up section) was also captured and saved. The reference and the look-up images were then viewed side by side on two colour computer monitors. Counting frames (Fig. 4.2) which correspond to 42436µm² on the tissue sections were placed over the reference and look-up images. Leydig cells (Fig. 4.3) were recognised by their oval to spherical nucleus with a thin rim of heterochromatin and prominent nucleolus (Ariyaratne et al, 2000c; Hardy et al, 1989; Teerds et al, 1989). They were easily distinguished from interstitial macrophages, as macrophages have a kidney-shaped nucleus with darkly stained peripheral chromatin and also PAS-positive cytoplasm (Duckett et al, 1997). Leydig cells were counted using the



Figure 4.2: Example of counting frame.



Figure 4.3: Testis section stained with PAS and Gill's haematoxylin. Leydig cells are marked with arrows (↗) while macrophages are marked with arrowheads (↖). Picture was taken with 100× objective.

‘unbiased counting rule of Sterio’ (Sterio, 1984) and the counting frame rule (Gundersen, 1977). Nuclear profiles of Leydig cells fulfilling the following criteria were counted (Fig. 4.4): (1) Appear in one section only (either in the reference or look-up section). Cells that appeared in both sections were not counted; (2) Completely or partially lie within the counting frame and do not intersect the exclusion line. In order to increase speed and efficiency, sections were counted both ways with the look-up section becoming the reference section, and vice-versa (Howard & Reed, 1998; Pakkenberg & Gundersen, 1988). For each treatment group, 4 animals were used for counting and a total of 48 disector areas were counted for each animal. At least 100 cells were scored for each animal.

The estimation of the numerical density of Leydig cells within the testis could be calculated using the formula (Wreford, 1995):

$$N_v = \frac{Q^-}{a \times h}$$

where Q^- = number of nuclei that fulfilled the unbiased counting rule of Sterio and also the counting frame rule; a = total area of disectors examined; h = thickness of tissue sections (i.e. 5 μ m).

4.2.4 Measurement of seminiferous tubules diameter

Paraffin-embedded testes were cut into 5 μ m sections using a Leica RM2025 rotary microtome (Leica, Wetzlar, Germany). To ensure that sections selected represent the whole testis, sections were taken from different parts of the testis and random sections selected were at least 15 sections apart. Random sections were mounted on superfrost slides, stained with H&E (see section 2.3.5). Images were captured from six different parts of each section. Microscope pictures were captured on the Cell^F program using a 10 \times

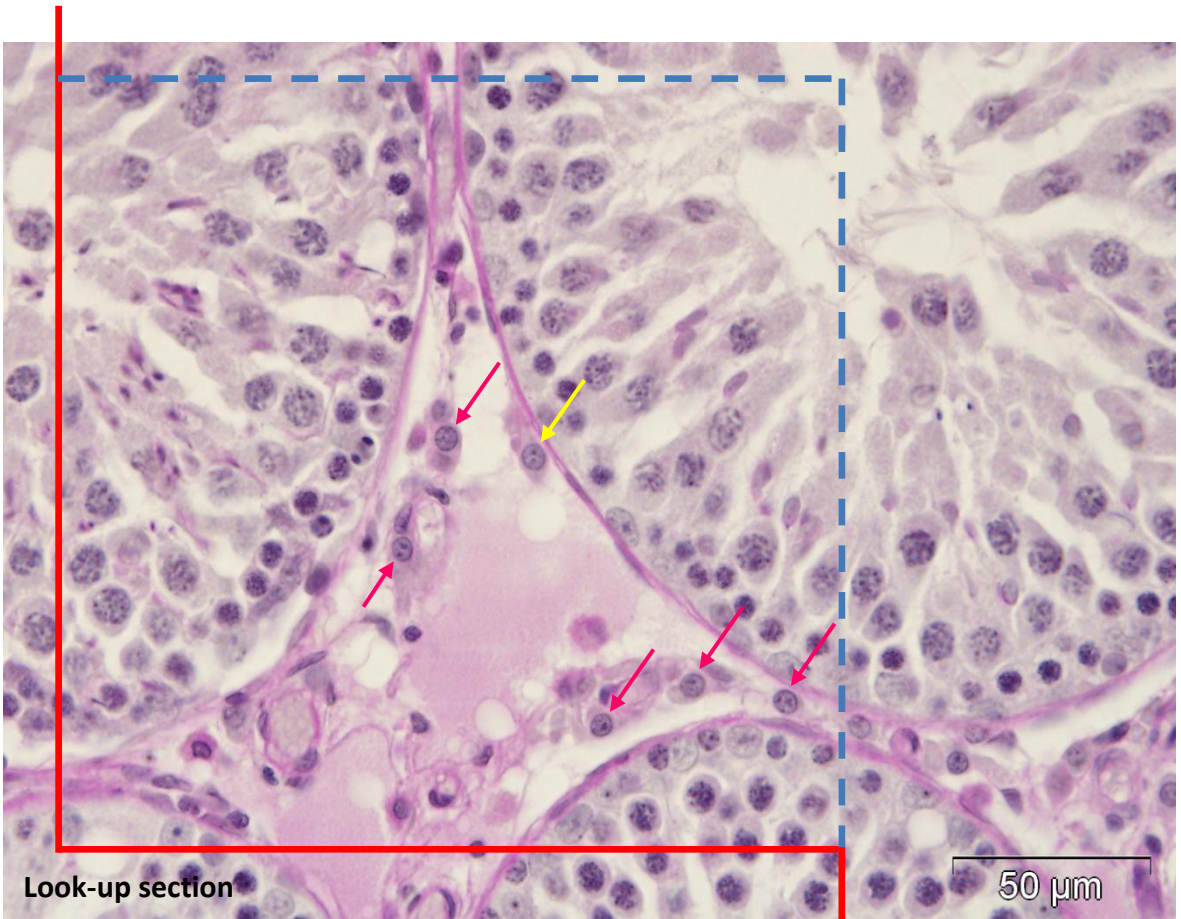
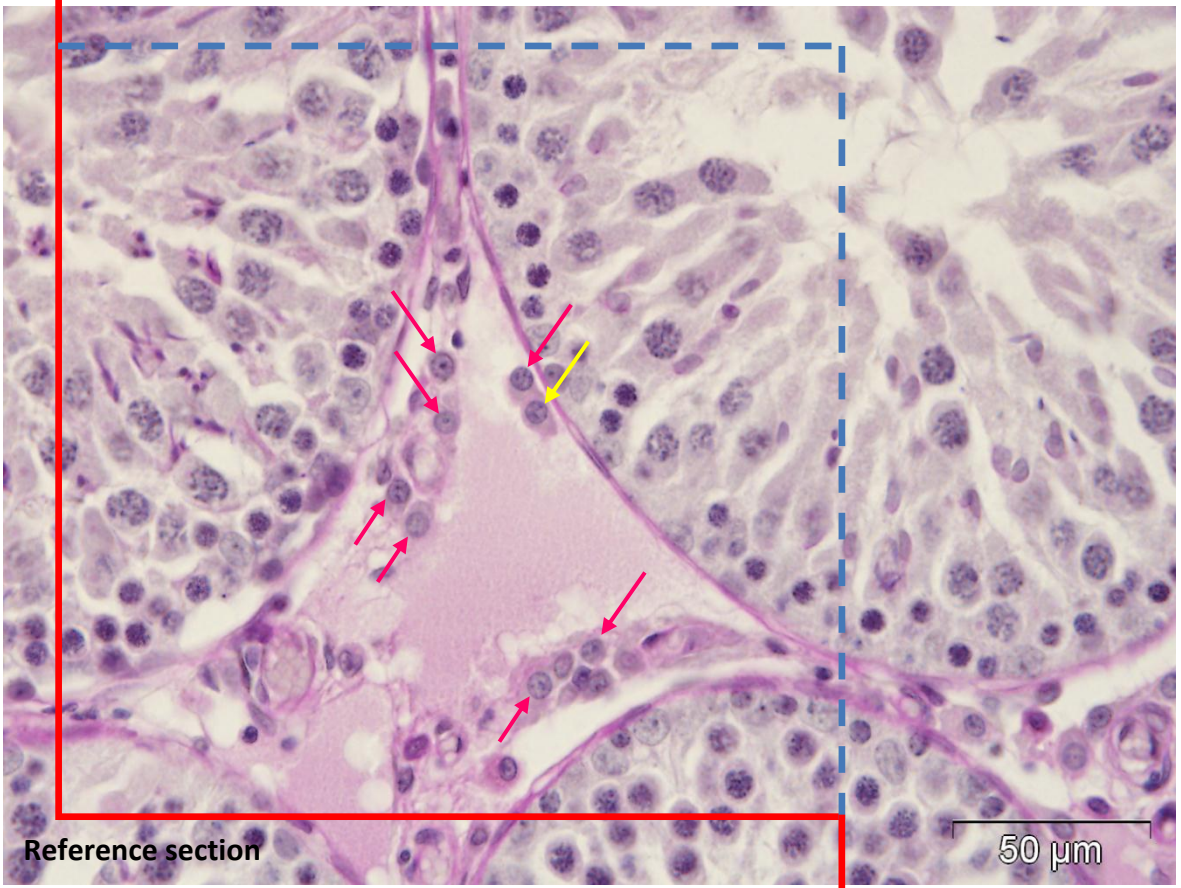


Figure 4.4: The physical disector. Nuclear profiles of Leydig cells fulfilling the following criteria were counted: (1) Appear in one section only (either in the reference or look-up section). Cells that appeared in both sections were not counted; (2) Completely or partially lie within the counting frame and do not intersect the exclusion line. In both sections, Leydig cells indicated by red arrows were counted. Leydig cells indicated by yellow arrows in both sections represented the same cell and were therefore excluded in the counting. Therefore, the total number of cell count from this disector pair is 7 (reference section) + 5 (look-up section) = 12.

objective on an Olympus BX-51 microscope attached with an Olympus DP72 digital camera. The diameter of tubular cross-sections was measured using the Image J program (version 1.44, NIH). For each animal, the diameter of at least 30 tubules was measured and 4 animals were used per group for each time-point (i.e. at least 120 measurements/group/time-point).

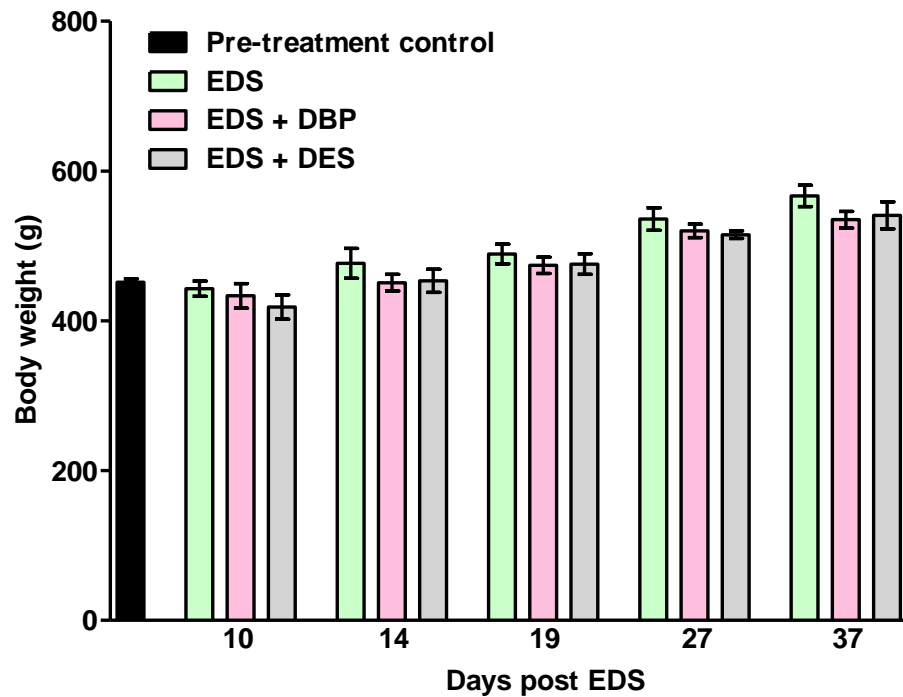
4.3 – Results

4.3.1 Body and testis weight

Two studies with different dosing regimen and sampling period were carried out (Fig. 4.1). In both studies DBP or DES treatments had no effect on the body weight throughout the experiments (Fig. 4.5). All animals had body weight gain comparable to normal growth.

On the other hand, the testis weights were significantly reduced in all EDS-treated rats compared to the pre-treatment control (Fig. 4.6). In the first study, the testis weight in all groups reduced from day 10 onwards after EDS treatment and reached a nadir on day 19. Recovery of testis weight was apparent from day 27 onwards. On day 10, DBP significantly reduced testis weight (Fig. 4.6A). Similar to the first study, DBP appeared to reduce testis weight on day 10 in the second study, although the effect was not significant, with this smaller number of animals. In the second study, testis weight reduction following EDS treatment was comparable to the first experiment. However, the more extensive treatment with DES significantly reduced testis weights on day 14 and 27 compared to control (Fig. 4.6B).

(A)



(B)

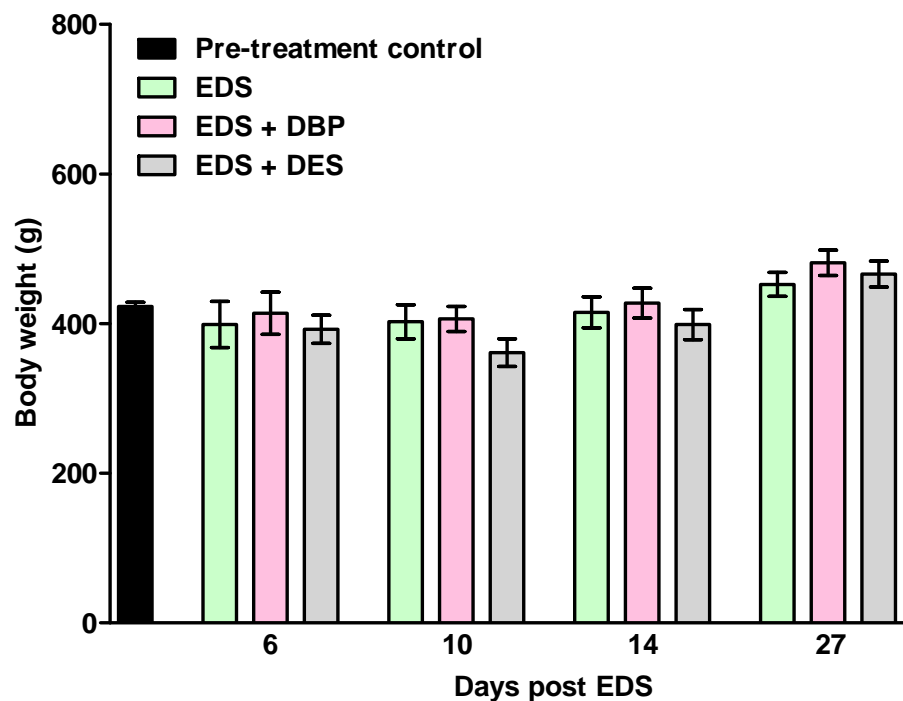


Figure 4.5: Body weight of adult SD rats following EDS and/or DBP or DES treatment from first(A) and second(B) studies. Values are mean \pm SEM for 6 animals (A) or 4 animals (B) at each sampling time-point. Treatment had no significant effect on body weight in both studies.

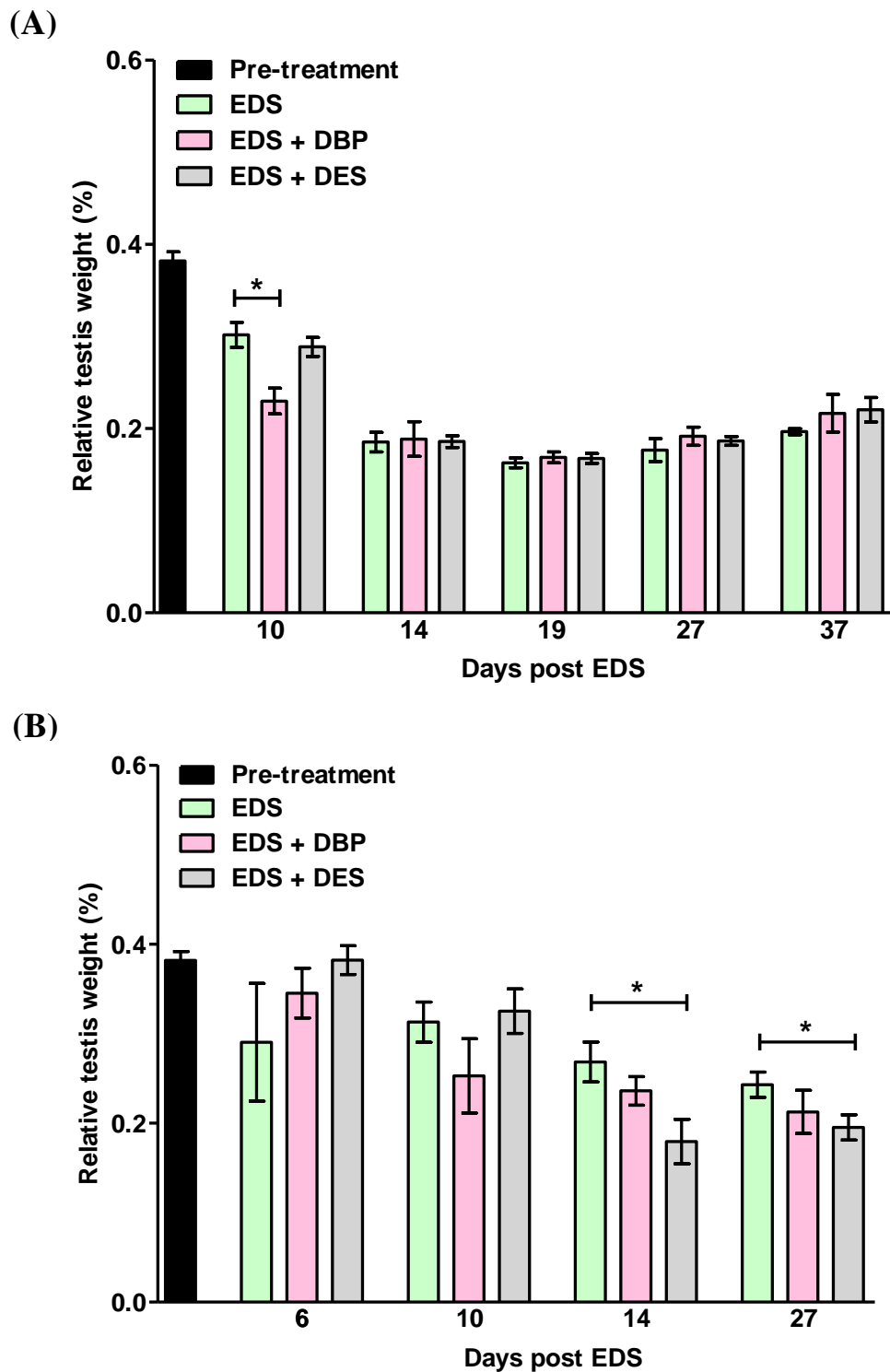
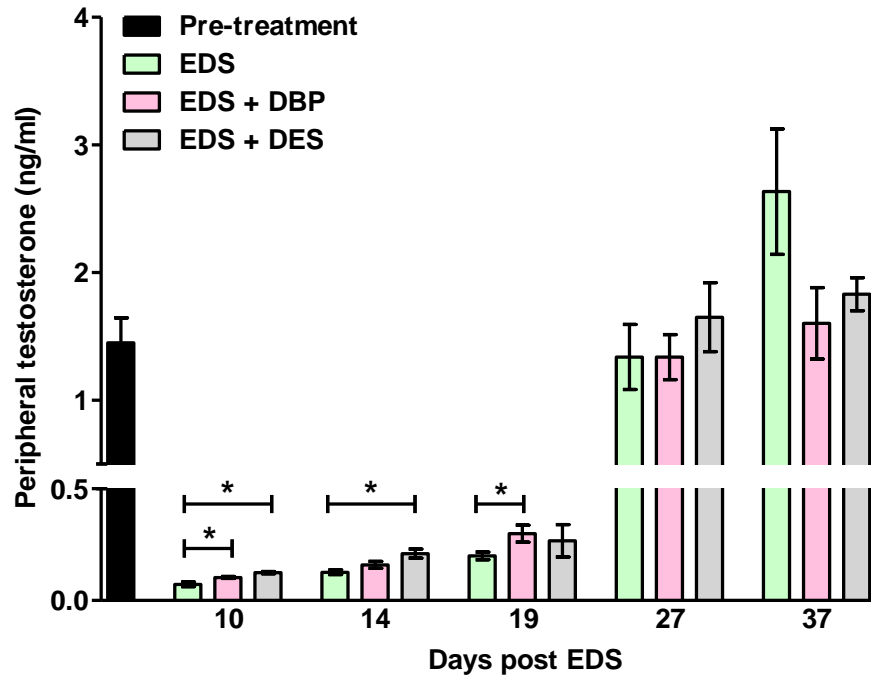


Figure 4.6: Relative testis weight of adult SD rats following EDS and/or DBP or DES treatment from first(A) and second(B) studies. Values are mean \pm SEM for 6 animals(A) or 4 animals(B) at each sampling time-point. Relative testis weight = testis weight/body weight \times 100%. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p \leq 0.05$ in comparison with respective control.

(A)



(B)

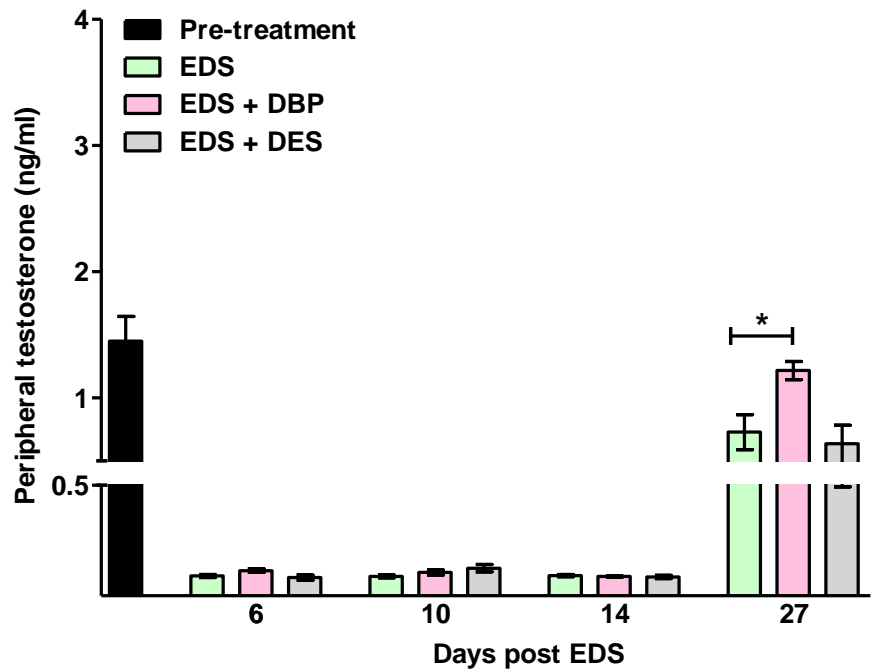


Figure 4.7: Testosterone level in peripheral plasma of adult SD rats following EDS and/or DBP or DES treatment from first(A) and second(B) studies. Values are mean \pm SEM for 6 animals(A) or 4 animals(B) at each sampling time-point. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p \leq 0.05$ in comparison with respective control.

4.3.2 Circulating testosterone and LH

Testosterone in plasma was determined as a measure of steroidogenic capacity of the regenerating Leydig cells. As expected, the destruction of mature Leydig cells in the testes led to a sharp decrease in circulating testosterone in both studies (Fig. 4.7). In the first study, DBP increased the level of testosterone on days 10 and 19 while testosterone level was increased by DES treatment on days 10 and 14 (Fig. 4.7A). The effects of DBP and DES were abolished at the later time-points on days 27 and 37. In the second experiment, however, testosterone production was only enhanced by DBP on day 27 (Fig. 4.7B). DES had no significant effect on the testosterone production. In addition, LH concentration in peripheral plasma from the second study was determined to see whether the effects on testosterone level caused by DBP and DES treatment might be due to a differential regulation of the HPG axis (Fig. 4.8). On day 10, DES appeared to up-regulate the level of LH, though this did not reach statistical significance. Otherwise no significant difference in the circulating level of LH was observed at any time-point.

