Regulation of Leydig Cell Differentiated Function by the Industrial Chemical Bisphenol A (BPA)

by

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Abstract

Exposure of the population to bisphenol A (BPA) is significant due to its extensive use in the manufacture of a wide variety of consumer products, including polycarbonate plastics and epoxy resins. However, there is concern that early-life exposure to BPA may alter developmental programming and predispose individuals to adverse health outcomes such as infertility and obesity in later life. This dissertation describes the adverse effects of early-life exposure to BPA on Leydig cell function using the rat model.

Testicular Leydig cells are the predominant source of the male sex steroid hormone testosterone, which supports the male phenotype. The objectives were to investigate the effects of developmental BPA exposure on (i) Leydig cell development; (ii) adipose tissue derived molecules (e.g., adiponectin) and their regulation of Leydig cells; and, (iii) estrogen metabolism and antioxidant capacity in Leydig cells.

Exposure of male rats to BPA by maternal gavage at doses of 2.5 and 25 μg/kg body weight once a day from gestational day 12 to day 21 postpartum stimulated Leydig cell division in the prepubertal period and increased Leydig cell numbers in the testes of adult male rats at 90 days. BPA-induced proliferative activity in the prepubertal period was associated with induction of cell cycle protein

expression, up-regulation of growth factor receptors (e.g., insulin-like growth factor 1 receptor and epidermal growth factor receptors), activation of mitogen activated protein kinase 3/1 and possibly decreased anti-Mullerian hormone (AMH)-mediated signaling. Furthermore, *in vitro* experiments confirmed that BPA acted directly as a mitogen in Leydig cells. On the other hand, developmental BPA exposure had an inhibitory effect on Leydig cell androgen secretion by decreasing protein expression of the luteinizing hormone receptor (LHR) and the 17beta-hydroxysteroid dehydrogenase 3 (HSD17B3) enzyme.

A separate study investigated the interaction of developmental BPA exposure with high fat diet (HFD) feeding from postnatal day 70 to 98 on adipose tissue and Leydig cell function. This is important because use of high fat diets and the incidence of obesity in the population are on the increase. Following perinatal BPA exposure, i.e., gestational day 12 to postnatal day 21, male rats were maintained on normal or high fat diet from PND 70 to 90 days of age. Animals were sacrificed within 48 h of day 98 postpartum when serum, gonadal fat tissue and Leydig cells were obtained for further analysis. Results showed that BPA exposure alone and/or in combination with HFD feeding decreased serum adiponectin levels compared to control animals on normal fat diet (NFD) ($P<0.01$). Similarly, BPA exposure and HFD feeding decreased Leydig cell adiponectin and its receptor (AdipoR2) expression. However, serum testosterone levels were paradoxically greater in HFD-fed male rats exposed to the 25 μg/kg dose of BPA compared to control HFD animals ($P<0.05$). The decrease in androgen secretion was associated with reduced Leydig

cell HSD17B3 enzyme protein expression. In contrast, HFD feeding had the effect of up-regulating HSD17B3 enzyme expression in Leydig cells.

Finally, the effects of developmental BPA exposure and HFD feeding on estrogen metabolism and antioxidant capacity in Leydig cells were investigated. In general, maintenance of male rats on HFD decreased serum 17β-estradiol levels in all groups compared to age-mates on NFD ($P<0.05$). BPA exposure increased estrogen sulfotransferase expression in adipose tissue and Leydig cells but this effect was reversed by maintenance on the HFD. Also, HFD feeding increased serum levels of thiobarbituric acid reactive substance, a marker for oxidative stress, in male rats exposed to BPA. Exposure to BPA at the 25 μg/kg dose and/or HFD feeding increased reactive oxygen species generation in Leydig cells. Analysis of enzyme antioxidant capacity showed that superoxide dismutase activity was decreased in Leydig cells in the HFD groups, whereas catalase and glutathione peroxidase activities were increased by exposure to BPA and HFD feeding. *In vitro* experiments confirmed that BPA acts directly to induce oxidative stress in Leydig cells. This is important because oxidative stress is known to inhibit Leydig cell androgen biosynthesis.