4.3.3 Circulating INSL3

INSL3 is a major peptide hormone secreted by differentiated Leydig cells. Unlike testosterone, its acute secretion is independent of the HPG axis (Anand-Ivell et al, 2006a; Bay et al, 2005). Therefore, it serves as an excellent marker that directly reflects the differentiation status and/or number of Leydig cells. As expected, the EDS treatment leads to a sharp drop in the circulating INSL3 concentration in both studies compared to the pre-treatment control (Fig. 4.9). DES appeared to cause a further decrease in INSL3 on day 14 in the first experiment. The recovery of INSL3 concentration in the circulation is first evident on day 27 and INSL3 level was significantly enhanced by DBP treatment although this effect was later lost by day 37 (Fig. 4.9A). In the second experiment, both DBP and DES treatments were able to enhance the circulating INSL3 level on day 14 (Fig. 4.9B). A

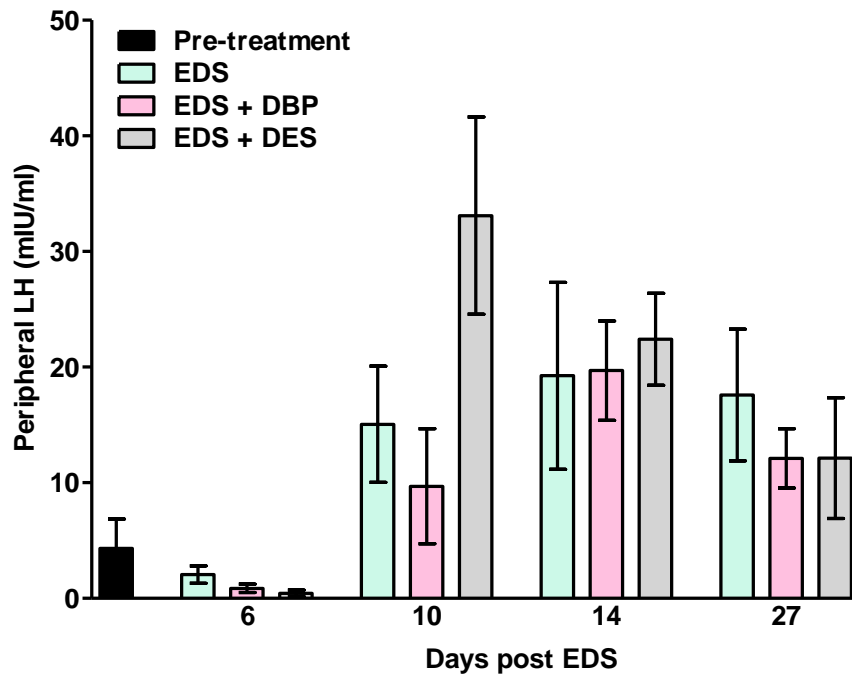


Figure 4.8: Luteinizing hormone level in peripheral plasma of adult SD rats following EDS and/or DBP or DES treatment from the second experiment. Values are mean \pm SEM for 4 animals at each sampling time-point. The LH level was not statistically different among treatment groups at all time-points.

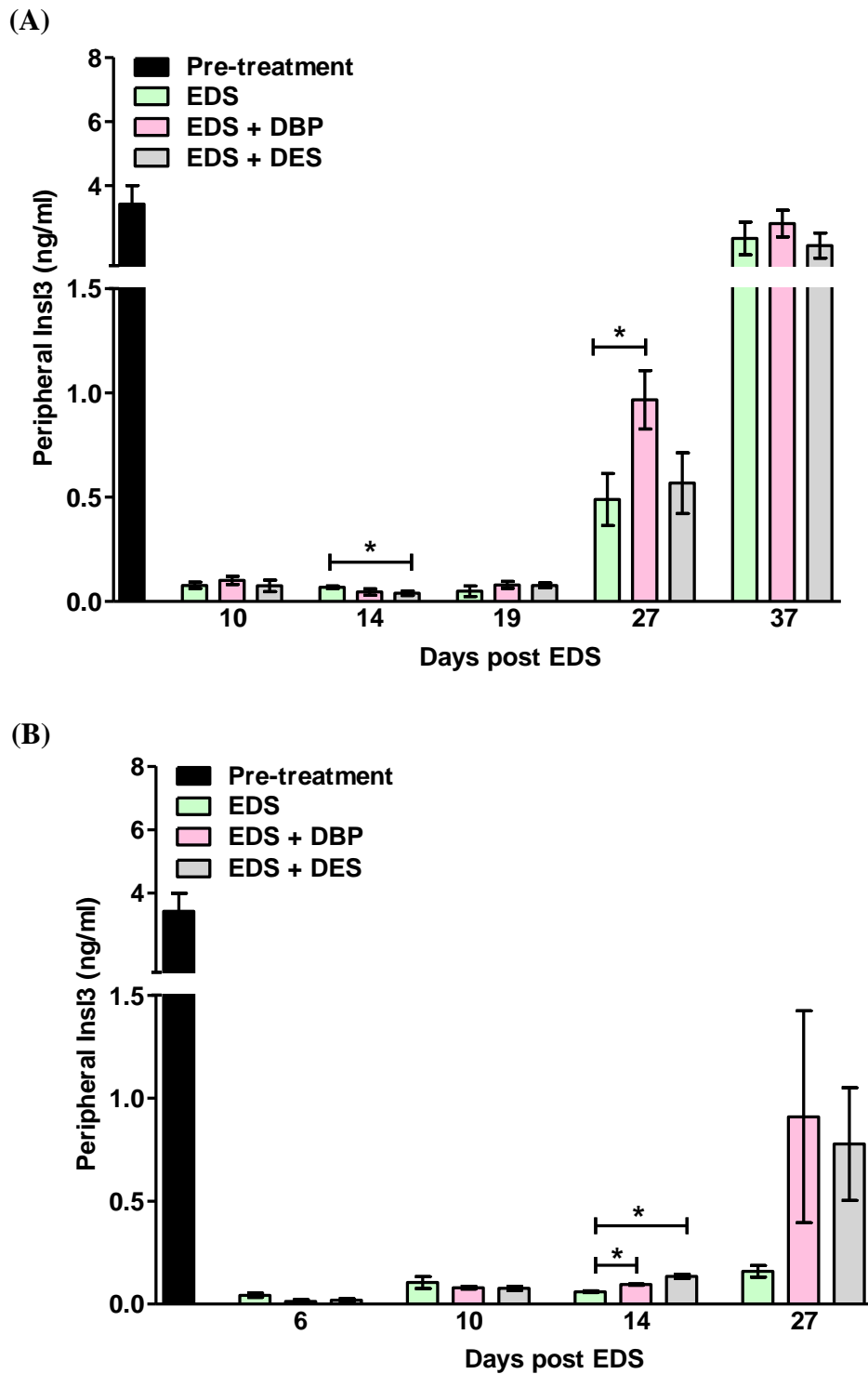


Figure 4.9: INSL3 level in peripheral plasma of adult SD rats following EDS and/or DBP or DES treatment from first(A) and second(B) studies. Values are mean \pm SEM for 6 animals(A) or 4 animals(B) at each sampling time-point. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p \leq 0.05$ in comparison with respective control.

similar trend was also observed for day 27, however, due to high variability in the sample group, the p value obtained was only $p=0.19$ (for DBP) and $p=0.06$ (for DES).

4.3.4 Quantitative real-time PCR analysis for various Leydig cell markers

To examine the differentiation status of Leydig cells, quantitative real-time PCR for various Leydig cells markers was performed on testes samples collected from the second study (Fig. 4.10). Leydig cell markers chosen included steroidogenic enzymes (Cyp17a1, Cyp11a1, StAR and Hsd17b3), LHR, Insl3, Hsd11b1, nicotinic acetylcholine receptor alpha 4 (Chrna4), nuclear receptor subfamily 4 group A member 1 (Nr4a1) and 17-beta-hydroxysteroid dehydrogenase type 10 (Hsd17b10). These markers were chosen due to their differential expression at different stages of Leydig cell development (Anand-Ivell et al, 2009; Ge et al, 2005; Ge & Hardy, 2007; Ivell et al, 2003; Mendis-Handagama & Ariyaratne, 2001).

The LHR, Insl3, Cyp11a1 and Cyp17a1 were chosen as late differentiation markers. As expected, the expression of these markers were decreased below detection level following a bolus EDS injection and the gene expression only recovered from day 27 onwards. The LHR is a well-characterized marker in the EDS model. The primers were designed to select specifically the expression of full-length functional receptor, rather than any of the truncated forms (Apaja et al, 2006; Dufau et al, 1995; Tsai-Morris et al, 1990; Veldhuizen-Tsoerkan et al, 1994). The expression of LHR remained low after EDS treatment until day 27 where DBP significantly enhanced its expression (Fig. 4.10). DES also showed a similar effect although this did not reach statistical significance. Similarly, expression of Insl3 and Cyp11a1 remained undetectable until day 27 post EDS where the expression of both markers was significantly increased by DES treatment. The expression of transcripts for another steroidogenic enzyme Cyp17a1 had a similar profile of recovery to those for

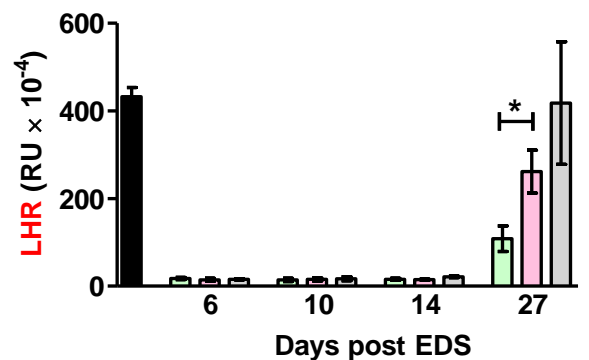
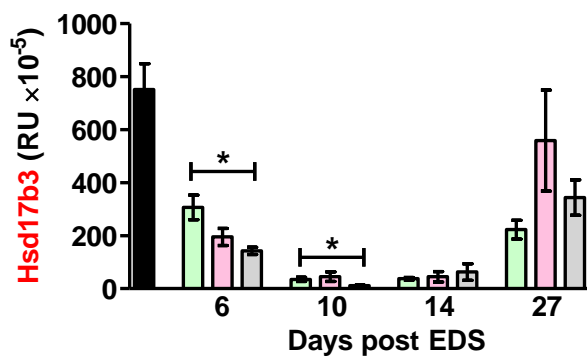
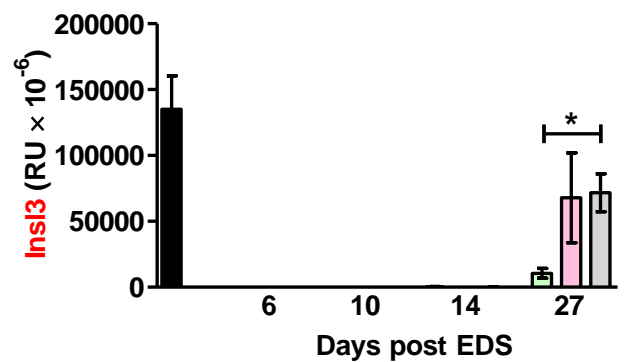
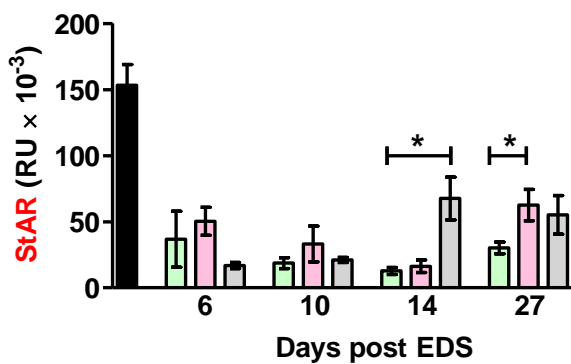
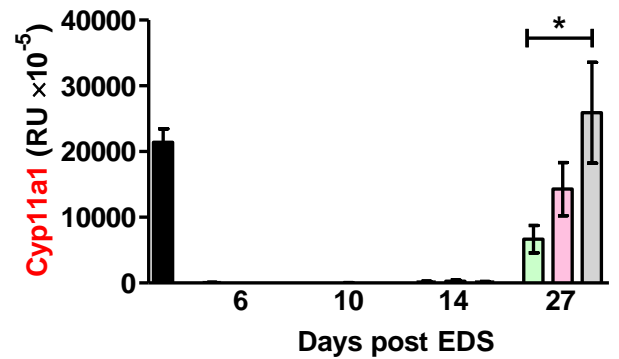
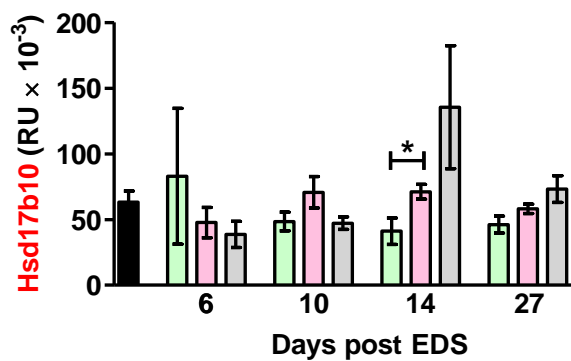
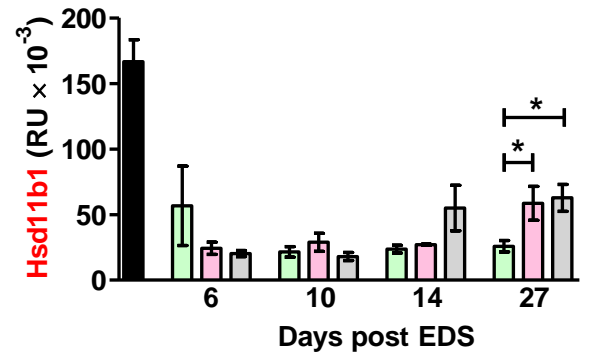
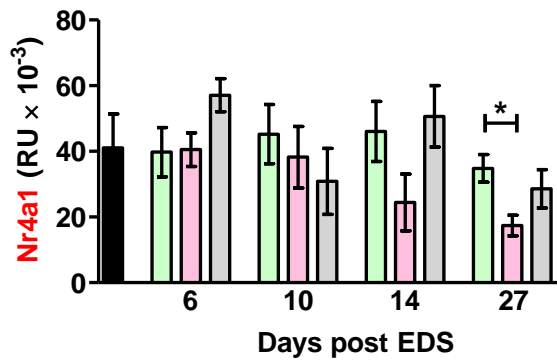
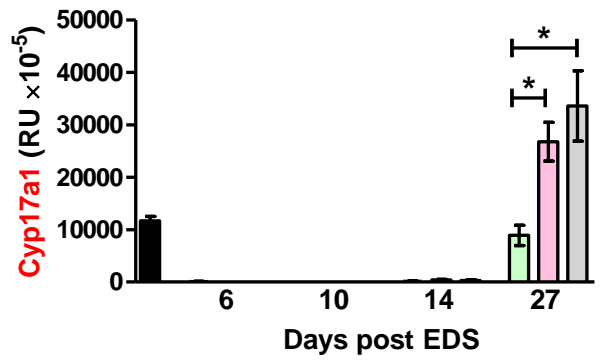
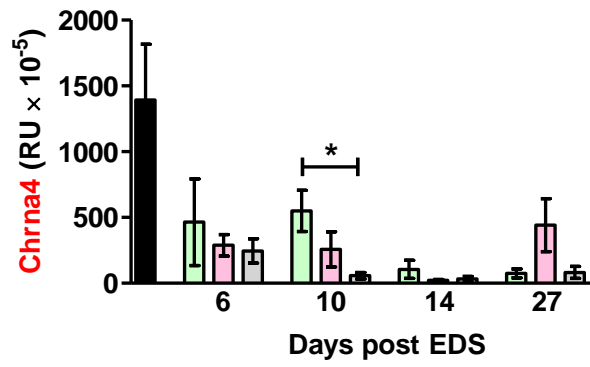


Figure 4.10: mRNA expression of various Leydig cell markers in the adult SD rat testis following EDS and/or DBP or DES treatments from the second experiment.

Values are mean \pm SEM for 4 animals at each sampling time-point. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p \leq 0.05$ in comparison with respective control.

Chrna4: nicotinic acetylcholine receptor alpha 4; Nr4a1: nuclear receptor subfamily 4 group A member 1; Hsd17b10: 17-beta-hydroxysteroid dehydrogenase type 10; StAR: steroidogenic acute regulatory protein; Hsd17b3: 17-beta-hydroxysteroid dehydrogenase type 3; Cyp17a1: cytochrome P450 17 α -hydroxylase/17,20 lyase; Hsd11b1: 11-beta-hydroxysteroid dehydrogenase type 1; Cyp11a1: cytochrome P450 cholesterol side chain cleavage enzyme; Ins13: insulin-like factor 3; LHR: luteinising hormone receptor.

the LHR. The expression of Cyp17a1 mRNA was enhanced by both DBP and DES treatment, to a level that is even higher than the pre-treatment control, on day 27 after EDS treatment.

On the other hand, expression of mRNA for Chrna4, StAR, Hsd17b3 and Hsd11b1 was evident already from day 6 onwards. Both DBP and DES significantly increased the expression of Hsd11b1 on day 27. Similarly, StAR expression was enhanced on day 14 and 27 by DES and DBP treatment, respectively. Chrna4 belongs to a superfamily of ligand-gated ion channels that play a role in fast signal transmission at synapses. It was previously reported that Chrna4 expression increases as Leydig cells differentiate (Ge et al, 2005). DBP and DES had no effect on Chrna4 expression during the later time-points. However, DES significantly reduced the Chrna4 expression on day 10. Finally, DES treatment caused significant reduction in the expression of mRNA for the steroidogenic enzyme Hsd17b3 on days 6 and 10.

In contrast to the markers above, the level of Nr4a1 and Hsd17b10 was not reduced by EDS treatment, suggesting that testicular cell types other than adult-type Leydig cells probably also expressed these markers. Therefore, the significant effect caused by DBP on day 27 for Nr4a1 expression and on day 14 for Hsd17b10 transcript expression may not reflect the effect of DBP on Leydig cell maturation.

4.3.5 Numerical density of Leydig cells

Unbiased stereological cell counting was only applied in the second study for day 27 samples, a time-point when upon preliminary visual inspection of sections, effects of treatment on the regenerating Leydig cell population appeared to be most evident. In DBP and DES treatment groups, Leydig cells were present in larger clusters as compared to



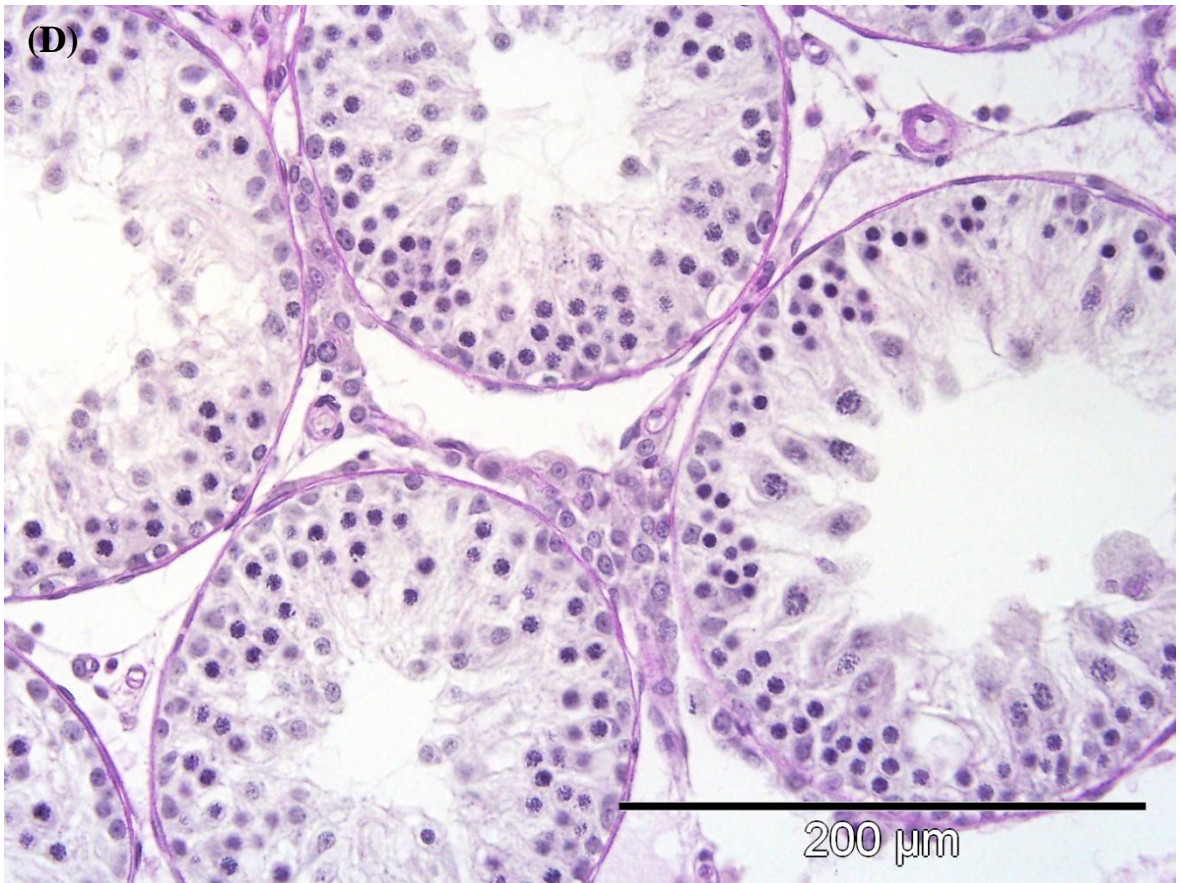


Figure 4.11: PAS and Haemotoxylin staining of rat testes showing Leydig cell clusters on day 27 following treatment in the second experiment. (A) Pre-treatment control (B) EDS only (C) EDS + DBP (D) EDS + DES.

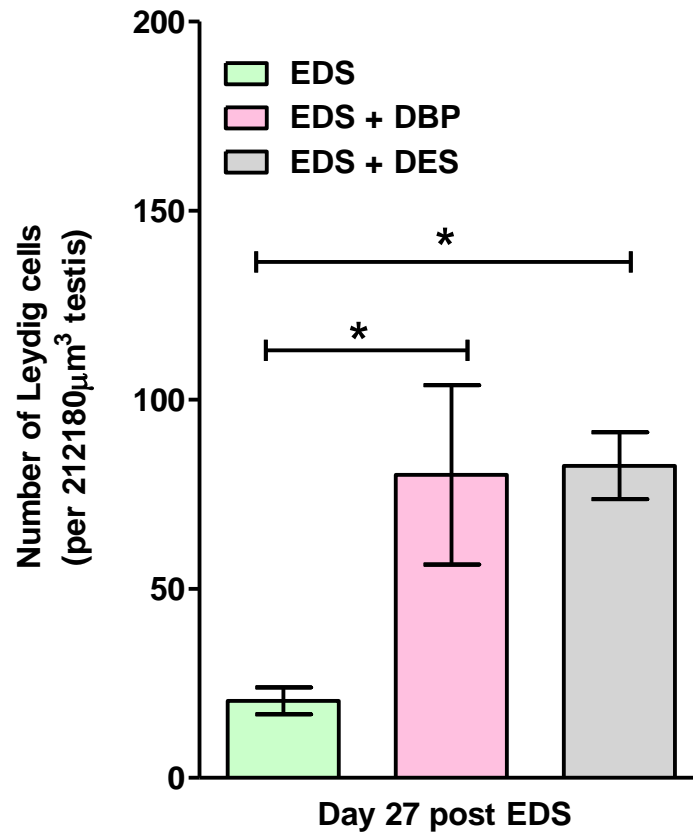


Figure 4.12: Cellular density of Leydig cells on day 27 following EDS and/or DBP or DES treatments from the second study. Values are mean \pm SEM for 4 animals at each sampling time-point. * $p < 0.05$ in comparison with respective control.

control (Fig. 4.11). Indeed, cell counting using the physical disector method confirmed that both DBP and DES treatments significantly increased the numerical density of Leydig cells within the rat testes, suggesting that the treatments caused an increase in proliferation of Leydig cells (Fig. 4.12). The number of Leydig cells could only be expressed per fixed volume of testis here since the testis has already been sectioned before being analyzed by physical disector. Hence, we could not estimate the volume of testis by using the Cavalieri method (Andrade et al, 1995; Kiki et al, 2007). From the tubule diameter data, although there is significant increase or decrease in DBP and DES tubules, respectively, on day 27, the change is relatively small compared to other time-points ($p=0.02$).

4.3.6 Histology of testis

To investigate the possible underlying reasons that caused significant loss in testis weight, testes samples from day 10 from the first experiment and also testes from days 14 and 27 from the second study were examined for any structural differences. In the first study, DBP treatment appeared to cause quicker germ cell sloughing in the seminiferous tubules on day 10 (Fig. 4.13). In the second study, the diameter of seminiferous tubules in the DES group appeared to be smaller on days 14 and 27 due to progressive loss of germ cells (Figs. 4.14 and 4.15). Indeed, the diameters of seminiferous tubules from DBP and DES groups were significantly smaller than the EDS-only group on days 10, 14 and 27 following EDS treatment, except for a small but significant increase in the DBP group on day 27 (Fig. 4.16). In general, the diameter data followed the same trend as the testis weight (Fig. 4.6), suggesting that reduction in testis weight could be primarily due to the loss of germ cells. Upon examining the testis sections, we noticed that one animal (which had a smaller testis at culling) from the EDS-only group had higher loss of germ cells, thereby causing a marked decrease in the average tubule diameter in the EDS-only group on day 6 (Fig.4.16).

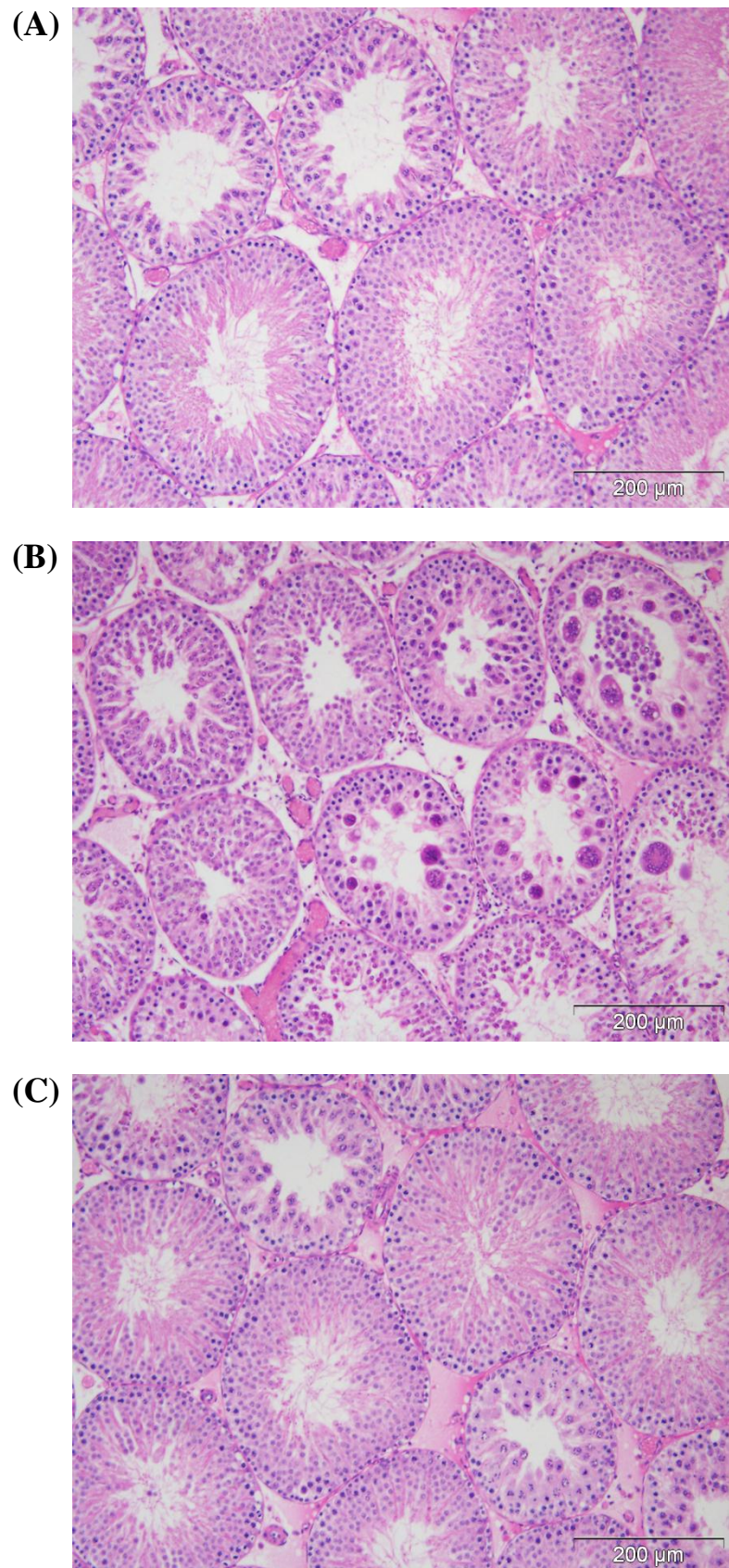


Figure 4.13: Haematoxylin and eosin staining of rat testes on day 10 following treatment in the first experiment. (A) EDS only (B) EDS + DBP (C) EDS + DES.

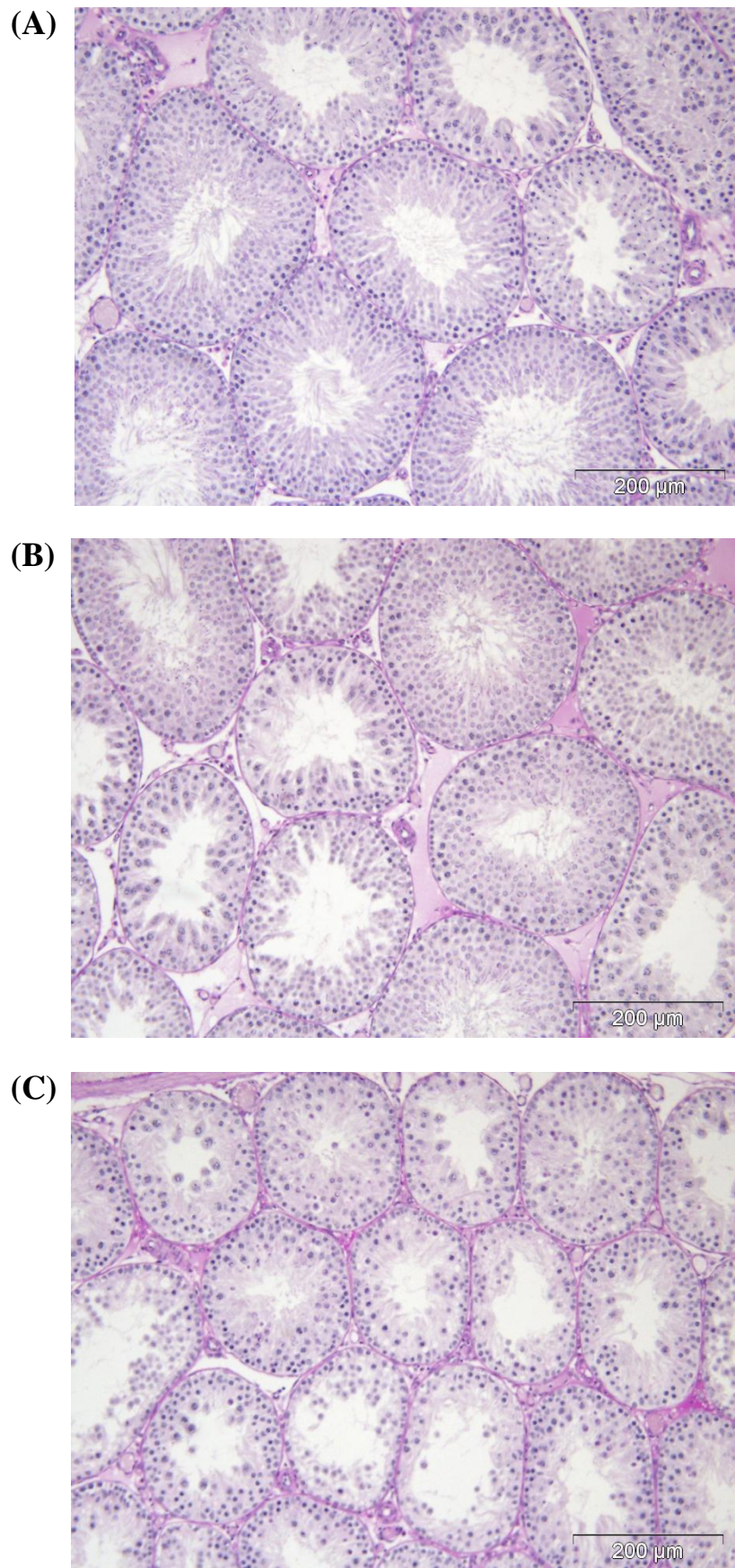


Figure 4.14: PAS and haematoxylin staining of rat testes on day 14 following treatment in the second experiment. (A) EDS only (B) EDS + DBP (C) EDS + DES.

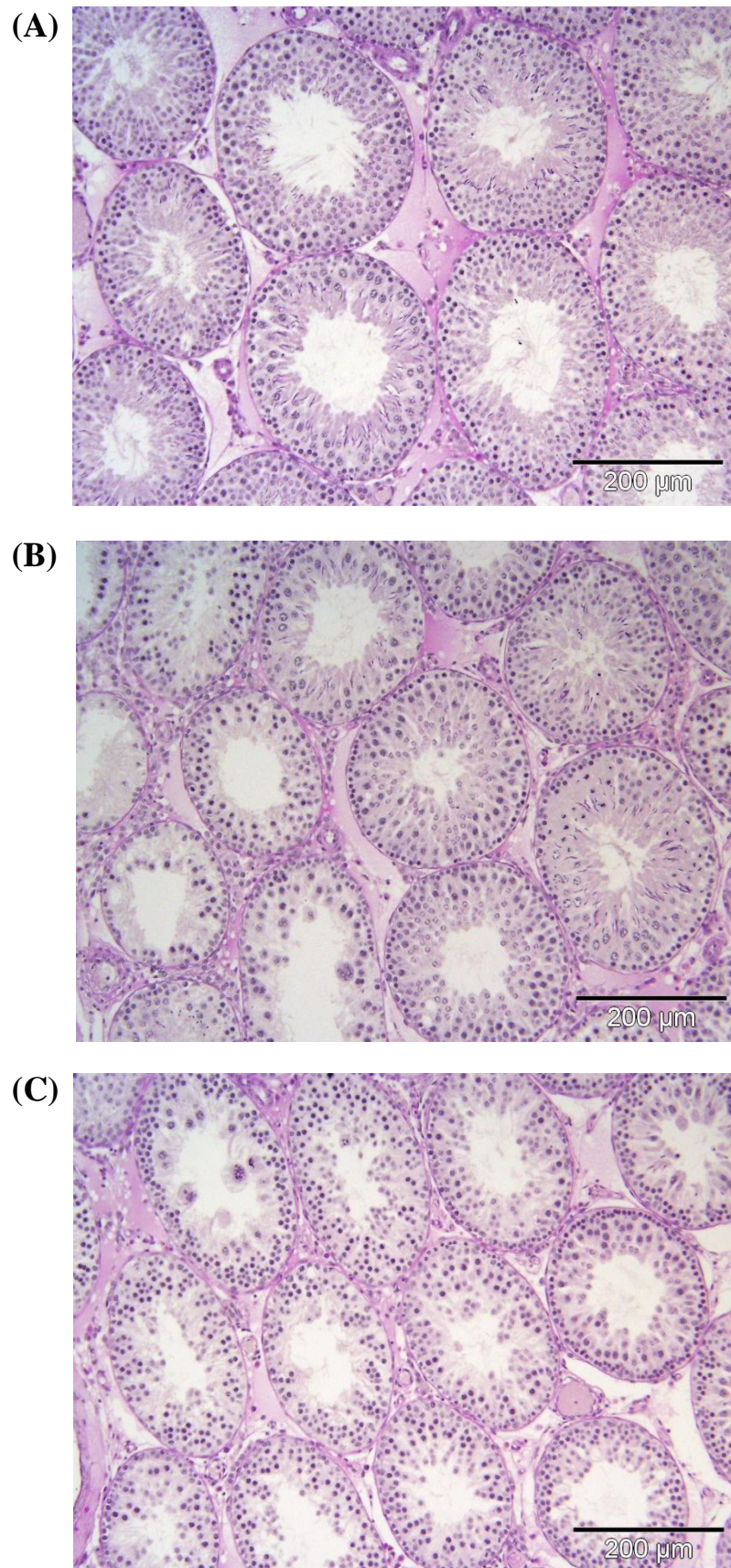


Figure 4.15: PAS and haematoxylin staining of rat testes on day 27 following treatment in the second experiment. (A) EDS only (B) EDS + DBP (C) EDS + DES.

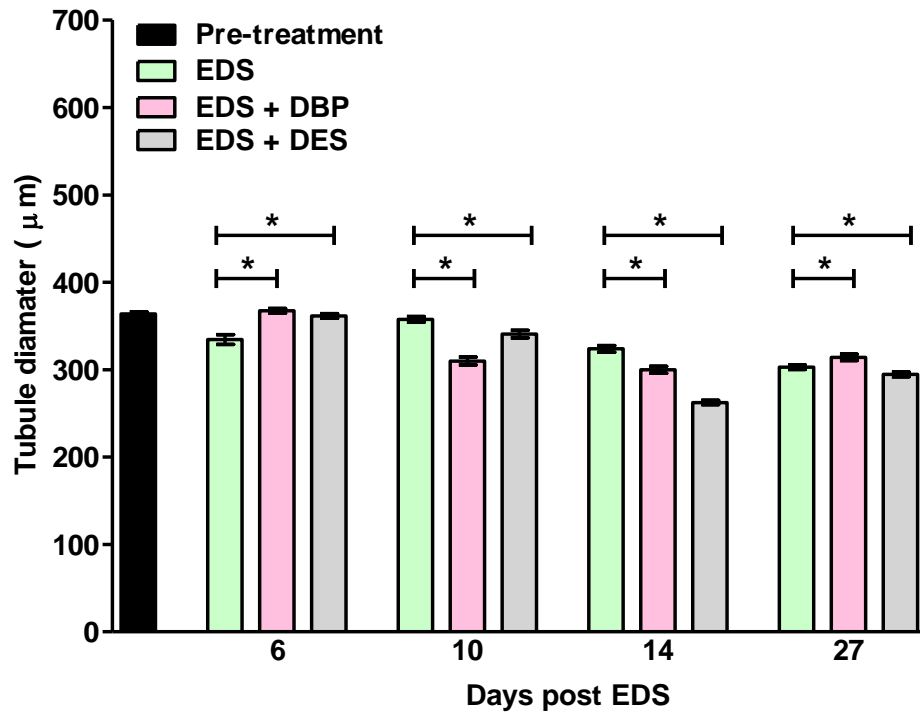


Figure 4.16: Diameter of seminiferous tubules of adult SD rats following EDS and/or DBP or DES treatment from the second experiment. Values are mean \pm SEM for at least 120 tubule measurements (i.e. at least 30 measurements per animal for 4 animals) at each sampling time-point. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ in comparison with respective control.

However, when this animal was eliminated (data not shown), no difference in the tubule diameter was observed between different treatment groups on day 6.

4.4 - Discussion

A single dose of ethane dimethane sulfonate is able to selectively eliminate all mature Leydig cells in the adult rat testes, but otherwise causes little effect in the animals (Teerds, 1996). Mature Leydig cells are eliminated within 48 hours after the treatment. It is believed that following the EDS treatment, mesenchymal stem cells within the testis, probably from around the seminiferous tubules, proliferate and differentiate to repopulate the interstitial compartment. The present exploratory study aimed to investigate whether treatment of DBP or DES during the early phase of Leydig cell regeneration could have any effect on the Leydig cell differentiation process.

DBP and DES had no effect on body weight throughout the experiment compared to control, indicating that neither endocrine disruptors had any systemic toxicity at the dosage given. Over the duration of the study, all animals had body weight gain comparable to normal growth. Survival of germ cells is primarily maintained by the production of testosterone by Leydig cells (Bakalska et al, 2001; Nandi et al, 1999; O'Leary et al, 1987) in the presence of sufficient pituitary hormones. The reduction in testis weight after EDS treatment observed was due to loss of germ cells following testosterone withdrawal as previously reported (O'Leary et al, 1987; Woolveridge et al, 1999). In the first study, DBP appeared to increase the rate of germ cell sloughing, thereby causing a greater loss in testes weight on day 10. However, this effect was not observed in the second study, probably because the kinetics of germ cell loss were changed with the more extensive treatment. On the other hand, extensive DES treatment in the second study appeared to retard testis

recovery as seminiferous tubules were still devoid of postmeiotic germ cells on days 14 and 27, compared to control, resulting in lower testis weight in the DES group on both days.

Circulating total testosterone and INSL3 were used as indices of Leydig cell maturity. In both studies, the testosterone level decreased significantly after EDS treatment and recovery was observed from day 27 onwards, consistent with previous reports where increased testosterone could be detected 20 days after EDS treatment (Ariyaratne et al, 2000c; Tena-Sempere et al, 1999; Tena-Sempere et al, 1997; Yan et al, 2000). There is little effect of DBP or DES on the testosterone level in both studies, except that the testosterone level was significantly elevated by DBP on day 27 in the second study. In addition, neither DBP nor DES appeared to have any significant effect on serum LH. Following androgen withdrawal by EDS treatment, serum LH was elevated due to the negative-feedback mechanism of the HPG axis. The level of serum LH was reported to increase gradually, reach its peak value around days 10-20 and return to baseline level around day 40 after EDS injection (O'Leary et al, 1987; Tena-Sempere et al, 1997; Yan et al, 2000). In our study, we detected considerable variability in the serum LH level. Although efforts have been taken to ensure that the rats were sacrificed at the same time of the day, the natural pulsatility of LH secretion into the blood stream might be the cause of such variability. Nevertheless, the measured level of serum LH in the control group was still within the previously reported range (O'Leary et al, 1987; Tena-Sempere et al, 1997; Yan et al, 2000). The absence of a peak in LH level could be due to the time-point chosen in this study. To address the issue of effects directly on the HPG axis would require a much more intensive sampling strategy, because of LH pulsatility, than was possible within the present study. The lack of any effect of DBP and DES on the circulating LH level suggests that DBP might be acting directly on the Leydig cells themselves rather than on other

components of the HPG-axis. Exposure of a closely related phthalate, DEHP, during the young adult period (PND62-PND90) in rats also had no effect on the levels of serum testosterone and LH (Akingbemi et al, 2001). It is thus likely that a well-established HPG axis in adult animals is insensitive to phthalate treatment compared to developing animals.

In this context, INSL3 may be a better marker to reflect the differentiation status of Leydig cells. It was previously reported that INSL3 secretion is not acutely regulated by the HPG axis (Anand-Ivell et al, 2006a; Bay et al, 2005). Therefore, the circulating level of INSL3 secreted by Leydig cells directly reflects the maturity and functionality of those cells, as well as their total number. Making use of the time-resolved fluorescent immunoassay that we recently developed for rat INSL3 (Anand-Ivell et al, 2009), for the first time we have been able to report the serum level of INSL3 in rats after EDS treatment. In the first study, DBP appears to increase INSL3 production on day 27 although this effect was later lost on day 37. In the second study, both DBP and DES were able to increase INSL3 production on day 14. A similar trend was shown for day 27, however, due to variance within the group, the results did not reach the level of statistical significance. It is important to note that the concentration of INSL3 found in the blood reflects its mRNA level within the testis. Previously, colleagues in our lab showed that INSL3 is constitutively expressed (Sadeghian et al, 2005). Therefore, the effect of DBP and DES on INSL3 is likely to be due to its long term effect on Leydig cell differentiation, rather than any acute effect on the gene expression.

Besides *Insl3*, mRNAs encoding the LHR and two steroidogenic enzymes, *Cyp17a1* and *Cyp11a1*, also showed expression profiles that closely resembled that for the serum INSL3 concentration. It is interesting to note that those genes that were affected by DBP and DES treatment were among those classified as the late Leydig cell differentiation markers which

were only detectable on day 27 after EDS. Truncated forms of LHR that encode regions of the extracellular domain of the receptor have been reported to be expressed persistently after EDS treatment (Tena-Sempere et al, 1994; Veldhuizen-Tsoerkan et al, 1994). The full-length functional LHR was shown to be expressed much later as Leydig cells differentiate. Hsd11b1 is only expressed in Leydig cells within the adult rat testis (Waddell et al, 2003). This enzyme is first detectable in newly formed adult Leydig cells on PND21 rat testis; its expression increases as Leydig cells differentiate into the mature ALCs (Ge et al, 2005). After EDS treatment, Hsd11b1 protein expression was first detected on day 21 using immunohistochemistry (Ariyaratne et al, 2003). Again, similar to the late Leydig cell differentiation markers, enhancement of Hsd11b1 expression by DBP and DES on day 27 suggested that the Leydig cell differentiation might be accelerated by these treatments.

In contrast to the late differentiation markers, DBP and DES appeared to have little effect on the expression of *Chrna4*, *StAR* and *Hsd17b3*. In 2005, Ge et al reported the expression of *Chrna4* in rat Leydig cells for the first time (Ge et al, 2005). Similar to *Hsd11b1* mRNA, expression of *Chrna4* mRNA increases as the Leydig cells differentiate, with its expression highest in the ALCs in PND90 rat testis (Ge et al, 2005). A possible explanation why no effect was observed in the present study could be that the Leydig cells have not yet reached the stage of differentiation when *Chrna4* expression is maximal.

In the initial literature search for possible Leydig cell markers, we found that *Nr4a1* appeared to be one of the most suitable early Leydig cell markers for use in real-time PCR for the following reasons: (1) Its expression is the highest in PLCs isolated from PND21 rat testis, and its expression decreases as Leydig cells differentiates into ILCs and mature ALCs (Ge et al, 2005). (2) There was no information suggesting expression of *Nr4a1* in testicular cell types other than Leydig cells (Johnson et al, 2007; Martin & Tremblay,

2010; Song et al, 2001; Song et al, 2004). However, the real-time PCR results here show that the opposite might be true. Since the level of Nr4a1 did not decrease with EDS treatment, it is likely that Nr4a1 is being expressed also by cells of the seminiferous compartment within the adult rat testis. Further real-time PCR using isolated Leydig cells and seminiferous tubules needs to be carried out to clarify this issue.

Similarly, the consistent level of Hsd17b10 gene expression after EDS suggested that it might be expressed in other testicular cells. In postnatal rat testis, Hsd17b10 protein expression became detectable on PND20 in Leydig cells and reaches its maximal expression around PND35 (Ivell et al, 2003). In addition, expression of this protein was also detected in late spermatids from PND45 onwards. During aging, the protein expression of Hsd17b10 was markedly reduced in Leydig cells only (Ivell et al, 2003). To our surprise, the expression of Hsd17b10 was not affected at all by EDS treatment, even after considerable germ cell apoptosis. The reason for this is unclear at the moment and this needs to be clarified with further investigation.

The relative increases in serum INSL3 and various Leydig cell transcript markers suggested that DBP and DES were able to accelerate Leydig cell proliferation and/or differentiation. Stereological analysis was carried out to investigate if the increase caused by DBP and DES was due to (1) increase in differentiation rate or (2) increase in Leydig cell number. Stereological assessment showed that both DBP and DES caused an approximate 4-fold increase in Leydig cell number per $212180\mu\text{m}^3$ of testis, and also appeared to result in the formation of larger Leydig cell clusters compared to control. It is important to note that this large increase in Leydig cell number was not reflected in the level of Leydig cell markers and serum INSL3. Therefore, it is likely that DBP and DES increased the proliferation rate of Leydig cells but also slowed down the differentiation

rate at the same time. Although the level of late Leydig cell markers were elevated by DBP and DES treatments, the capacity of each Leydig cell expressing these markers or producing INSL3 might be lower compared to control. Due to the experimental design, it was not possible for us to measure the testosterone and INSL3 production per Leydig cell in this study.

Taken together, the results showed that endocrine disruptors DBP and DES, when given during the early stage of the Leydig cell regeneration process after EDS treatment, were able to increase gene expression of Leydig cell markers by increasing the proliferation of Leydig cells but probably slowing the differentiation rate at the same time. It is currently unclear at which stage the proliferation is enhanced by these endocrine disruptors. Due to limitation of the experimental design in this exploratory study, we were not able to measure the level of alternative proliferation markers in the testes using methodology such as real-time PCR or thymidine labeling since these markers are likely to be expressed in other testicular cell types.

Chapter 5

**Long term effects of *in utero* DBP
or DES treatments on Leydig cell
development in male progeny**

5.1 – Introduction

Over the last 50 years, reproductive problems in humans appear to have increased, with a rising incidence of cryptorchidism and hypospadias in male new-borns and testicular cancer in young men (Skakkebaek et al, 2001). These reproductive problems are collectively termed the testicular dysgenesis syndrome (TDS) and are thought to have a fetal origin. Because of the geographical difference in the occurrence of TDS, where it is more prevalent in the West, studies have suggested that exposure to environmental toxins might be one cause of this syndrome. Indeed, treating rodents with different endocrine disruptors such as vinclozolin, phthalates, estrogens and flutamide in the laboratory have shown similar effect to those symptomatic of the TDS (Auharek et al, 2010; Emmen et al, 2000; Gray et al, 2000; Uzumcu et al, 2004; van der Schoot, 1992; Wolf et al, 2004).

Previously, many studies have reported that *in utero* exposure to DBP was able to cause various malformations of the reproductive system in male fetuses, including cryptorchidism, reduction in testis and sex accessory organ weight, reduced AGD, occurrence of Leydig cell aggregates, reduction in testosterone level and presence of multinucleated gonocytes (MNG) (Drake et al, 2009; Macleod et al, 2010; Mylchreest et al, 2002). Fetuses in these studies were exposed to DBP mostly from the second half of gestation and reproductive malformations were mainly assessed in male fetuses at the end of gestation. However, it still remains unclear if these malformations, especially the effects on Leydig cells, persist through to adulthood and if the fertility of the male progeny is affected.

Scarano et al (2010) reported that exposure to 100mg/kg body weight/day of DBP from gestation day 12 (GD12) to PND21 caused the occurrence of Leydig cell aggregation and

MNG in the fetal testis. However, at adulthood, testis weight and sperm parameters in the DBP-treated animals were similar to controls (Scarano et al, 2010). In contrast, Barlow and Foster (2003) showed that although the Leydig cell aggregates found in the fetal testis were resolved during the early postnatal period following *in utero* DBP exposure, there was severe degeneration of the seminiferous epithelium that did not manifest until PND70 (Barlow & Foster, 2003). A study by Mahood et al (2007) reported that male progeny that were exposed to DBP prenatally had a high incidence of cryptorchidism, reduced fertility and the presence of Sertoli-cell only (SCO) tubules at adulthood (Mahood et al, 2007).

Although reproductive deformities have been reported in these studies, it was not very clear if the differentiation of Leydig cells is affected. The present study aimed to look at the long term effect of *in utero* and lactational DBP exposure on testicular development, especially Leydig cell development, in the male progeny by assessing the number of Leydig cells and expression of various Leydig cell markers.

5.2 – Methods

5.2.1 Treatment of rats

9-week old female SD rats were mated overnight and checked the next morning for the presence of sperm in vaginal smears. The morning when sperm were observed in the vaginal smears was designated as gestation day 0.5 (GD0.5). The day when the pups were delivered was designated as postnatal day 1 (PND1). To eliminate possible estrogenic effects from the standard diet, pregnant rats and their offspring in this study were maintained on the soy-free standard AIN93G rodent diet (Specialty Feeds, Western Australia, Australia) throughout the experiment. Pregnant rats were divided into 3 groups,

using randomization of body weights to ensure equal weight distribution among groups, and dosed as following:

- (1) Control group (n =8) : Orally gavaged with corn oil 1ml/kg body weight/day every second day from GD14.5 to PND6 and subcutaneously (s.c.) injected with corn oil containing 0.5% ethanol on GD14.5 and GD16.5.

- (2) DBP group (n =9) : Orally gavaged with DBP 500mg/kg body weight/day in corn oil every second day from GD14.5 to PND6 and s.c. injected with corn oil containing 0.5% ethanol on GD14.5 and GD16.5.

- (3) DES group (n =12) : Orally gavaged with corn oil 1ml/kg body weight/day every second day from GD14.5 to PND6 and s.c. injected with DES 125µg/kg body weight/day in corn oil containing 0.5% ethanol on GD14.5 and GD16.5 only.

On PND2, excess pups were culled so that only 10 pups (female pups: male pups \cong 1:1) were retained per litter. On PND21, both male and female pups were weaned and caged with their littermates of the same gender. Male and female progeny were culled on PND10, 24 and 90. Testes and blood were collected as described in section 2.3.1.1.

5.2.2 Measurement of anogenital distance (AGD) in offspring

The anogenital distance is the distance between the anus and the genital opening (Fig. 5.4A). To investigate the effect of indirect DBP or DES exposure, either *in utero* or through lactation, the AGD was measured for all pups before they were culled on PND2 and PND10. Briefly, pups were photographed from the ventral aspect on PND2 and PND10 with a precise 1mm scale included in each picture as reference point. Subsequently, all pictures were imported to the same computer and the same computer

screen was used for all measurement. Before measuring the AGD distance, the ratio of enlargement was first determined (this varies from screen to screen). The distance between the tip of the genital opening and anus was measured and converted to the actual distance using the enlargement ratio determined previously. This video measurement of AGD was repeated a few days later for the same images. The AGD for each animal was the average of the two values obtained from both measurements. All measurements were carried out by an Honours student, Mr. Damien Hunter, who was blinded to the identity of the treatment groups.

5.2.3 Repetitive collection of blood using the tail bleed technique

To determine the level of INSL3 in peripheral blood postnatally following prenatal and perinatal exposure to DBP or DES, blood was collected by tail bleeding from male offspring (n = 6-8) at different postnatal ages (i.e. PNDs30, 35, 40, 45 and 55) in addition to the PND10, PND24 and PND90 time-points. Briefly, rats were restrained in a clean towel. To dilate the lateral tail vein, the tail was immersed in warm tap water for around 3 minutes. Blood was collected from the lateral tail vein (initial collection was done from the tip of the tail, and the collection point was moved upwards towards the base of the tail for subsequent collections) using heparinised 26G needles, and approximately 300µl blood was collected each time. Following collection, blood was kept on ice until being centrifuged in the Eppendorf Centrifuge 5415R at 10,000rpm at 4°C for 20 minutes. Plasma was collected from the top clear layer and stored at -20°C until further analysis with rat INSL3 TRFIA.

5.2.4 Estimation of total number of Leydig cells using the optical disector method

The total number of Leydig cells per testis was estimated using the fractionator and optical disector method (Gundersen, 1986; Wreford, 1995). This method has been shown to be a

precise method to estimate the total number of various testicular cell types within the whole testis (Wreford, 1995). The procedure was carried out as previously described (Gould et al, 2007).

Briefly, paraffin-embedded testes were fractionated into 10-15 pieces of similar thickness from which three slices were randomly selected (i.e. the testis is cut into around 10-15 pieces depending on testis size using a razor blade. At this step, it is best to cut the testis into pieces of similar thickness; the thickness of each testis slice is not measured directly, since it will be measured in terms of number of 30 μ m sections when the testis slice is sectioned in the next step). The randomly selected testes slices were then sectioned exhaustively into 30 μ m sections throughout. The number of 30 μ m sections resulting from each selected testis slice was counted. Summation of the number of 30 μ m sections from all selected testis slices = Number of total sections for f_2 , which is the proportion of 30 μ m sections used (i.e. when we select 3 pieces for sectioning into 30 μ m sections, if slices #1, #2 and #3 give 50, 75 and 61 sections, respectively, then the number of total sections (for f_2) will be 50+75+61 = 186, see below for f_2 equation.). One section was randomly selected from each testis block and mounted on HistoBond® adhesive microscope slides. For each animal, a total of three sections were stained with PAS and haematoxylin (see section 2.3.4) and used for Leydig cell counting.

Microscope images were captured on Spot analysis software (Diagnostic Instruments Inc., Sterling Heights, MI, USA) using a 100 \times oil free objective (NA 0.95, for PND24 testes) or 40 \times oil free objective (NA 0.65, for PND90 testes) on an Olympus BX-51 microscope attached with a Spot RT colour camera and OptiScan™ Motorized Stage Systems. The OptiScan™ motorized stage was programmed to move across the testis section in steps of 400 μ m (for PND24 testis) or 1mm (for PND90 testis) across the x-axis, then 400 μ m (for

PND24 testis) or 1mm (for PND90 testis) in the y-axis, randomly and systematically to traverse the entire testis section to ensure that each cell has an equal chance of being counted (Fig. 5.1D). A 10cm × 10cm counting frame (Fig. 4.2) was placed over the microscope image on the computer screen. The section is imaged through the z-axis, initially incorporating a guard zone of 2µm. To avoid artifacts of the cutting surface, no Leydig cells were counted within the guard zone. The movement in the z-axis was measured by the attached microcator. Leydig cells fulfilling the counting frame rule (Gundersen, 1977) were counted as they come into focus when the section was sectioned optically. To avoid the same cell being counted twice, counting was done on the computer screen on a piece of acetate transparency. The nuclei of Leydig cells were marked on the transparency as they came into focus on the screen, if they lay within the counting frame without intersecting the exclusion line. Leydig cells (Fig. 4.3) were recognised by their oval to spherical nucleus with a thin rim of heterochromatin and prominent nucleolus (Ariyaratne et al, 2000c; Hardy et al, 1989; Teerds et al, 1989). Finally, the testis sections were captured with 1.25× objective for determination of the surface area of each testis section.

The total number of Leydig cells within the whole testis could be determined using the formula below (Howard & Reed, 1998):

$$\hat{N} = Q \times 1/f_1 \times 1/f_2 \times 1/f_3$$

where

- \hat{N} = Estimated number;
- Q = Number of Leydig cells counted in disector;

- $f_1 = \text{fraction of tissue} = \frac{\text{Number of randomly selected slices}}{\text{Number of total slices}}$

For example, if a testis was fractionated into 13 slices and 3 slices were randomly selected then $f_1 = 3/13$ (Figs. 5.1A & 5.1B);

- $f_2 = \text{fraction of sections} = \frac{\text{Total number of randomly selected sections}}{\text{Number of total sections}}$

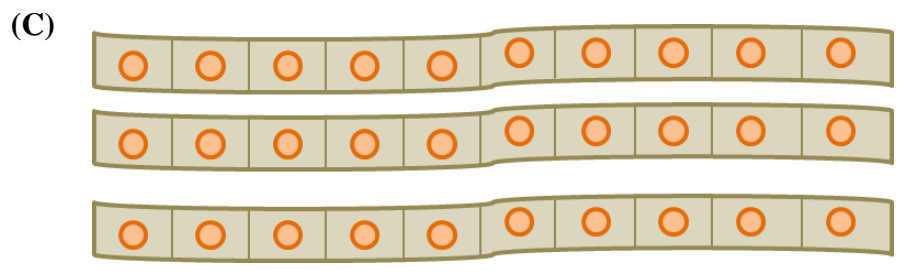
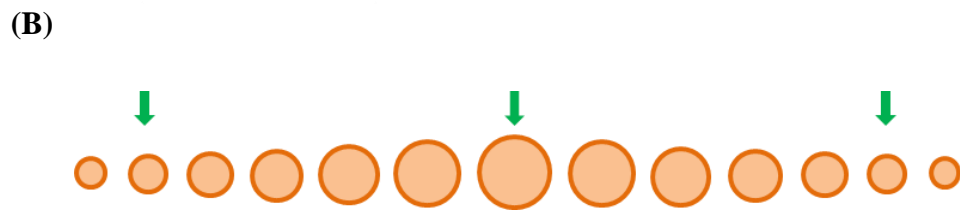
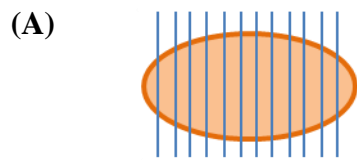
For example, if a total of 186 sections resulted from exhaustive sectioning of the 3 randomly selected testis slices from which three 30 μm sections were randomly selected then $f_2 = 3/186$ (Fig. 5.1B);

- $f_3 = \text{sampling fraction} = \frac{\Sigma \text{Volume of disectors}}{\Sigma \text{Volume of the entire testis section}}$

Under the oil-free 100 \times objective, the 10cm \times 10cm disector square was equivalent to 37 μm \times 37 μm on the testis section. Under the oil-free 40 \times objective, the 10cm \times 10cm disector square was equivalent to 92.5 μm \times 92.5 μm on the testis section.

Hence, the total sampling volume = area of disector square \times total height of section at all sampling point (from all selected testis slices).

Volume of the entire testis section = average thickness of the testis section \times surface area (from all selected testis slices) (Fig. 5.1D).



M240
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26
27
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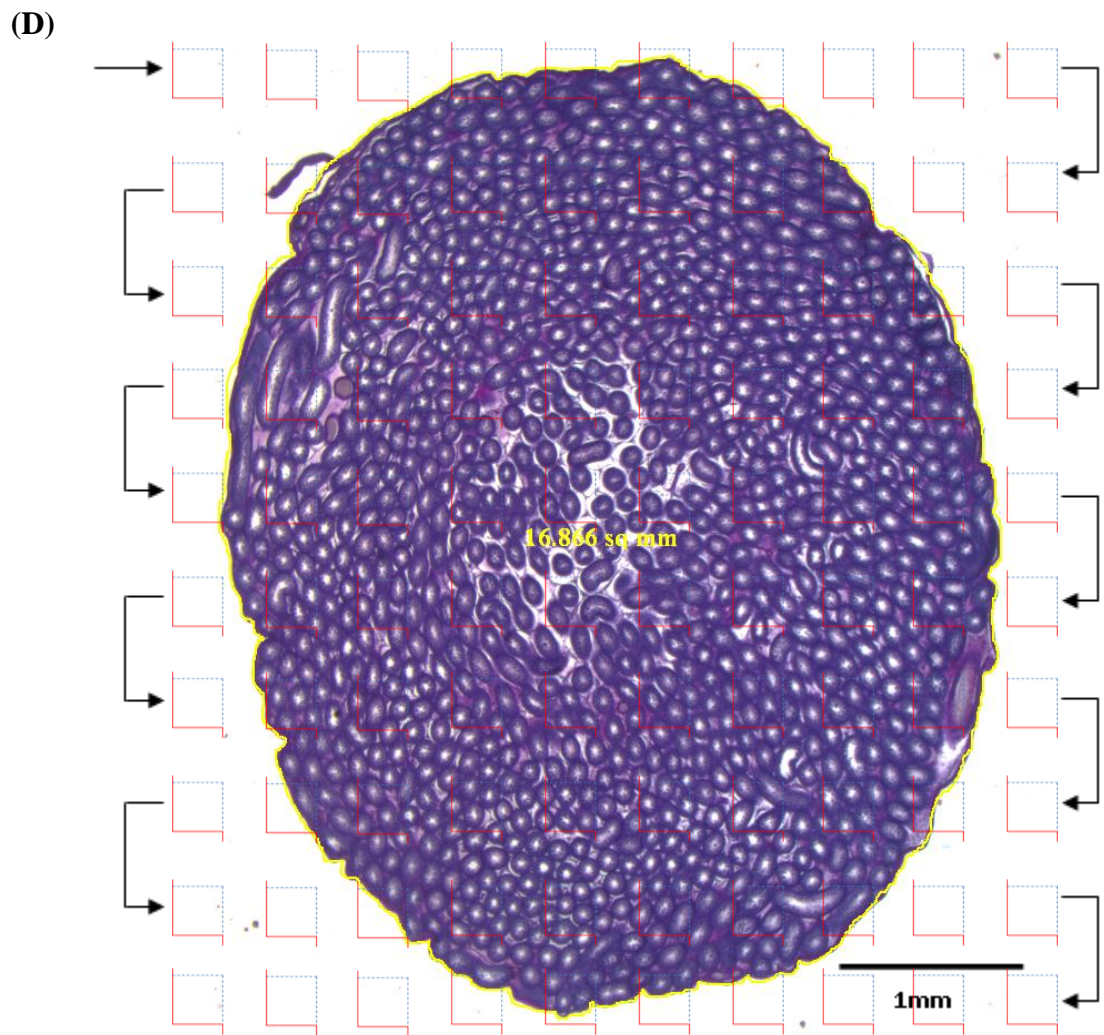


Figure 5.1: An illustration of the fractionator and optical disector. (A) Each testis was cut into slices of even thickness. (B) The slices were laid out according to their position in the testis. Using a randomly selected number, 3 slices were selected. (C) Randomly selected slices from (B) were exhaustively sectioned into 30 μ m sections throughout. The number of 30 μ m sections from each testis slice was counted. Summation of the number of sections from all selected testis slices = Number of total sections for f_2 (see section 5.2.4). Using a randomly selected number, one section was selected to be mounted, stained with PAS and counted. Sections before and after the selected sections were also mounted as back-ups. Therefore, for each testis sample, three 30 μ m sections were used for Leydig cell counting using the optical disector method. (D) The testis section was captured with a 1.25 \times objective and the surface area was measured (yellow line) and used in f_3 (see section 5.2.4). Starting at a random point, the motorized microscope stage was programmed to traverse the whole testis section at defined distance in a snake-like pattern for the random selection of counting field.

5.2.5 Vaginal opening in female offspring

The onset of sexual maturity in female pups was determined by the separation of the vaginal membrane (i.e. vaginal opening). The female progeny were checked daily from PND29 onwards for the day when the vaginal membrane separated.

5.3 – Results

5.3.1 Overall wellbeing of the pregnant rats and offspring

To investigate if indirect exposure to DBP during late gestation and lactational period (i.e. every second day from GD14.5 to PND6) could affect later testicular development in male progeny, pregnant rats were given either corn oil (control), DBP or DES treatments. DES was used as the positive control in this study because it was previously reported that fetuses which were exposed to DES *in utero* had a phenotype similar in many ways to those who had *in utero* DBP exposure (Emmen et al, 2000). Administration of DES to rats in excessive doses has previously also been associated with abortion of fetuses (Kawaguchi et al, 2009). In order to prevent abortion of fetuses, the pregnant rats were dosed only on GD14.5 and GD16.5. Pregnant rats in all groups looked healthy throughout the duration of the experiment. However, the weight gain in DES group was significantly lower compared to the control group on GD20.5 (Fig. 5.2).

All of the pregnant rats in the control (n=8) and DBP (n=9) groups delivered live pups. 2 out of 12 ($\cong 16.67\%$) of the pregnant rats in the DES group produced still-births. For these 2 pregnant rats, all pups were underdeveloped and born dead. The number of total pups per litter in the DES group appeared to be lower than the other 2 groups [i.e. if the 2 rats with still-birth were included then $p=0.05$ (i.e. take total number of pups born = 0 for these

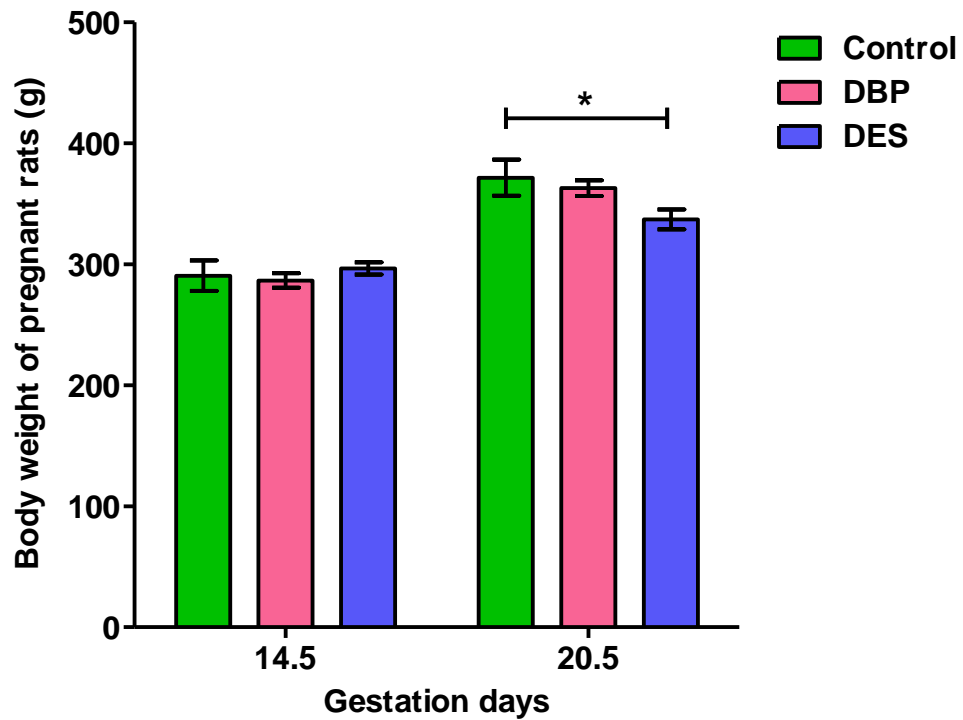


Figure 5.2: Body weight of pregnant rats at the start of treatment (GD14.5) and at the end of gestation (GD20.5). The weight gain by pregnant female rats in the DES group was significantly lower compared to control group [n=8 (control), 9 (DBP) and 12 (DES)]. Data were presented as mean \pm SEM. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ in comparison with respective control.

pregnant rats); otherwise there was no significant difference from control if these 2 rats were excluded ($p=0.16$), see Table 5.1]. In addition, the mortality rate of pups per litter in the DES group was also the highest among the 3 treatment groups (Table 5.1). Pups exposed to DBP indirectly also appeared to have a higher mortality rate compared to control animals, however this did not reach the level of statistical significance with the p -value attaining 0.132 (Table 5.1). Pups in the DES group appeared to be smaller at birth (data not shown) but their growth caught up later by PND21 at weaning (Fig. 5.3A). To determine whether maternal treatment influenced the growth of the progeny, pups were weighed before being culled on PND10, PND24 and PND90. Male and female progeny from all groups have comparable growth on PND10 and PND24. However, both maternally DBP-treated male and female pups had lower body weight compared to controls on PND90 (Fig. 5.3B).

5.3.2 Anogenital Distance (AGD)

AGD is a measure of perinatal disruption of androgen-dependent physiology and has widely been used to confirm endocrine disruption (Carruthers & Foster, 2005; Macleod et al, 2010; Welsh et al, 2008). To see if the male pups were affected by indirect DBP exposure from GD14.5 to PND6, the AGD measurement was performed on all viable pups on PND2. On PND2, DBP or DES did not appear to have an effect on AGD for both male and female offspring (Fig. 5.4B). Consistent with previous studies (Welsh et al, 2008; Wolf et al, 2002), the AGD in the control male was roughly twice as long of those in the control female (Fig. 5.4A). On PND10, the AGD in male offspring from the DBP group was significantly shorter compared to that from the other treatment groups, implying that the experimental paradigm employed was successfully able to reproduce symptoms of endocrine disruption through indirect maternal exposure (Fig. 5.4C). None of the treatments had an effect on the AGD in the female pups at both time-points.

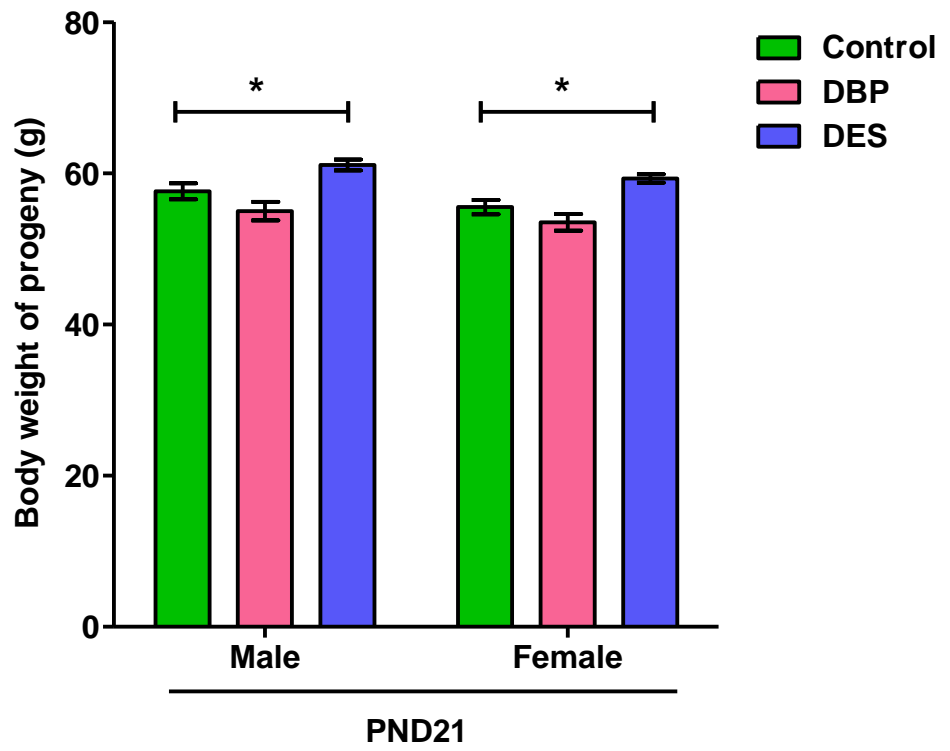
Table 5.1: Reproductive parameters in rats treated with DBP or DES during pregnancy and lactation.

	Control	DBP	DES
Number of rats which delivered live pups on PND1	8/8	9/9	10/12
Number of rats which had still-birth	0/8	0/9	2/12
Total number of pups per litter ^a	13.63 ± 0.98	12 ± 0.82	9 ± 1.75* (10.80±1.52)
Mortality rate of pups per litter (%) ^b	0.89 ± 0.89	15.87 ± 8.82	40.00 ± 16.33*

^a Data were represented as mean ± SEM. For DES groups, when the 2 litters with still-birth were included, the total number of pups/litter = 9±1.75 (p=0.05). However, if the 2 litters with stillbirth were excluded, then then total number of pups/litter = 10.80±1.52 (p=0.16).

^b Data were represented as mean ± SEM. The mortality rate per litter is the difference between number of pups born and number of pups found dead on PND2. Since we could not identify the number of stillborn pups born by the 2 pregnant rats in DES group, these 2 rats were excluded for mortality rate analysis (i.e. n=10 for DES group) (p= 0.132 for DBP; p= 0.049 for DES when compared with control group). * $p < 0.05$ in comparison with respective control.

(A)



(B)

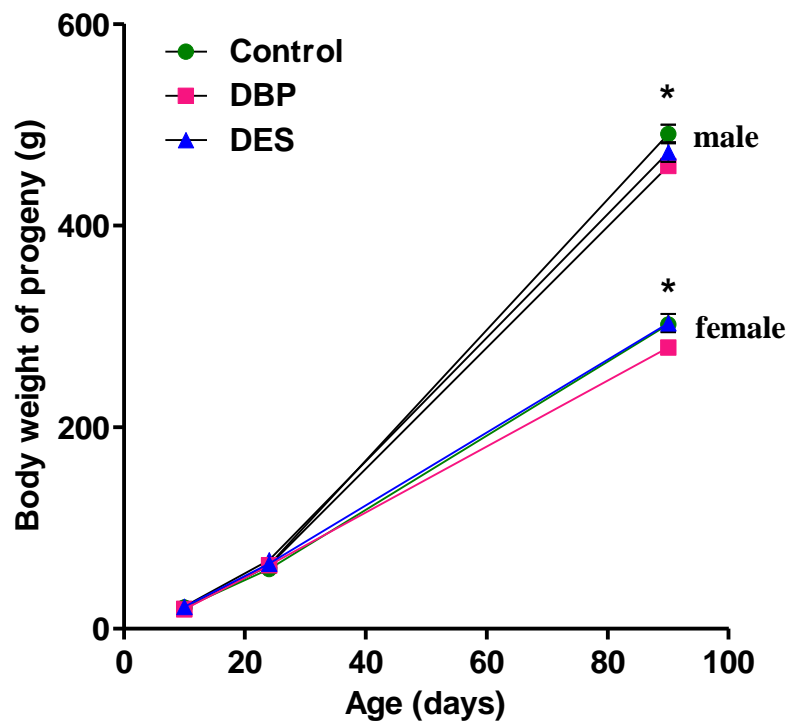


Figure 5.3: Body weight of male and female progeny during early development and as young adults. (A) The pups were weighed at weaning on PND21 [For males, n=24 (control), 27 (DBP) and 21 (DES); for females, n=24 (control), 26 (DBP) and 19 (DES)]. Progeny from DES-treated pregnant rats had a higher body weight compared to the control group. **(B)** Body weight of male and female progeny from control, DBP and DES groups on PND10, PND24 and PND90. Progeny in DBP groups had lower body weight on PND90 compared to control. Data were presented as mean \pm SEM [For males, n=16 (control PND10), 12 (DBP PND10) and 10 (DES PND10), 8 (control PND24), 9 (DBP PND24) and 9 (DES PND24), 16 (control PND90), 18 (DBP PND90) and 11 (DES PND90); for females, n=11 (control PND10), 13 (DBP PND10) and 11 (DES PND10), 8 (control PND24), 8 (DBP PND24) and 7 (DES PND24), 16 (control PND90), 17 (DBP PND90) and 12 (DES PND90)]. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ in comparison with respective control.

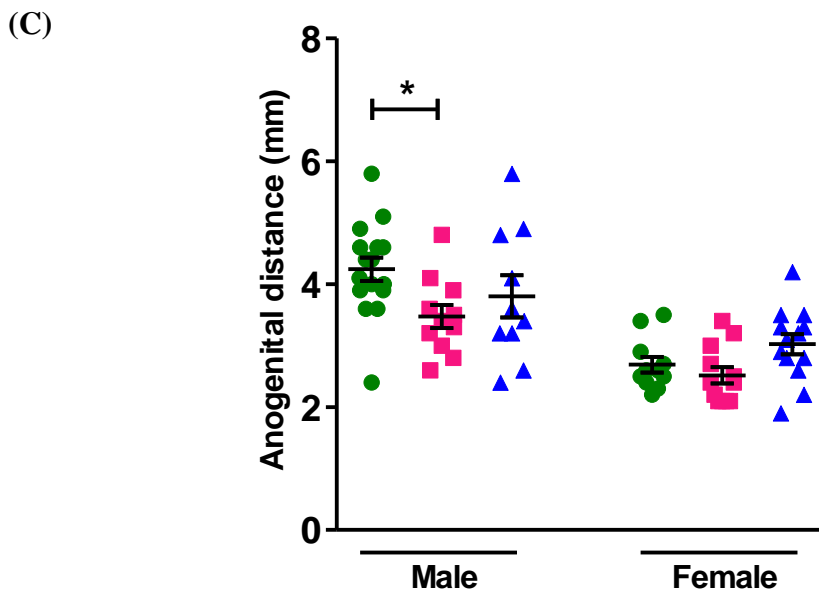
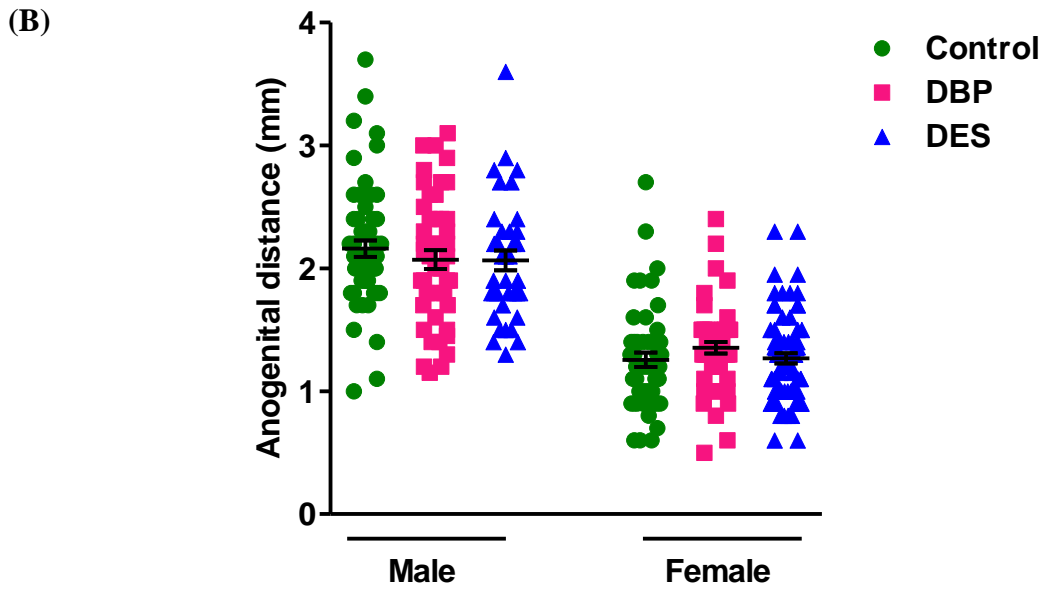
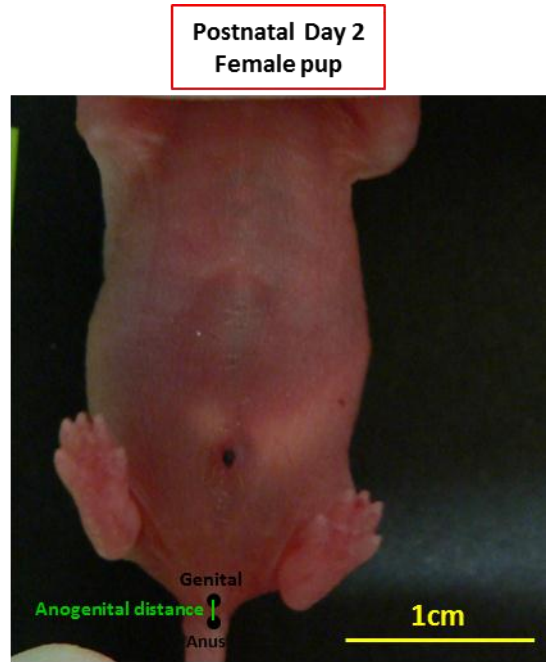
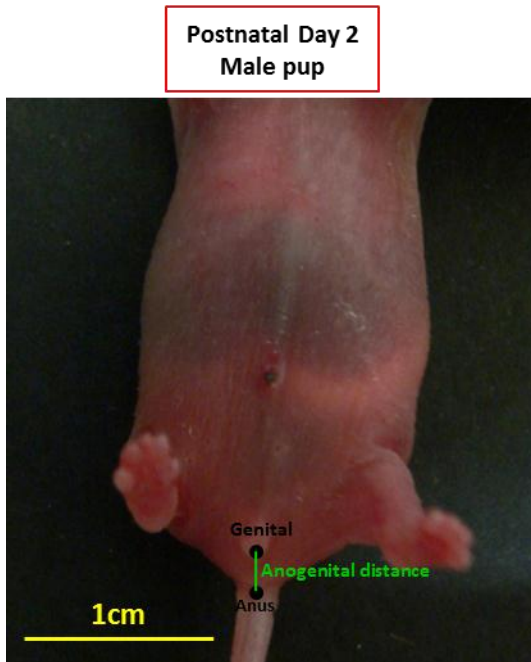


Figure 5.4: Anogenital distance(AGD) in male and female progeny following *in utero* and lactational exposure to control, DBP or DES treatments. (A) The distance between the anus and genital, designated the anogenital distance, was measured for all male and female progeny on PND2. In control group, AGD of male progeny was approximately twice the length of those in female progeny. (B) The AGD in both male and female progeny did not differ between treatment groups on PND2. [For male, n=58 (control), 47 (DBP), 38 (DES); for female, n= 50 (control), 53 (DBP) 73 (DES).] (C) On PND10, AGD of male progeny in DBP group was significantly reduced, while treatments had no effect on AGD in female progeny. [For male, n= 16 (control), 11 (DBP), 10 (DES); for female, n= 11 (control), 12, (DBP) 13 (DES).] Results were presented as mean \pm SEM. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ in comparison with respective control.

5.3.3 Testis abnormalities in male progeny

The gross appearance and location of the testes in the male progeny were inspected upon tissue collection for any obvious abnormalities (Table 5.2). On PND24, 22% of male progeny in the DBP group and 11% of progeny in the DES group had unilateral cryptorchidism, where one of the testes was found mid-way between the kidney and the scrotum. In addition, one testis was found missing (testicular agenesis) in 33% of the male progeny in the DBP group on PND24. No animals from the control group showed any signs of abnormality on PND24. All animals had scrotal testes on PND90 at culling. However, some animals were found to have one undersized testis, with 6%, 22% and 9% occurrence in control, DBP and DES groups, respectively. Overall, the incidence of testis abnormalities was higher in DBP group compared to other treatment groups.

When the testis weight was normalized against the body weight, it was observed that DBP treatment decreased the relative testis weight on PND10 and PND24, while inducing an increase on PND90. On the other hand, DES treatment caused an increase in testis weight on PND24, compared to the control group (Fig. 5.5), with no effect evident at PND90.

5.3.4 Peripheral testosterone and LH level in male progeny

To examine whether DBP and DES treatments could affect the functionality of the testes, peripheral total testosterone was measured using a time-resolved fluorescent immunoassay. Consistent with the development of Leydig cells within the testes, the concentration of testosterone in plasma remained low on PND10 and PND24. The level of testosterone was significantly increased on PND90 in all adult male progeny. There was no significant difference in the testosterone level among the treatment groups, suggesting that any effect of DBP and DES did not persist through to adulthood (Fig. 5.6A), or was always

Table 5.2: Testis abnormalities found in male progeny on PND24 and PND90 following maternal treatment with corn oil, DBP or DES. The incidence of abnormalities were scored for each treatment group and presented as a percentage of the total number of animals in the group.

	Control	DBP	DES
Number of male progeny with unilateral cryptorchidism on PND24	0/8 (0%)	2/9 (22%)	1/9 (11%)
Number of male progeny with only one testis on PND24 ^a	0/8 (0%)	3/9 (33%)	0/9 (0%)
Number of male progeny with one undersized testis on PND90 ^b	1/16 (6%)	4/18 (22%)	1/11 (9%)

^a In these animals, only one testis could be found. The other testis was absent due to reasons that is still unclear.

^b These animals had one testis of similar weight to testes in other animals within the same treatment group. The undersized testis weighed at least 35% lighter than the other testis from the same animal.

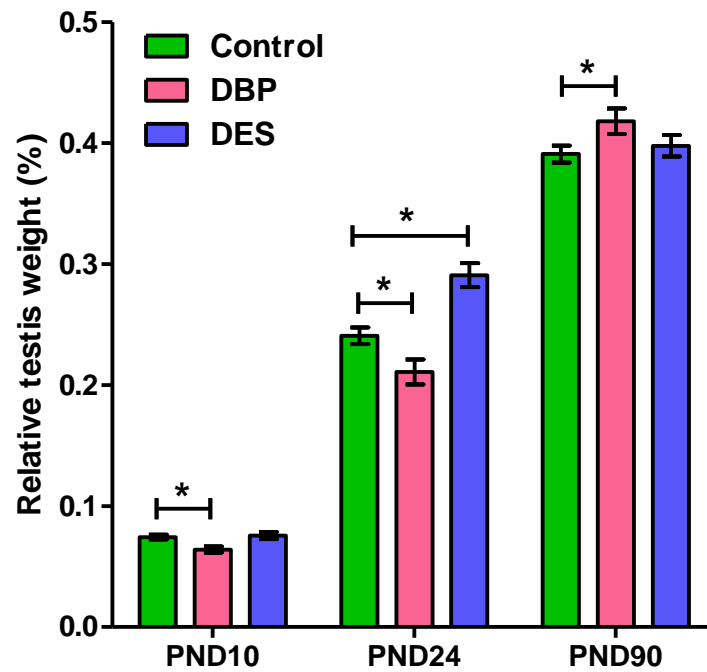


Figure 5.5: Relative testis weight of male progeny from control, DBP and DES groups [On PND10, n= 16 (control), 12 (DBP), 10 (DES); On PND24, n= 8 (control), 4 (DBP) 8 (DES); On PND90, n= 15 (control), 14 (DBP), 10 (DES)]. The relative testis weight of each animal was calculated as a percentage of its body weight and was presented as mean \pm SEM for each treatment group. Animals with testis abnormalities on PND24 and PND90 (as defined in Table 5.2) were not included in the graph. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ in comparison with respective control.

compensated by negative feedback in the HPG axis.

This latter notion was supported by the observation that DES appeared to increase the LH level in peripheral blood on PND10, however, due to large variability, the p-value ($p=0.08$) did not reach the level of significance (Fig. 5.6B).

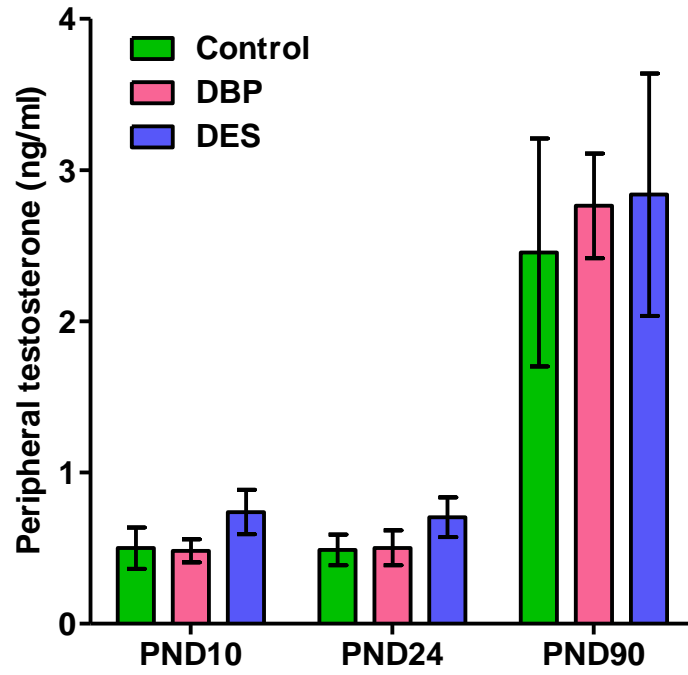
5.3.5 Peripheral INSL3 concentration in male progeny

As previously mentioned in Chapter 4, INSL3 is the peptide hormone that is specifically produced in a constitutive manner by mature Leydig cells, and acts as a marker of Leydig cell functionality and number. The peripheral INSL3 level in the male progeny was measured to assess whether the postnatal ALC population is affected by *in utero* and lactational DBP or DES treatments. The INSL3 level in serum increased gradually from PND24 onwards, reaching a peak value around PND40-45, and decreased thereafter to reach a stable value typical of older adult animals (Fig. 5.7). DBP did not appear to have much effect on the INSL3 level in serum throughout postnatal life in the male progeny, except to cause a significant increase on PND35. On the other hand, there was a clear DES effect which appeared to be biphasic. On PND24 and PND30, there was evidence for an increase in circulating INSL3 caused by the DES treatment, whereas the same treatment later led to a marked decrease in INSL3 on PND45.

5.3.6 Total Leydig cell numbers in male progeny

The fractionator method and optical disector were used to assess the effects of *in utero* and lactational exposure to DBP or DES on postnatal ALC numbers within the testis. Since the morphologically identifiable postnatal ALCs could be observed from around PND12 onwards (Ariyaratne et al, 2000b), only testes from PND24 and PND90 were evaluated. For each time-point, 3-5 animals were randomly selected for Leydig cell counting. The

(A)



(B)

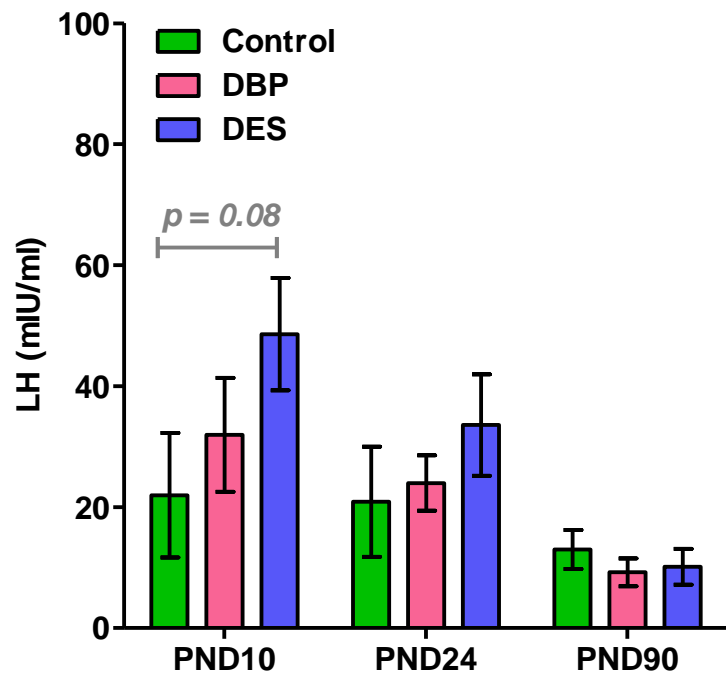


Figure 5.6: Effects of *in utero* and lactational exposure to DBP or DES on peripheral testosterone and LH levels in male progeny. Testosterone(A) and LH(B) in the peripheral blood was measured using TRFIA and ELISA respectively. The testosterone and LH levels in all treatment groups did not differ significantly at the time-points chosen. Data are presented as mean \pm SEM (n = 6).

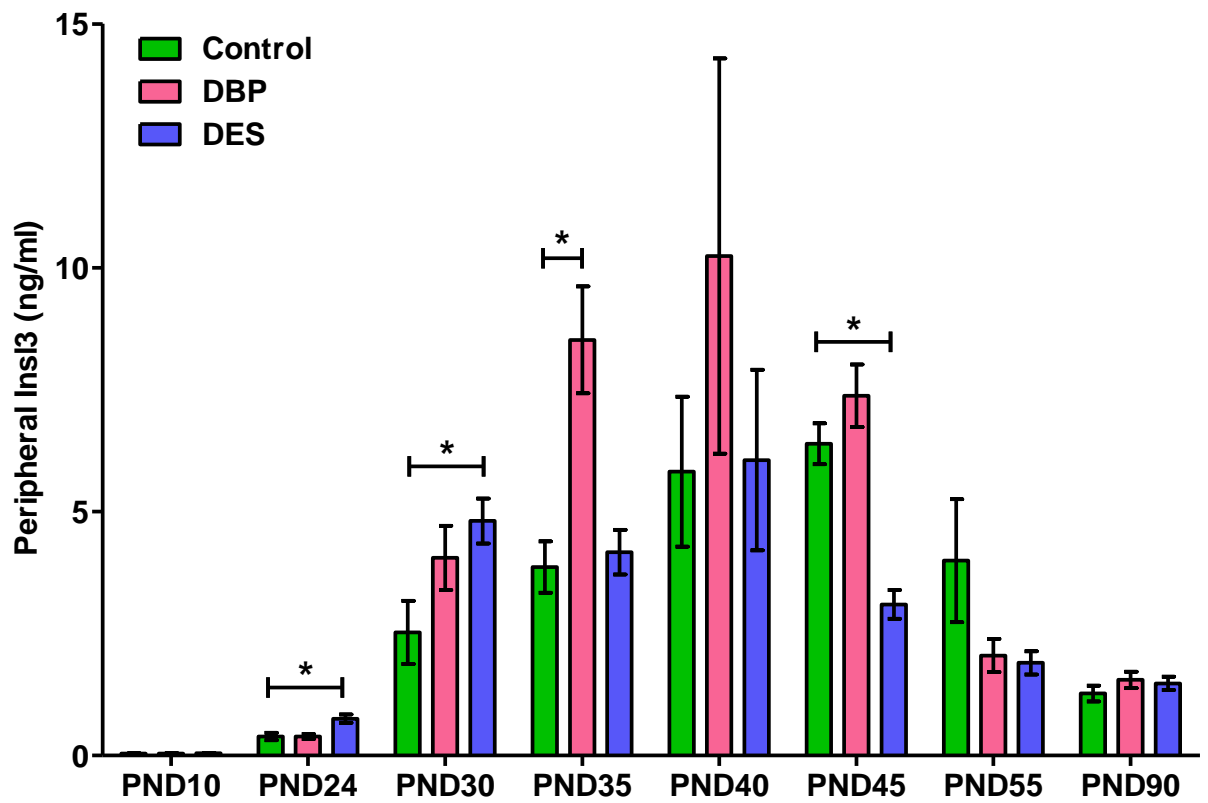


Figure 5.7: Effects of *in utero* and lactational exposure to DBP or DES on peripheral INSL3 level in male progeny. INSL3 in the peripheral blood was measured using TRFIA. Data were presented as mean \pm SEM (n=6). Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ in comparison with respective control.

Leydig cells are counted based on their nuclear morphology as described in section 4.2.3. Since slides were only stained with PAS and haematoxylin, and the thick sections could not be stained employing specific antibodies, it was not possible to distinguish immature from mature Leydig cells. Consequently, it is likely that those Leydig cells that were counted on PND24 were mostly newly-formed Leydig cells and on PND90 they were mostly mature ALCs, both, however, committed to the Leydig cell lineage. The number of Leydig cells was significantly increased by DES treatment on PND24 when compared to the control group (Fig. 5.8). In contrast, DBP treatment had no effect on Leydig cell number on PND24. On the other hand, the number of Leydig cells on PND90 was not altered by either DBP or DES treatments.

5.3.7 Quantitative real-time PCR analysis for various Leydig cell markers

Seven Leydig cell marker transcripts were selected for quantitative real-time PCR analysis in order to better understand the differentiation status of postnatal Leydig cells in the maternally-DBP or DES exposed male progeny (Fig. 5.10). The expression level of these markers within the testis was also evaluated in non-treated male SD rats (obtained from the puberty series in Chapter 3) and used as reference points (Fig. 5.9).

In normal development, the relative transcript expression levels for *Insl3*, *Hsd11b1*, *Cyp11a1* and *LHR* all increase as Leydig cells differentiate during puberty, with the highest level of expression in mature ALCs. On the other hand, transcript expression for *Cyp17a1* appeared to be highest in newly-formed ALCs and immature Leydig cells during the period PND20-PND40. *Hsd17b3* was also highly expressed at a very early stage of postnatal life in normal male SD rats, presumably due to expression in persisting fetal-type Leydig cells, as earlier shown for *Hsd17b10* (Ivell et al, 2003). *Hsd17b3* expression decreased dramatically on PND10. As the animals develop through puberty, we could not

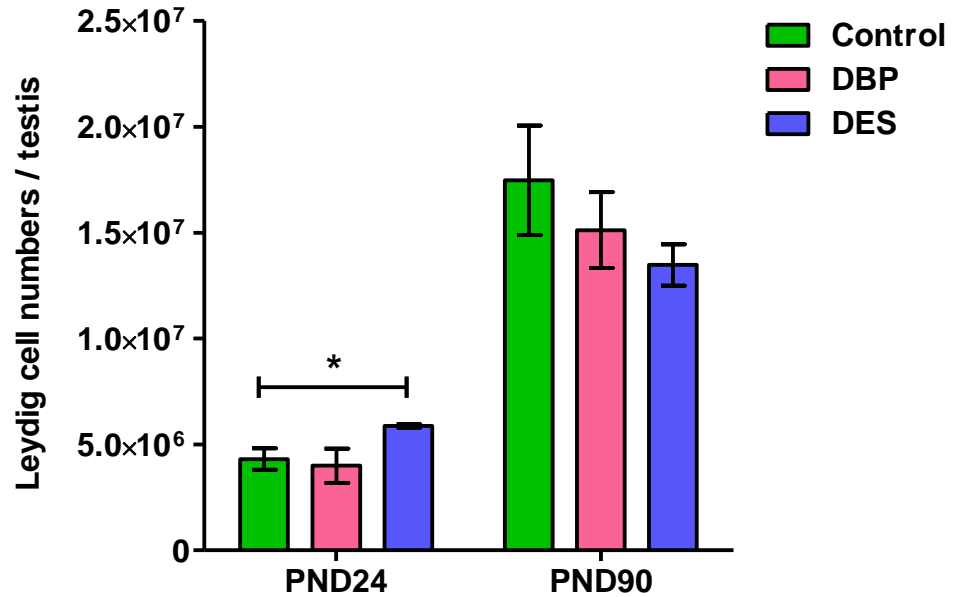


Figure 5.8: Total Leydig cell number in male progeny following *in utero* and lactational DBP and DES exposure. Leydig cell numbers were estimated using the fractionator and optical disector method. Data were presented as mean \pm SEM (n=3-5). Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ in comparison with respective control.

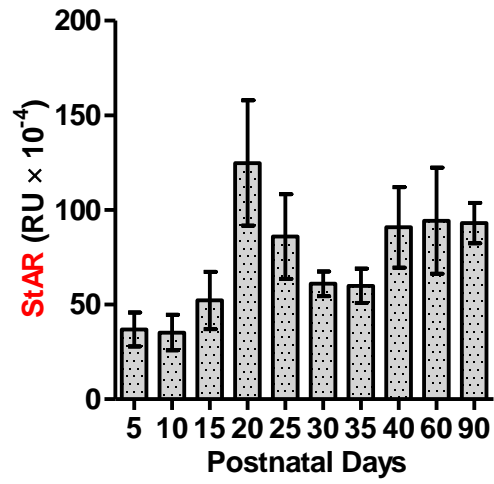
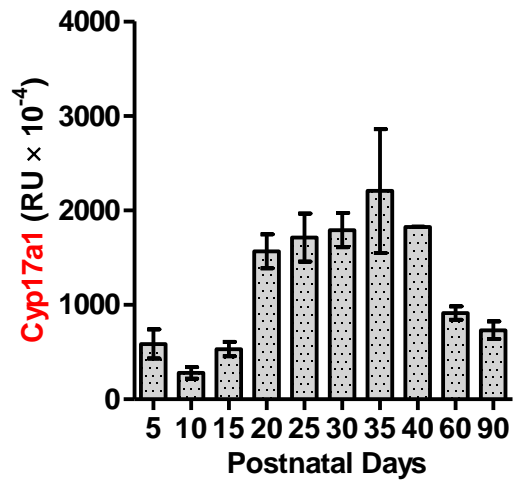
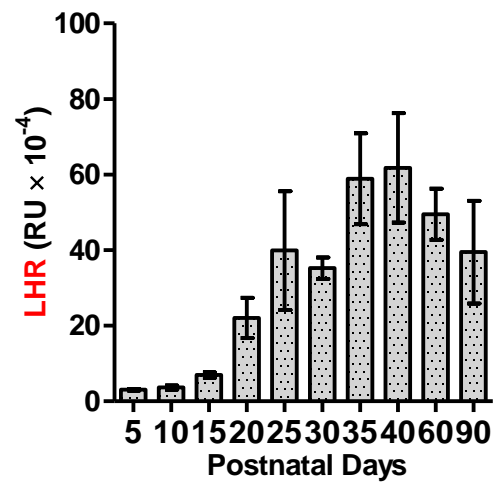
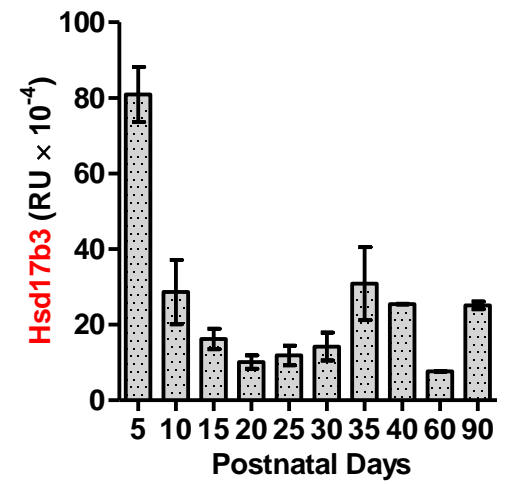
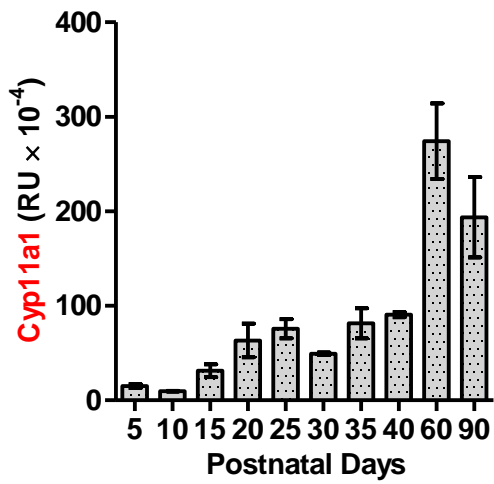
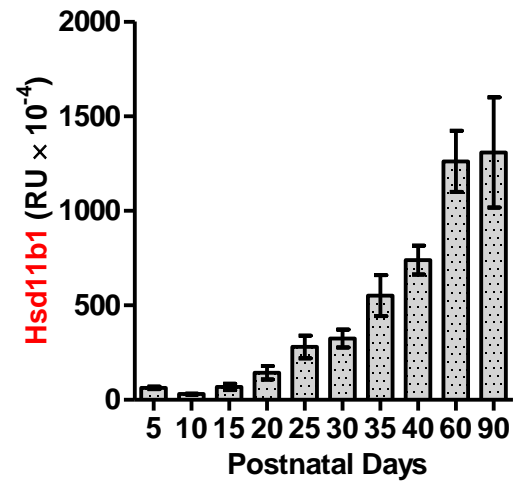
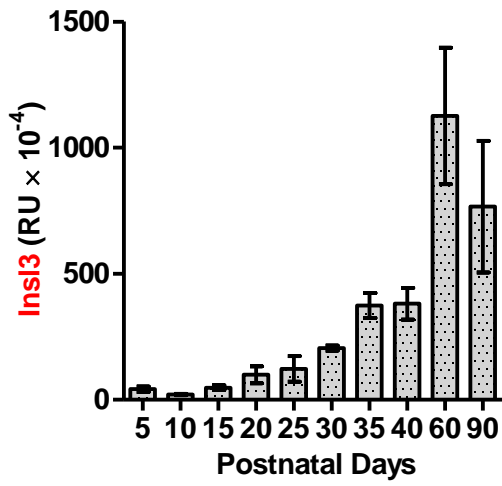


Figure 5.9: Normalised mRNA expression of various Leydig cell markers during development of male SD rats. Data were normalized against expression of housekeeping gene *Rps27a* and presented as mean \pm SEM for 4 animals at each sampling time-point.

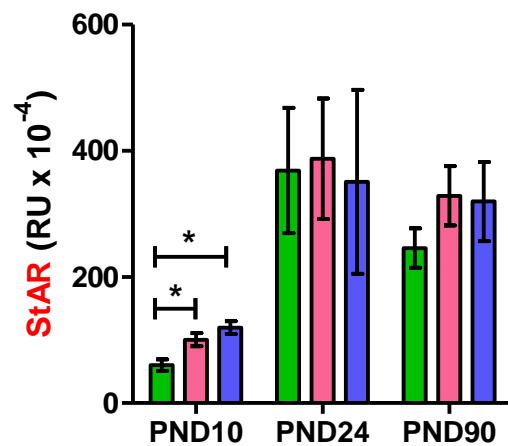
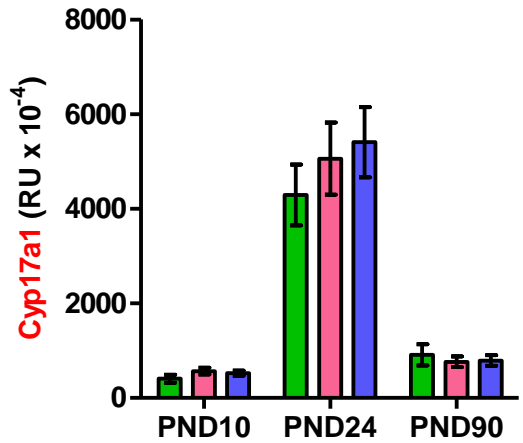
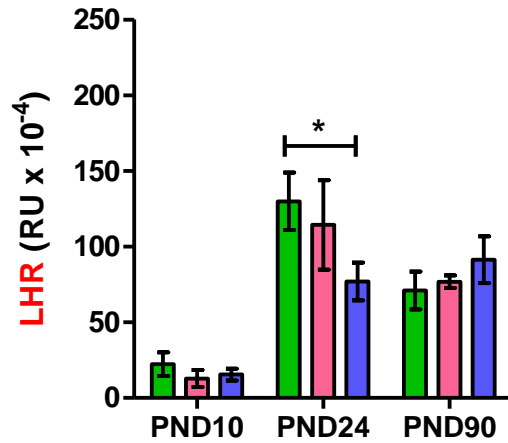
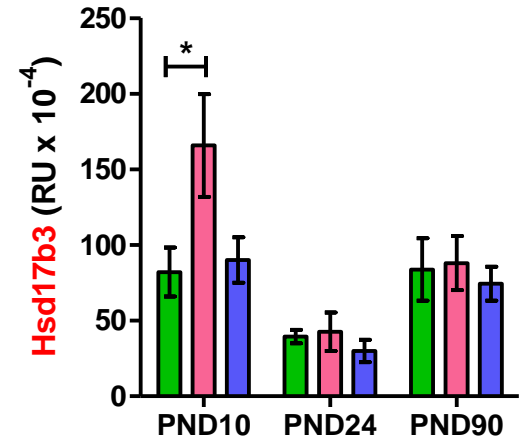
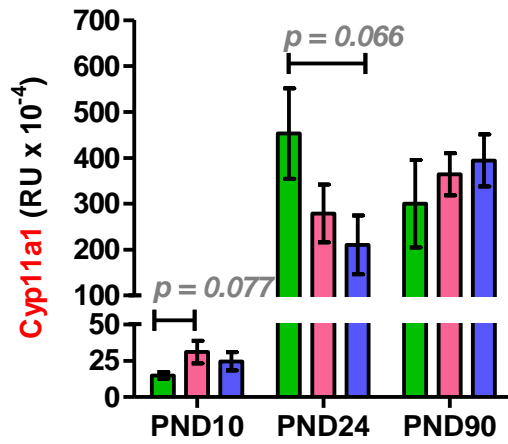
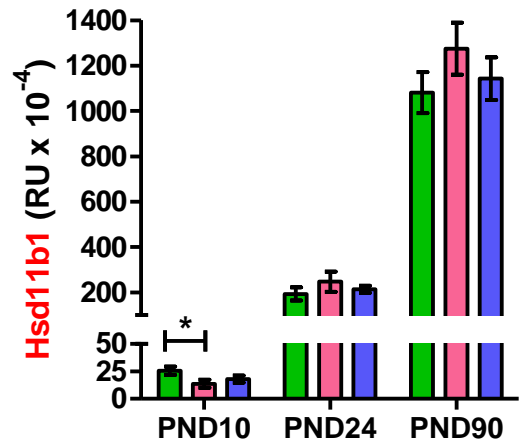
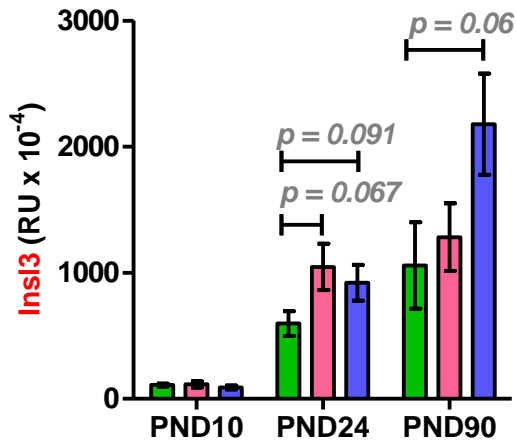


Figure 5.10: Normalised mRNA expression of various Leydig cell markers in male progeny following *in utero* and lactational exposure to control (■), DBP (■) or DES (■) treatments. Data were normalized against expression of housekeeping gene Rps27a and presented as mean \pm SEM for 6 animals at each sampling time-point. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ in comparison with respective control.

observe any obvious trend in Hsd17b3 mRNA expression with the age of the animals. Similar to Hsd17b3, we also did not observe any obvious trend in StAR mRNA expression with the age of the animals.

The level of mRNA expression of the Leydig cell markers in the male progeny on PND10, PND24 and PND90 (Fig. 5.10) from our experimental groups with different maternal xenobiotic exposures were essentially comparable to what we observed in normal male rat development (Fig. 5.9). When expression of these Leydig cell markers was analyzed in the male progeny, we did not observe any significant effect of DBP or DES treatment on Leydig cell marker expression on PND90. On PND24, only LHR expression was significantly decreased by DES treatment compared to control. Out of the 3 experiment time-points, PND10 was the time-point that was most affected by the treatments. On PND10, the expression levels for StAR and Hsd17b3 transcripts were significantly increased by DBP treatment. On the other hand, DES caused a significant increase in the expression of StAR mRNA while producing a significant reduction in Hsd11b1 mRNA expression on PND10.

5.3.8 Vaginal opening in female progeny

Previous studies suggested that prenatal and perinatal exposure to phthalates could affect the onset of puberty in female progeny (Salazar et al, 2004). Ma et al (2006) reported that exposure to DEHP through inhalation during the pre-pubertal period could advance puberty in female rats (Ma et al, 2006). To examine whether the DBP or DES treatments used in the present study might influence the timing of puberty in female rats, the female progeny was checked daily from PND29 onwards for vaginal opening. There was no significant difference in the age at vaginal opening, suggesting that our treatment paradigms had no effect on the onset of female puberty (Fig. 5.11).

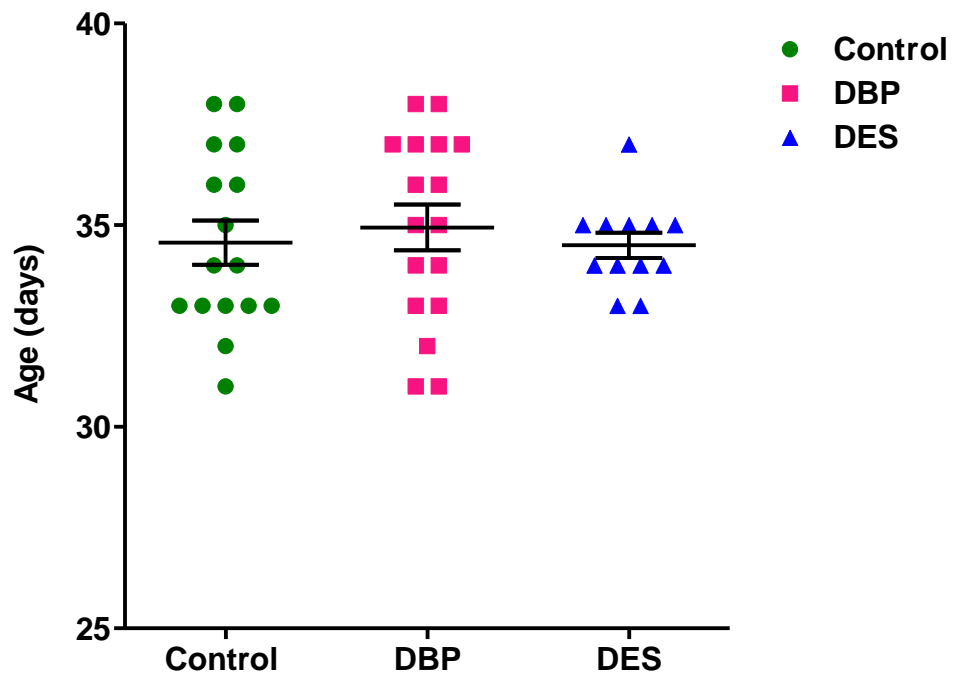


Figure 5.11: Age at vaginal opening in female progeny. Age at vaginal opening was not significantly different between treatment groups. Data were presented as mean \pm SEM.

5.4 - Discussion

The present study aimed to investigate whether indirect maternal exposure to DBP, during both the late gestational and lactational periods, could have a long term effect on postnatal adult Leydig cell development in male progeny. In this study, DBP was administered at 500mg/kg body weight/day every second day from GD14.5 to PND6. This dosage was chosen in order to maximize fetal response to phthalate effect without causing overt maternal toxicity. At this dosage, DBP was previously reported to cause cryptorchidism, Leydig cell aggregation, presence of MNGs, reduction in testosterone level and AGD in male fetuses when exposed *in utero* (Drake et al, 2009; Mahood et al, 2007; Mylchreest et al, 2002). Although the dosage administered is much higher than the maximum level of DBP that the human population is likely to be currently exposed to, the higher penetrance of the DBP effect at this concentration allows the experiment to be carried out with an ethically acceptable lower number of litters and animals.

Pregnant rats in all groups appeared healthy throughout the experiment, suggesting that the dosage of DBP or DES given had no systemic toxicity. Consistent with previous reports (Howdeshell et al, 2007; Mylchreest et al, 2000), DBP treatment had no effect on the litter size. However, the mortality rate in the DBP group observed in this study appeared to be higher than what has been previously reported (Howdeshell et al, 2007; Mylchreest et al, 2000). On the other hand, pregnant rats in the DES group had minimal weight gain throughout pregnancy, which was also reflected in smaller litter sizes at birth. The incidence of still-birth observed in this study was thought to be linked to the DES effect to induce abortion in rats during late gestation (Kawaguchi et al, 2009). Although born smaller in size, the overall development in the DES progeny was not compromised, as indicated by a comparable growth to that of the control group.

The adverse effect of *in utero* DBP exposure on the reproductive tract of male progeny is generally considered to be a secondary consequence of lower testosterone production by FLCs. The AGD is a measure of androgen-dependent development, with a reduction in AGD being linked to lower testosterone production by the fetal testis in male fetuses exposed to DBP *in utero* (Carruthers & Foster, 2005; Ema et al, 2000; Macleod et al, 2010; Mylchreest et al, 1998). Since in the present study we also observed a decrease in AGD in the indirectly DBP-exposed male progeny, this would suggest that the testosterone produced by the fetal testes in our experimental paradigm was also probably lower, thus validating the approach taken. Similar to previous reports (Auharek et al, 2010; Kim et al, 2010; Mylchreest et al, 2000), maternal DBP treatment caused a decrease in relative testis weight on both PND10 and PND24. On PND90, there was no significant difference in absolute testis weights across the groups (data not shown), however, since the DBP group had a lower body weight on PND90, there was a significant increase in the relative testis weight in the DBP group, when testis weight was corrected against body weight. In our study, DBP also caused a 22% incidence of cryptorchidism in the male progeny on PND24. In addition, we found that 33% of the DBP male progeny only had one testis on PND24. This phenotype was reported in one earlier study (Mylchreest et al, 1998) although the cause of this phenotype is unclear. Although all testes appeared to be scrotal at adulthood, 22% of the DBP-exposed male progeny had one testis smaller than the other, which might be attributable to cryptorchidism that occurred in the early postnatal period. In rats, the inguinoscrotal phase (i.e. second phase) of testicular descent normally occurs around the 3rd week after birth (Shono et al, 1996; Stoker et al, 2000). Consistent with previous studies, the prevalence of cryptorchidism in male progeny at adulthood following *in utero* DBP exposure is markedly lower in SD rats (<10%) compared to that in Wistar rats (~100%) (Fisher et al, 2003; Mahood et al, 2005; Mylchreest et al, 1998; Mylchreest et

al, 1999). In the current study, cryptorchidism was only observed in 22% of DBP rats on PND24 and there is no evidence of cryptorchidism on PND90. The incidence of cryptorchidism observed at adulthood in our study is somewhat lower, again reinforcing the generally mild nature of the experimental paradigm chosen here.

Abnormal aggregation or hyperplasia of FLCs has been well-documented for the male fetuses that were prenatally exposed to DBP (Mahood et al, 2005; Mahood et al, 2007). These Leydig cell aggregates in the DBP-exposed males were mostly found in focal areas of the testis on embryonic days E21.5 (Mahood et al, 2005; Mahood et al, 2007). Barlow and Foster (2003) reported previously that the Leydig cell aggregation observed in the fetal testis following *in utero* DBP exposure appeared to be resolved in the early postnatal period, although the severity of seminiferous tubule degeneration (focal atrophy) was significantly increased as animals developed (Barlow & Foster, 2003). In addition, mild Leydig cell hyperplasia was reported in adult SD rats that were exposed to DBP prenatally (Mylchreest et al, 1998; Mylchreest et al, 2000). In these studies, Leydig cell hyperplasia was defined as an increase in the number of Leydig cells (i.e. Leydig cell aggregates) with focal or irregular distribution. The total number of Leydig cell per testis has never been evaluated. However, when we assessed the testis sections for ALC number using the optical disector methodology, no obvious abnormalities of Leydig cell localization or seminiferous tubule disruption were observed, either on PND24 or PND90. Mahood et al (2007) reported that the occurrence of Sertoli-cell only (SCO) tubules and dysgenetic areas in cryptorchid and scrotal testes were different. They observed a 100% occurrence of dysgenetic areas in cryptorchid testes compared to only ~50% occurrence in scrotal testes at adulthood in prenatally DBP-treated male progeny (Mahood et al, 2007). Therefore, the reason why I failed to observe any obvious testes abnormalities, as described in fetal testes,

may have been because the testes selected randomly for histological examination in the present study were all scrotal testes and had normal testis weight.

The fractionator and optical disector method has previously been shown to be a reliable method for the estimation of the total number of Leydig cells in mice and rats (Duckett et al, 1997; Gould et al, 2007). In this study, we reported that there were approximately 4 million and 17 million ALCs per testis in the control rat testis on PND24 and PND90, respectively. Our results were consistent with the number of Leydig cells in developing rat testis reported in other studies (Hardy et al, 1989; Tapanainen et al, 1984; Zirkin & Ewing, 1987). Using the physical disector method, Hardy et al reported that there were around 2 million Leydig cells per testis on PND21, and the Leydig cell number increased steadily to 13 million and 25 million on PND28 and PND56, respectively (Hardy et al, 1989). Another study by Zirkin and Ewing showed that Leydig cell numbers per testis increased from around 3 million in 3-week old rats to 18 million in adult rats (Zirkin & Ewing, 1987). On PND24, we observed a small but significant increase in Leydig cell number in DES male progeny, suggesting an increase in the preceding Leydig cell proliferation. However, the proliferation rate in the DES group appeared to slow down as the animals develop into adulthood, since no difference in Leydig cell number per testis was observed at adulthood on PND90.

To determine whether the rate of development of the Leydig cells was affected, serum testosterone, serum INSL3 and various Leydig cell markers were assessed. In rats, plasma testosterone remains low in the first 2 weeks of postnatal age. Thereafter, testosterone levels increase steadily and attain the adult level by 60 days of age (Resko et al, 1968; Smith et al, 1977; Zapatero-Caballero et al, 2003). Although there was an increase in Leydig cell number in DES group on PND24, this increase was not reflected in the serum

testosterone concentration among all groups at any time-point. However, this could be interpreted to indicate that the acute HPG axis was effectively compensating for any reduction in the Leydig cell capacity to make testosterone. However, just as for total testosterone, we did not observe any significant difference in the concentration of serum LH using a species-specific immunoassay although there was a trend ($p=0.08$) towards an increase in LH for the DES treatment group on PND10. Similar to our observation, Howdeshell et al (2007) also reported that serum testosterone in 7-month old adult male rats was not affected by the prenatal DBP treatment, although testosterone production by the fetal testis was markedly reduced (Howdeshell et al, 2007).

Unlike testosterone, *Insl3* mRNA appears to be constitutively expressed (Sadeghian et al, 2005); and secretion of INSL3 into the circulation is not dependent on the acute regulation of the HPG axis (Anand-Ivell et al, 2006a; Bay et al, 2005). Therefore, it serves as a better index that reflects the differentiation status of the Leydig cells and their number. In the current study, the serum INSL3 concentration was up-regulated on PND24 by maternal DES treatment. This increase could be explained by the increase in Leydig cell number. The effect of DBP or DES treatments were more pronounced on peripheral INSL3 concentrations at the time when Leydig cells were actively differentiating and these effects subsequently disappeared when the animals attained sexual maturity. Similarly, quantitative real-time PCR analysis for different Leydig cell marker transcripts also suggested that the time when Leydig cells were actively differentiating/proliferating probably represented the most sensitive window towards the effects of both treatments, since we did not observe any difference for all markers on PND90.

Taken together, our results suggested that *in utero* and lactational DBP or DES treatment was able to affect subsequent adult-type Leydig cell development, at the time when these

Leydig cells are actively differentiating or proliferating. This was clearly indicated by the peripheral INSL3 level. The results from this study suggested that although Leydig cell function and/or number during development might be affected by earlier DBP or DES treatments, the effect of such treatments did not appear to persist through to adulthood. Here it would appear that mechanisms are in place which, by local feedback within the testicular niche or long-term via the HPG axis, Leydig cell capacity can be regulated about a consistent norm value. As a corollary to this, it would therefore seem that when Leydig cell capacity does change, as in aging, this is likely to reflect either an intrinsic age-dependent property of the Leydig cells themselves and/or changes to the Leydig cell niche and/or changes to the HPG axis.

Chapter 6

**Development of an *in vitro*
immature rat Leydig cell
differentiation model**

6.1 - Introduction

The phenotypically anti-androgenic effects of DBP have been demonstrated in various *in vivo* experiments in the recent years. In order to delineate the mechanism of action of phthalates specifically on Leydig cells, some investigators have made use of *in vitro* models such as fetal testis explants, primary cells and also secondary cell lines (Chauvigne et al, 2009; Clewell et al, 2010; Hallmark et al, 2007; Jones et al, 1993; Lambrot et al, 2009; Svechnikov et al, 2008; Thompson et al, 2004). The use of an *in vitro* model allows exposure of phthalates in a micromolar range which might be closer to our daily exposure level to such compounds, compared to *in vivo* experiments.

Previous *in vivo* experiments showed that MBP, the active metabolite of DBP, was present in the fetal rat testis at a concentration of 186 μ M following *in vivo* maternal DBP application from GD12-19 (Clewell et al, 2010). Mild inhibitory effects of phthalate monoesters on hCG-stimulated steroidogenesis have previously been demonstrated in primary rat Leydig cells and mouse Leydig tumour MA-10 and MLTC-1 cell lines, at concentrations similar to that found in the fetal testis following maternal exposure (Dees et al, 2001; Ge et al, 2007; Jones et al, 1993; Wang et al, 2006; Wang et al, 2007). Marked inhibition of hCG-stimulated steroidogenesis could only be observed following MBP or MEHP treatment at a concentration of around 1mM (Ge et al, 2007; Jones et al, 1993). The effect of MBP appeared to be biphasic. Using the mouse Leydig cell tumour cell line, MLTC-1, Wang et al showed that low levels (i.e. 100-1000nM) of MBP were able to cause a slight up-regulation of steroidogenesis, whereas higher doses (i.e. > 200 μ M) of MBP had a mild inhibition on the hCG-stimulated progesterone production (Wang et al, 2006; Wang et al, 2007). High doses of MBP appeared to disrupt steroidogenesis by inhibiting cholesterol uptake into the mitochondria through inhibition of StAR expression (Wang et

al, 2006; Wang et al, 2007). Cyp11a1 and Hsd3b1 did not appear to be responsible since the effect of MBP on steroidogenesis could be reversed by addition of 22(R)-hydroxycholesterol or pregnolenone to the culture medium (Wang et al, 2006; Wang et al, 2007). In addition, Svechnikov et al (2008) also showed that MEHP was able to down-regulate hCG-stimulated testosterone at 250 μ M through down-regulation of StAR in primary Leydig cells from day 40 and day 60 rats (Svechnikov et al, 2008).

Almost all of the studies carried out *in vitro* looked at acute effect of phthalate monoesters on steroidogenesis in differentiated Leydig cells. It was not clear if phthalate treatment could have a long term effect on processes such as Leydig cell differentiation *in vitro*. Similarly, the methodology used for primary rat Leydig cells mostly employed short term culture, with only one article referring to a 2-week long term culture (Teerds et al, 2007). Therefore, the aim of this study was to establish a long term *in vitro* Leydig cell differentiation model that could be used to investigate the effect of MBP/DBP, using adult-type Leydig cells and beginning at a stage of differentiation which would include also early stages of Leydig cell development. In this way it was hoped to have a model which more closely reflected the known negative effects of phthalates on fetal Leydig cell development but which offered a more robust and accessible system than to use explant cultures of fetal testis (Chauvigne et al, 2009; Hallmark et al, 2007; Lambrot et al, 2009).

6.2 – Methods

6.2.1 Isolation of Leydig cells from immature rats

Time-mated pregnant SD rats were followed through pregnancy and the exact date of delivery was recorded. For each preparation, 20-25 SD male rats at PND10 were euthanized by CO₂ asphyxiation. The still abdominal testes were removed from the

animals and transferred to transport medium containing 1:1 mixture of Dulbecco's modified Eagle media (containing 4,500 mg/L D-glucose, 1mM L-glutamine, and 25mM HEPES buffer) and nutrient mixture F-12 HAM (containing 14mM NaHCO₃ and 1mM L-glutamine) supplemented with penicillin (100U/ml) and streptomycin (100µg/ml).

The decapsulated testis mass was suspended in 20ml of transport medium and dispersed gently through a syringe, up and down 20 times, in order to mechanically separate the Leydig cells from the seminiferous tubules without the use of collagenase. Subsequently, 30ml of transport medium was added to the tube containing the testicular mass. The tube was inverted once. Larger tubular fragments were allowed to settle under unit gravity for 10 minutes (i.e. unit sedimentation). The supernatant was then transferred to a new tube. A further 30ml of transport medium was added to the tube and unit sedimentation was repeated. The unit sedimentation procedure was performed 3 times in total. The final supernatant collected from unit sedimentation was then filtered through a 100µm nylon mesh and centrifuged at 300 ×g for 3 minutes. The cell pellet was resuspended in approximately 5ml culture medium and cells were counted using a haemocytometer.

Isolated cells were cultured in medium containing 1:1 mixture of Dulbecco's modified Eagle media (containing 4,500 mg/L D-glucose, 1mM L-glutamine, and 25mM HEPES buffer) and nutrient mixture F-12 HAM (containing 14mM NaHCO₃ and 1mM L-glutamine) supplemented with 0.1% bovine serum albumin (BSA), penicillin (100U/ml), streptomycin (100µg/ml), 1× antibiotic-antimycotic solution, 100µM non-essential amino acids and 1mM sodium pyruvate.

In initial experiments, 1,400,000 cells were seeded per well in 12-well plates. The cells were cultured at 37°C with 5% CO₂ for 1, 3, 5, 8, 12, 15 or 17 days with medium replaced

every third day. The cells were allowed 1 hour to attach to the plates before addition of 5ng/ml hCG and/or supernatant medium from cultured Sertoli SK11 cells (added at 0.1 volume of total volume in each well). Supernatants were collected from each well approximately 48 hours following media change (except on day 1 where supernatant was collected 24 hours after the cells were seeded).

For all subsequent experiments assessing the effect of phthalates, the cells were seeded in 24-well plates at a density of 450,000 cells per well. The cells were allowed 1 hour to attach to the plates before stimulation with 0.1% DMSO, 5ng/ml hCG, 30 μ M MBP or 30 μ M DBP. The cells were cultured at 37°C with 5% CO₂ for 1, 4, 8, 12, 14 or 16 days with medium replaced every second day. Approximately 24 hours following medium change, 300 μ l supernatant was collected from each well. All supernatant was kept at -20°C until analysis for rat INSL3.

6.2.2 SK11 culture

The mouse immortalized Sertoli cell line, SK11 (Walther et al, 1996) was cultured in medium containing 1:1 mixture of Dulbecco's modified Eagle media (containing 4,500 mg/L D-glucose, 1mM L-glutamine, and 25mM HEPES buffer) and nutrient mixture F-12 HAM (containing 14mM NaHCO₃ and 1mM L-glutamine) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100U/ml), streptomycin (100 μ g/ml), 1 \times antibiotic-antimycotic solution and 100 μ M non-essential amino acids. The cells were cultured at 34°C with 5% CO₂. 10ml of SK11 supernatant cell media was collected per 4.5 million cells before the cells were passaged. The supernatant was kept at -20°C until use in experiments in section 6.2.1.

6.2.3 WST-1 cell proliferation assay

The cell proliferation reagent WST-1 was utilized in order to investigate the proliferation of the cells in culture. WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) is a tetrazolium salt which could be cleaved into formazan by mitochondrial dehydrogenases. Increase in the number of viable cells results in an increase in the activity of mitochondrial dehydrogenases in the sample, and thus an increase in the amount of formazan formed, which could be measured at 450nm. The amount of formazan directly correlates to the number of metabolically active cells in the culture.

Briefly, isolated cells were seeded in 96-well plates at a density of 30,000 cells per well. The cells were cultured at 37°C with 5% CO₂ for 1, 4, 8, 12, 14 or 16 days with medium replaced every second day. On the day when supernatant was collected from 24-well plates, media in 96-well plates were changed. 0.1 volume of WST-1 was added to all wells followed by an incubation of 90 minutes at 37°C. Plates were read after 90 minutes at 450nm in a Victor³ 1420 multilabel counter (Perkin Elmer, Boston, MA, USA).

6.3 – Results

6.3.1 Development of a Leydig cell differentiation model *in vitro*

In order to investigate the effect of phthalates on Leydig cell differentiation, I first needed to establish an *in vitro* Leydig cell model where undifferentiated Leydig cells isolated from young prepubertal SD rats could be differentiated *in vitro*. Although the absolute number of Leydig cells in the PND10 rat testis is lower (Hardy et al, 1989), there is also a lower chance of germ cell contamination in the culture, as at this age only spermatogonia are present. For each preparation (n= 9 preparations), approximately 0.53±0.09 million cells per testis could be isolated.

In the initial experiments, the PND10 Leydig cells were cultured in the presence or absence of hCG. INSL3 in culture media was measured as an indicator of Leydig cell maturation. The production of INSL3 was significantly increased by the addition of hCG in the culture with time (Fig. 6.1). Conversely, the INSL3 level secreted by non-treated cells remained significantly lower throughout the experiment. Previous studies have suggested that factors from Sertoli cells, such as anti-Mullerian hormone (AMH), might have regulatory effects on Leydig cell development (Behringer et al, 1990; Behringer et al, 1994; Salva et al, 2004). To investigate whether Leydig cell differentiation could be influenced by factors from Sertoli cells, medium supernatant from Sertoli SK11 cell culture was added to the cells. Similar to the AMH effects previously reported, the Sertoli conditioned medium significantly inhibited hCG-stimulated INSL3 production (Fig. 6.1).

6.3.2 Effect of phthalates on Leydig cell differentiation *in vitro*

In order to investigate the effect of MBP on the rate of differentiation of PND10 primary Leydig cells, 30 μ M of MBP was incubated with the cells with or without 5ng/ml hCG. It took around 8 days before INSL3 became detectable in culture media in the presence of hCG. For all experiments, the level of INSL3 was below detection level after 1 and 4 days in culture. MBP treatment had no consistent effect on hCG-stimulated INSL3 production over time. Whereas MBP was inhibitory on hCG-stimulated INSL3 production in 2 separate experiments (Fig. 6.2); there was no significant effect in a third experiment (data not shown). Nor did addition of MBP have any significant effect on basal INSL3 expression in the absence of hCG. Although MBP is seen as the principal active metabolite of DBP *in vivo*, DBP is also cytoactive in its own right (Dr. Henrik Leffers, personal

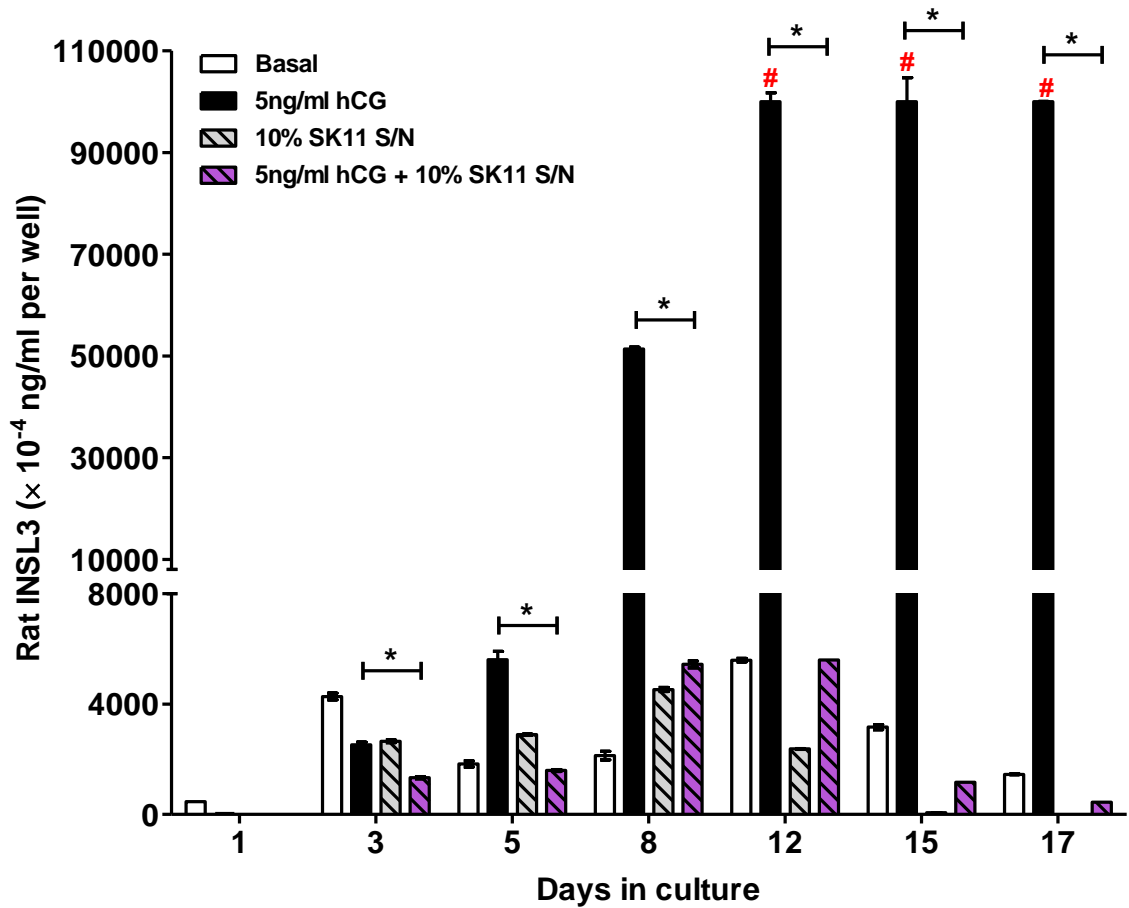


Figure 6.1: Effect of hCG and SK11 supernatant (S/N) on cumulative INSL3 production by primary differentiating rat Leydig cells. The supernatant was collected approximately 48 hours after media replacement (except for day 1 where supernatant was collected 24 hours after media replacement). The level of INSL3 was measured by rat INSL3 TRFIA. Bars marked with # were above upper detection limit of rat INSL3 TRFIA. The experiment was performed in triplicate and carried out twice in total. Representative figure was presented. Data were presented as mean \pm SEM. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.01$ in comparison with respective control.

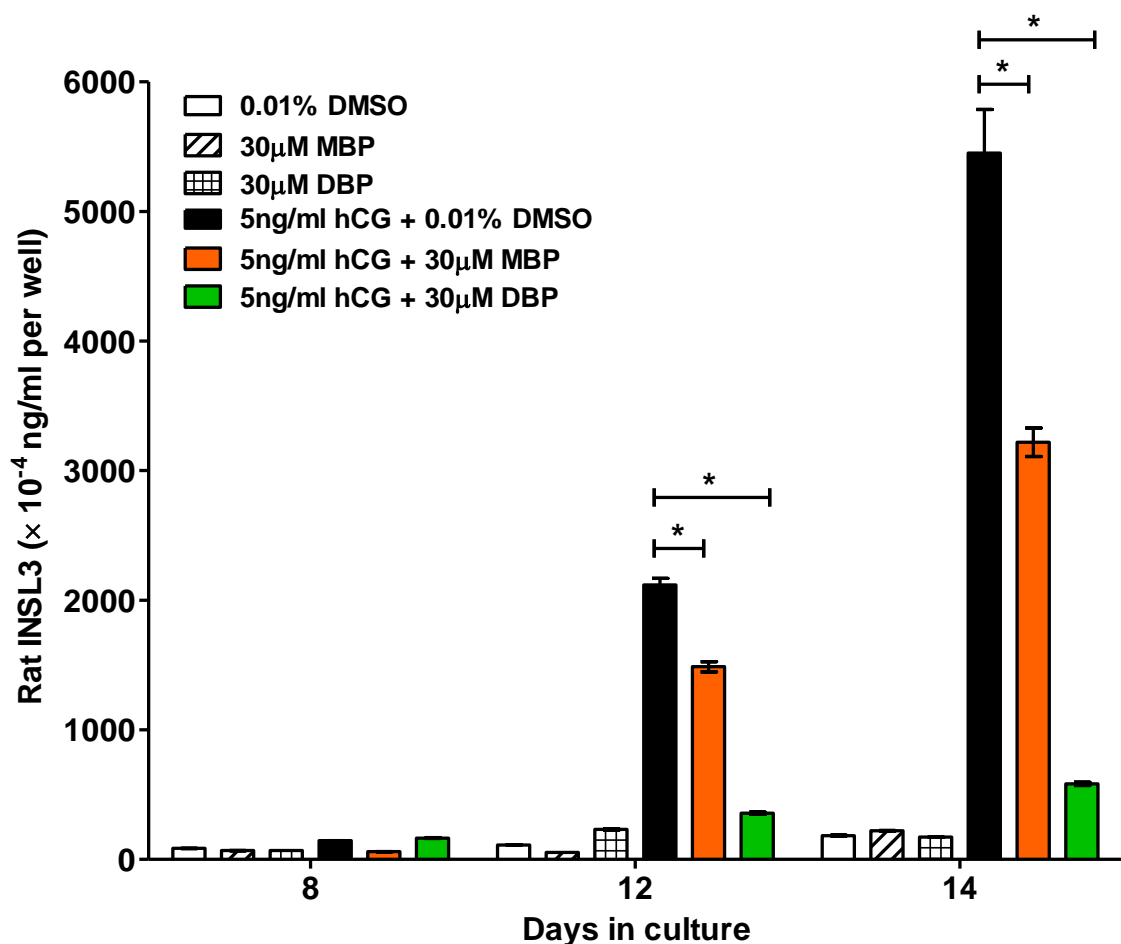


Figure 6.2: Effect of MBP and DBP on hCG-stimulated INSL3 production by primary differentiating rat Leydig cells. The supernatant was collected approximately 24 hours after media replacement. The level of INSL3 on days 1 and 4 were all below detection level. The experiment was performed in triplicate and carried out twice in total. Representative results are presented. Data are given as mean \pm SEM. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.01$ in comparison with respective control.

communication). To determine whether DBP had similar effect to MBP *in vitro*, DBP was added to the culture at 30 μ M. DBP consistently inhibited the level of hCG-stimulated INSL3 at all times while having no effect on basal INSL3 production (Fig. 6.2).

6.3.3 Proliferation of Leydig cells

Overall, primary PND10 Leydig cells isolated by the described procedure showed a relatively low proliferation rate. The proliferation of cells appeared to be somewhat variable in different independent experiments. Most of the time, there was no significant difference of cell viability between different treatment groups. However, in some instances (e.g. on day 8; Fig. 6.3), the proliferation rate of the cells appeared to be significantly accelerated by hCG treatment.

6.4 – Discussion

It is generally considered that the adult Leydig cells arise from spindle-shaped stem Leydig cells of mesenchymal origin during the early pubertal period (Haider et al, 1995; Mendis-Handagama & Ariyaratne, 2001). Stem Leydig cells have recently been identified and isolated from the 7-day-old rat testis (Ge et al, 2006). The authors were able to differentiate the platelet-derived growth factor alpha (PDGFR α -) and leukemia inhibitory factor receptor (LIFR-) positive and Hsd3b1- and LH receptor-negative stem Leydig cells into Hsd3b1 positive cells in the presence of differentiating medium containing LH, thyroid hormone, IGF-1 and platelet derived growth factor BB (PDGF-BB) (Ge et al, 2006).

It is unclear whether LH is essential for the onset of stem Leydig cell differentiation. This is because adult Leydig cells are first detected at a time when circulating LH is still very

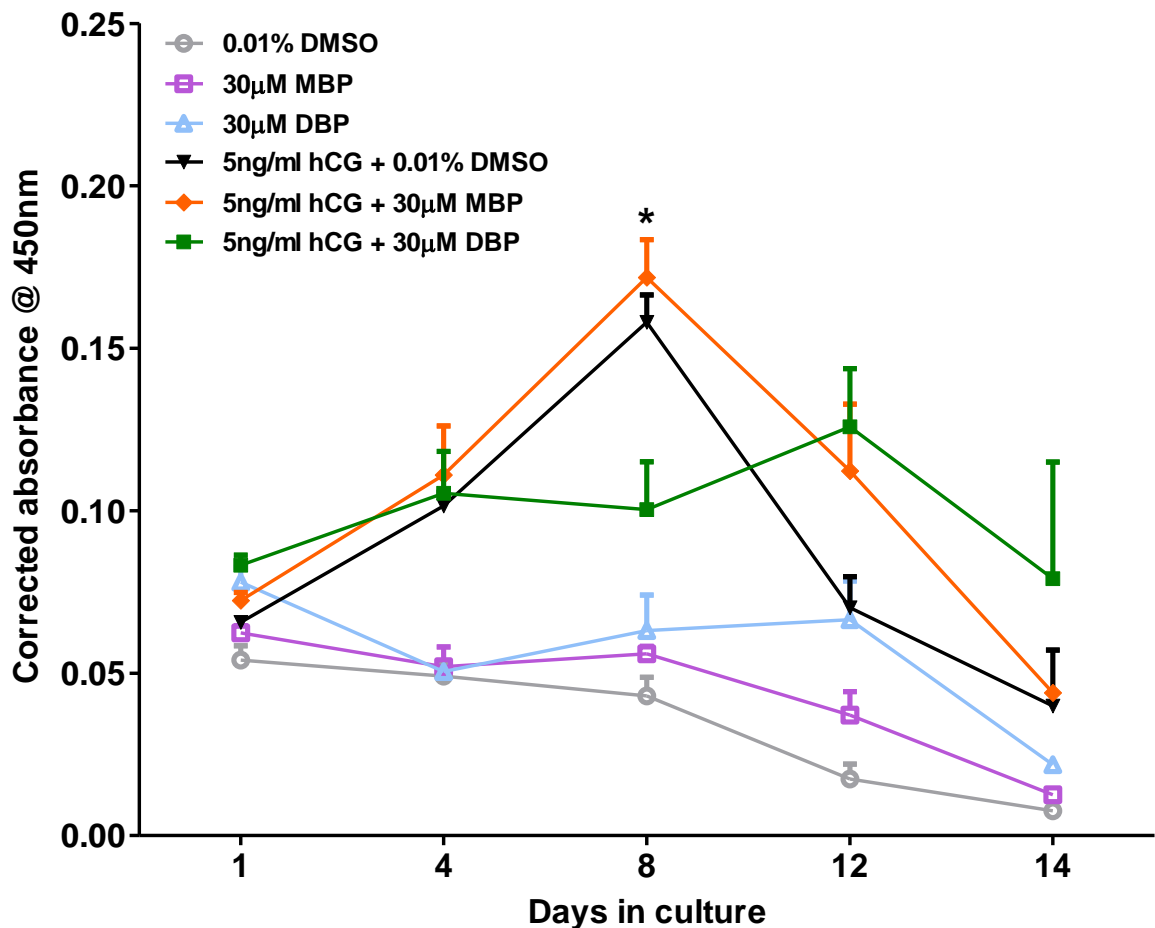


Figure 6.3: Effect of hCG, MBP or DBP on cell proliferation of primary differentiating rat Leydig cells. Proliferation of the differentiating rat Leydig cells was quantified using the cell proliferation reagent WST-1, a tetrazolium salt which could be cleaved into formazan by mitochondrial dehydrogenases. Increase in cell proliferation results in an increase in the activity of mitochondrial dehydrogenases in the sample, and thus an increase in the amount of formazan formed, which could be measured at 450nm. Absorbance presented was corrected with background absorbance. The experiment was performed in triplicate and carried out once in total. Data were presented as mean \pm SEM. Where data are significantly different, this is indicated by a bar and accompanying asterisk. * $p < 0.05$ (comparing hCG-treated groups with non-hCG treated groups).

low (Dohler & Wuttke, 1975; Lee et al, 1975). In addition, Leydig cells in hypogonadal (hpg) mice appear to be less differentiated since they exhibited lower INSL3 expression without exogenous LH (Balvers et al, 1998). However, in recent years it has become clearer that only a small subpopulation of stem Leydig cells within the testis have the ability to differentiate in an autonomous fashion in the absence of LH. Zhang et al (2004) showed by immunohistochemistry the expression of Hsd3b1 in a few peritubular Leydig precursor and mesenchymal cells of the testes at all ages in LH receptor knockout mice (Zhang et al, 2004). Furthermore, a recent study showed that a small population of mesenchymal/progenitor Leydig cells were able to differentiate *in vitro* and express Hsd3b1 in an autonomous fashion without LH supplementation, while the majority of stem Leydig cells indeed require the addition of LH for the initiation of differentiation (Teerds et al, 2007). Similarly, our data is in line with the requirement of LH for Leydig cell differentiation after PND10, since the level of INSL3 increased with culture time under the influence of 5ng/ml hCG, whereas INSL3 in basal conditions remained undetectable or just above the lower detection limit at all times. However, it is still not clear why LH was not able to initiate differentiation of stem Leydig cells on its own, but appeared to require a cocktail containing LH, thyroid hormone, IGF-1 and platelet derived growth factor BB (PDGF-BB) (Ge et al, 2006).

Hardy et al (1989) previously reported that 0.129 million and 0.4 million Leydig cells are present in 7-day-old and 14-day-old rats, respectively (Hardy et al, 1989). It is likely therefore that our culture may have contained some other interstitial cell types, since the total number of cells isolated here was somewhat higher. However, we deliberately did not purify the culture further, since paracrine factors secreted by testicular macrophages and Sertoli cells have been shown to be important for Leydig cell development (Gaytan et al, 1994a; Gaytan et al, 1994b; Mendis-Handagama & Ariyaratne, 2001; Wu & Muro, 2001).

1994) and our principal goal is to develop an *in vitro* model which accurately reflects *in vivo* Leydig cell differentiation. Since Leydig cells are the only testicular cell type that expresses the LH receptor (Ariyaratne et al, 2000a; Shan & Hardy, 1992; Teerds et al, 1999), we assumed that only mesenchymal/progenitor Leydig cells that arise postnatally would respond to the hCG treatment during the differentiation process. It is unlikely that the amount of INSL3 measured was produced by resident fetal Leydig cells for the following reasons: (1) The fetal Leydig cell population was decreased after birth; only 50-75% of the original fetal Leydig cell population persists in adult testis (Kerr & Knell, 1988); (2) Most fetal Leydig cells appear to involute irreversibly after birth (Codesal et al, 1990). The observation here that INSL3 secretion only becomes detectable after day 8 in culture under hCG treatment is strong support for the validity of our model, since *in vivo* I have shown earlier that INSL3 only becomes significantly elevated in peripheral blood after PND18 (see Chapter 3).

In the current study, phthalate dosage was chosen at 30 μ M, which is similar to the level of phthalate to which the fetal testis has been exposed in the various *in vivo* models. Previous *in vivo* experiments showed that monobutyl phthalate (MBP), the active metabolite of DBP, was present in the fetal rat testis at a concentration of 186 μ M following *in vivo* maternal DBP application from GD12-19 (Clewel et al, 2010). At most times, MBP appeared to slow down the differentiation process, indicated by lower INSL3. However, this inhibitory effect of MBP was not observed in one separate experiment. Due to time constraints, it was not possible to carry out further experiments to clarify this issue. It is somewhat surprising that DBP had a marked inhibitory effect on hCG-stimulated INSL3, as MBP was thought to be the active metabolite responsible for the phthalate action. Since DBP treatment had no inhibitory effect on cell proliferation, it would appear to decrease INSL3 production by slowing down the differentiation process.

One of the difficulties faced during the development of this model was to obtain enough Leydig cells for each experiment. Although the animals were time-mated, it was difficult to get all females to deliver on the same day. In future, one would require a larger breeding colony so that at least 6 litters are delivered on the same day (i.e. there would be at least 30 male PND10 rats available for each preparation). The other problem that we had was to keep the cells alive in culture for longer than the 2-week period. It would be interesting to see if addition of other growth factors could increase the survival time of those cells in culture.

In summary, we have developed an *in vitro* rat Leydig cell differentiation model which we have shown can be used to study the mechanism of action of phthalates *in vitro*. Indeed, both MBP and DBP appear to have direct actions on adult-type Leydig cell differentiation *in vitro*. Due to time limitation, the RNA extracted from the cells has not yet been analyzed using quantitative real-time PCR. It will be of our interest to look at the expression of other Leydig cell markers and also proliferation markers to determine the possible mechanism, such as PPARs or glucocorticoid pathways, that could be involved in the slowed down Leydig cell differentiation process. These are difficult experiments requiring extensive animal work, and it was thus not possible to complete sufficient experiments for a publication within the time-frame available. I show here the first sets of such experiments to indicate the potential that this methodology has.

Chapter 7

General discussion

There is growing concern about the adverse effect that a diverse range of environmental xenobiotics could bring to human health, as many appear to possess the ability to interfere with a number of hormonal systems in human, laboratory and wildlife populations. Phthalates, the plasticizers that are added to plastics to impart flexibility, are currently a group of chemicals being extensively subject to scrutiny. Numerous rodent studies have demonstrated that indirect exposure to phthalates such as DBP and DEHP, via the mother, especially during gestation and in the perinatal period could perturb the development of the internal and external male reproductive phenotype. However, the exact mechanism of action of phthalates is still very incompletely understood.

In the human population, partly because of relatively low symptom penetrance and because of a wide range of confounding factors, it has been difficult to establish a direct causal link between phthalates and male reproductive function. Nevertheless, there is increasing evidence to imply that this group of chemicals alone or in concert with others might be responsible for the reduced AGD in male newborns, reduced circulating testosterone, as well as altered behaviour in boys (Main et al, 2006a; Swan et al, 2010; Swan et al, 2005). Phthalates are metabolized and excreted quickly from the body within 24-48 hours; we are however still under constant exposure to phthalates due to their ubiquitous distribution in the environment. It is therefore important to understand how these environmental chemicals work so that an effective protective measure can be employed.

Several studies have suggested that testicular Leydig cells might be a primary target of phthalate action, where they often exhibit abnormal aggregation due to possible enhanced proliferation or abnormal cell translocation (Chapter 1.3.2). This is largely based on microarray studies of the fetal rat testis, whereby Leydig cell gene products are amongst the earliest to show significant changes upon maternal phthalate exposure (Johnson et al,

2007). However, such studies do not indicate whether such effects on Leydig cells are direct actions of the chemical substance or indirect effects via other cell types. The effect of phthalates on Leydig cells appears to depend predominantly on the dosage and time of exposure.

Early attempts to investigate effects of xenobiotics such as phthalates on Leydig cell function have been hampered by several methodological problems. Firstly, the obvious target tissue, the rodent fetal testis at E13.5 to E16.5, is very small and not easily accessible to conventional experimentation, though this has been attempted (Chauvigne et al, 2009; Hallmark et al, 2007; Thompson et al, 2004). In a collaboration with the laboratory of Professor Bernard Jégou in Rennes, France, we have attempted to assess INSL3 expression in fetal testis explants following phthalate exposure (not shown), though without significant results. Moreover, such studies would still not inform as to the primary mechanism of phthalate action. Secondly, several studies have looked for acute effects of phthalates on *in vitro* cultured mature Leydig cells or on cell-lines, mostly with modest effects on steroidogenesis (Ge et al, 2007; Jones et al, 1993; Wang et al, 2006; Wang et al, 2007). Such studies ignore, however, the strong likelihood that *in vivo* phthalate action is upon the differentiation of Leydig cells, rather than on their acute and mature functioning. Thirdly, the disruptive actions of phthalates *in vivo* can neither segregate effects on Leydig cells from effects on other cell types, especially since studies are looking at very dynamic time-points in organogenesis, when most testicular cell types are also differentiating and mutually interacting, nor has it been able to assess whether early effects on cell differentiation persist into later life, or the extent to which these are compensated, for example, by the regulatory actions of the HPG axis. This last point is extremely important in the larger context that we now refer to as the ‘Barker Hypothesis’ or ‘developmental origins of health and disease’ (DOHaD) (Barker, 1995). Indeed, endocrine disruption of

male reproductive function, in as much as it could lead to long-term alteration in Leydig cell status and hence steroidogenic capacity, could be a key factor in determining the health of the aging male, as has been already suggested in recent results from the Massachusetts Male Aging Study (Travison et al, 2007).

The aims of this thesis were therefore to better understand the effect on the Leydig cell differentiation process of a model phthalate, DBP, shown previously to exert disruptive effects on the development of the male reproductive system and commonly present in our environment. The research particularly aimed to develop better methodological paradigms with which to clarify the role and effect of Leydig cell differentiation in the endocrine disruptive action of phthalates.

The initial phase of the project set out to develop a time-resolved fluorescent immunoassay specific for rat INSL3, for the following reasons: (1) INSL3 is a circulating marker that uniquely and directly reflects the differentiation status and/or number of Leydig cells in both the fetal and adult testes in a range of mammals, including humans (Anand-Ivell et al, 2006a; Bay et al, 2005); (2) *Insl3* gene expression has been repeatedly reported to be suppressed in the fetal testis following *in utero* phthalate treatment, in fact being a very early marker for such endocrine disruption, and responsible for testicular maldevelopment (McKinnell et al, 2005; Wilson et al, 2004); (3) Currently, there is no good commercial immunoassay available to detect rodent INSL3 in body fluids or in cell culture medium. The data reported in Chapter 3 show that the newly developed rat INSL3 TRFIA was highly specific for measuring rat INSL3 in both serum and cell culture medium, and is also as effective in identifying mouse INSL3, due to their highly conserved sequences. Circulating INSL3 measured by our rat INSL3 TRFIA reflects directly the age and gender of the animals, also confirming its suitability as a marker for aging Leydig cells. The

circulating INSL3 was also found to be proportional to the amount of specific mRNA expressed. Moreover, we could report for the first time the circulating profile of INSL3 concentration in rat serum through puberty, which is precisely comparable to results previously obtained in our laboratory using immunohistochemistry of testis sections (Sadeghian et al, 2005).

Previous studies have suggested that rats are more susceptible to phthalate action during the time of active gonadal development, since the most pronounced adverse effects were observed in male offspring following *in utero* exposure to phthalates, within a relatively short time-window of exposure (Carruthers & Foster, 2005; Welsh et al, 2008). We hypothesize that phthalate treatment might be affecting the differentiation process of Leydig cells, rather than their acute function. In a first approach, we have made use of the adult EDS-treated rat model. In this paradigm all mature Leydig cells within the adult testis are eliminated and a new population of Leydig cells regenerate from resident stem cells following a precise time-kinetic which is very similar to that during puberty. The advantage of this model is that, unlike puberty, the cellular context is already representing an effectively adult endocrine milieu, whereby most other cell types (other than germ cells) in the testis will be in an already differentiated adult state. We investigated whether DBP treatment, given at an early stage of Leydig cell regeneration after EDS treatment, could alter the differentiation kinetics of those cells. Analysis of various Leydig cell markers by quantitative real-time PCR suggested that the xenobiotic treatment at an early stage of Leydig cell regeneration was indeed able to enhance Leydig cell differentiation. A similar trend was also observed for circulating testosterone and INSL3. In order to find out if the increase of Leydig cell marker expression was due to accelerated differentiation or proliferation, the total number of Leydig cells was quantified using the physical disector method. The results (Chapter 4) strongly suggest that the increase in Leydig cell marker

expression is most likely due to increased Leydig cell proliferation, since larger clusters of Leydig cells were observed in the EDS+DBP and EDS+DES groups, compared to the EDS-only group. Although the number of Leydig cells was only expressed as cellular density, we believe that the total number of Leydig cells per testis in the EDS+DBP and EDS+DES groups was also increased since animals that had higher circulating INSL3 also had a higher number of Leydig cells within the volume of testis assessed. Although Leydig cells were present in larger clusters in DBP and DES-exposed groups, unlike studies in fetal testis where fetal Leydig cells abnormally translocated to the center of testis following DBP treatment (Mahood et al, 2005), we observed a normal distribution of Leydig cells in different parts of the testis in all groups. Our findings in these preliminary experiments imply that adult-type Leydig cells are also sensitive to the action of endocrine disruptors and that the EDS rat model could be very useful in understanding the effect of various endocrine disruptors on adult Leydig cells.

As a second experimental paradigm, we also investigated the long-term effect of phthalates on the adult Leydig cell population. Currently, most studies have concentrated on the acute effects of phthalates, where animals were normally examined at the end of gestation, when typical symptoms of testicular dysgenesis (cryptorchidism, altered AGD, hypospadias, etc.) may be evident. Studies looking at long-term phthalate effects have produced somewhat mixed findings (Chapter 5.1). We were concerned to know if any effect that is caused by phthalates on Leydig cells during fetal life might persist through to adulthood. In other words, could such disruptive effects also influence ‘stem cells’ and hence differentiation of the adult Leydig cell population? In order to investigate this, the progeny received indirect maternal exposure (gestational and lactational) every second day from GD14.5 to PND6. Our findings in Chapter 5 provide evidence that the phthalate effects on Leydig cells that manifested early in life mostly did not persist through to adulthood. This

was illustrated clearly by the circulating INSL3 (Fig. 5.7) at PND90. We have shown elsewhere that circulating INSL3 is an accurate measure of Leydig cell functional capacity, comparable if not superior to the T/LH ratio (Anand-Ivell et al, 2006a). Exposure to DBP or DES during gestation and early postnatal life, however, appear to accelerate the kinetics of Leydig cell development, especially at the time when Leydig cells are actively proliferating or differentiating. Both treatments appeared to cause an earlier increase in circulating INSL3 compared to the control group. This increase in INSL3 level was most likely due to an increase in Leydig cell number (Fig. 5.8). Although some of the Leydig cell markers were affected by DBP or DES treatment at an earlier age (i.e. PND10 and PND24), the effect did not seem to be permanent, since by PND90, all Leydig cell parameters assessed were comparable in all treatment groups. It should be noted that there are marked differences in the occurrence of testicular lesions in cryptorchid and scrotal testes (Mahood et al, 2007). Findings presented in Chapter 5 were based solely on observations of scrotal testes, following random selection. For future studies, it would also be necessary to assess the cryptorchid testes from this study to see if any testis abnormalities found in young animals are resolved later in life. The results presented here would suggest that mechanisms are in place, either by local feedback or using the HPG axis, to regulate the Leydig cells to maintain a consistent norm value of product expression or functional capacity. In addition, our findings provide some insight into the ability of Leydig cells in the scrotal testis to recover from endocrine disruption. This is an important finding since cryptorchidism in the human is common, though normally corrected within the first year of life. This is not to state that early maternal exposure to phthalates is without effect in adulthood. In a parallel study to this one, we showed that there are indeed persistent effects in adulthood on both hypothalamic gene expression and on adult behaviour (unpublished).

The final aim of this thesis was to develop a long term *in vitro* Leydig cell differentiation model, using undifferentiated adult type Leydig cells isolated from day 10 rats (PND10, i.e. before puberty onset), in order to study the mode of action of DBP (Chapter 6). The rationale for using Leydig cells from day 10 rats was based on studies showing that Leydig stem cells first became committed to the Leydig cell lineage at around PND10 (Chapter 1.5.2.2). In this way, we aimed to develop a model that included the early developmental stage of Leydig cells which more closely reflected the situation in the fetal testis, but was more accessible than the fetal testis explant culture. We were able to maintain the cells in culture for a minimum of 14 days and showed that INSL3 secretion increased as the Leydig cells differentiated in the presence of hCG. Consistent to previous reports (Chapter 1.5), we showed that Leydig cells had only low proliferative capacity during the course of development until they attain their mature phenotype under the stimulation of hCG. Preliminary experiments with DBP and its main metabolite, MBP, showed that both compounds were inhibitory to Leydig cell differentiation in the presence of hCG. This important result provides the first direct evidence for an effect of phthalates on Leydig cell differentiation freed from any possible indirect effects on other cellular components within the testicular environment. On their own, however, DBP and MBP appeared to have little effect; in other words at the concentrations used, they were not cytotoxic. For future studies, we plan to analyze the expression of various Leydig cell markers, and intend to identify the molecular mechanisms leading to the retarded Leydig cell differentiation process in this model system. In addition, it will be advantageous to optimize the system further to prolong the survival time of the Leydig cells in culture.

In conclusion, the research reported in this thesis has provided novel and important findings highlighting that differentiating/developing adult-type Leydig cells are indeed potential targets of endocrine disruption. We have also provided evidence supporting the

view that phthalate effects on Leydig cells may be different depending on the dosage and exposure time.

Finally, studies such as these have been criticized, since effects of xenobiotics, such as DBP, are only achieved at concentrations which are many times higher than those prevalent in the natural world. Whilst superficially correct, such criticism requires comment. Firstly, in performing animal experiments, and also when using derived cell cultures, there is an ethical and financial obligation to reduce the numbers of animals and repetitions involved, and thereby to maximize the power of the experiment. This is done by using doses consistent with high symptomatic penetrance amongst the treated animals. To reproduce the level of penetrance typically seen in the human population (e.g. 1% incidence of cryptorchidism, 0.1% incidence of hypospadias) would involve animal experiments using many millions of rodents in order to achieve any statistical significance, clearly something which would be ethically unjustifiable. Secondly, in a recent study on AGD in newborn male infants from the normal human population, Swan and colleagues were able to show a statistically significant association with levels of phthalates in maternal urine which were well within the so-called normal range for this xenobiotic and at least 100-fold lower than the limit previously considered to represent a hazard to human health, based on previous animal experiments (Swan et al, 2005). One explanation is that in animal experiments we are assessing only exposure to a single chemical entity (or its metabolites) at any one time, whereas in nature exposures are always to many quite diverse compounds, for which the one measured (e.g. a particular phthalate ester) can be seen as a surrogate for the total chemical exposure. Recently, both *in vitro* and *in vivo* studies have shown that mixtures of xenobiotics, each at a very low level (well below previously measured risk concentrations), can have additive or even supra-additive (synergistic) effects leading to a highly significant impact on endocrine systems (Christiansen et al,

2008; Hass et al, 2007; Hotchkiss et al, 2004). It is in this light that experiments such as those described here are to be interpreted. I have been able to show that differentiating Leydig cells are indeed targets for phthalate action, effects which lead to a change in the rate of differentiation and/or proliferation of the cells, under circumstances which are statistically significant even given the relatively low power applied. Translating such effects to a human population would imply that lower concentrations of xenobiotic are indeed likely to impact on testis function with a symptom penetrance comparable to that observed in large epidemiological studies.

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