In conclusion, developmental exposure to BPA disrupted Leydig cell development and function. Further, the effects of BPA in Leydig cells were influenced by a HFD. Altogether, the present results showed that BPA causes biological effects at environmentally relevant exposure levels with implication for public health.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ALC	Adult Leydig cell
AMH	Anti-Mullerian Hormone
AMHR2	Anti-Mullerian Hormone Receptor 2
AR	Androgen receptor
BTB	Blood-testis-barrier
CYP11A1	Cytochrome P450 side-chain cleavage enzyme
CYP17A1	Cytochrome P450 17α-hydroxylase
DES	Diethylstilbestrol
dpc	Days post coitum
E_2	17β-estradiol
EDCs	Endocrine disruptor chemicals
EDS	Ethane dimethanesulfonate
EGF	Epidermal growth factor
EGFR	EGF receptor
EPA	Environmental Protection Agency
ESR	Estrogen receptor
EST	Estrogen sulfotransferase
FLC	Fetal Leydig cell
FSH	Follicle stimulating hormone

FSHR	Follicle stimulating hormone receptor
GD	Gestational Day
GnRH	Gonadotropic releasing hormone
GPCR	G-protein Coupled Receptor
GPx	Glutathione Peroxidase
GSH	Reduced glutathione
HFD	High fat diet
HSD17B3	17-beta hydroxysteroid dehydrogenase isoform 3
HSD3B	3-beta hydroxysteroid dehydrogenase
IGF-1	Insulin like growth factor-1
IGF-1R	IGF-1 receptor
ILC	Immature Leydig cell
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
NFD	Normal fat diet
NIEHS	National Institute of Environmental Health Sciences
NTP	National Toxicology Program
PLC	Progenitor Leydig cell
PND	Postnatal day
RIA	Radioimmunoassay
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SRY	Sex-determining region Y

StAR	Steroidogenic acute regulatory protein
T	Testosterone
TBARS	Thiobarbituric acid reactive substances

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1. Introduction

The term 'endocrine disruptor' was first coined at the conference on "Chemically-Induced Alterations in Sexual Development: The Wildlife/Human Connection" held at the Wingspread Centre, Racine, Wisconsin, in 26-28 July 1991. Theo Colborn published the first paper in 1993 describing the phenomenon of hormone disruption due to endocrine disruptor chemicals (EDCs) and their adverse health effects, which are often permanent if exposure occurred early in development (Colborn et al., 1993). Since then, a broader consensus has emerged among scientists that EDCs may cause adverse health effects in both wildlife and humans (Diamanti-Kandarakis et al., 2009).

According to the U.S. Environmental Protection Agency (EPA), EDCs are defined as "exogenous agents that interfere with the synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process" (Diamanti-Kandarakis et al., 2009). Among EDCs, exogenous sources of estrogens are classified as xenoestrogens if they interfere with endogenous estrogen action. Xenoestrogens are known to act through estrogen

receptors (ESR) (e.g., ESR1 and ESR2), which are expressed in the brain, bone, cardiovascular system, adipose tissue and reproductive tract. Studies of transgenic mice and natural cases of estrogen deficiency have shown that estrogen regulates normal fertility and adipogenesis in males (Akingbemi, 2005; Jones et al., 2007). Also, exposure of laboratory animals to xenoestrogens or supra-normal levels of estrogens were found to reduce gonad size, sperm count and quality, and caused genital abnormalities in the male (Akingbemi, 2005), and disturbances in carbohydrate and lipid metabolism, as seen in metabolic syndromes and obesity, were present in EDC-exposed animals (Alonso-Magdalena et al., 2006; Hugo et al., 2008; Miyawaki et al., 2007; Rubin et al., 2001; Rubin and Soto, 2009; Somm et al., 2009). The effects of xenoestrogens are relevant to public health because there have been reports of global deterioration in male reproductive health affecting sperm production and semen quality in many industrialized countries (Carlsen et al., 1992; Rubin and Soto, 2009; Swan et al., 2000). Furthermore, the incidence of developmental defects such as hypospadias and cryptorchidism in newborn male infants as well as testicular cancers in men has increased in the past two decades (Swan et al., 2000; Toppari et al., 1996). These observations are similar to those seen in male subjects exposed to diethylstilbestrol (DES) during pregnancy (Toppari et al., 1996).

A sedentary lifestyle, high calorie intake and genetics are thought to be contributing factors to development of obesity and metabolic syndromes in the population (Ebbeling et al., 2002). More recently, several reports suggest that exposures to environmental estrogens such as DES in the fetal and neonatal period

can alter developmental programming and predispose individuals to obesity later in life (Newbold et al., 2009). This is perhaps not surprising because reproductive capacity is closely linked to the nutritional status of an individual. In this regard, a growing number of adipocyte-derived molecules (adipokines), which regulate energy balance and metabolism, have been identified as putative regulators of reproduction in the male (Fernandez-Fernandez et al., 2006). Of interest to the present study, receptors for adipokines such as adiponectin, leptin, ghrelin, orexins and resistin have been identified in Leydig cells and possibly mediate the effects of energy balance on male reproduction (Barreiro et al., 2004; Caminos et al., 2008; Nogueiras et al., 2004; Tena-Sempere and Barreiro, 2002; Tena-Sempere et al., 2002). Indeed, BPA was found to inhibit adiponectin secretion from human adipose tissue explants (Hugo et al., 2008). Therefore, xenoestrogens may cause reproductive deficiency by regulating adipokine secretion.

BPA is a known estrogenic chemical used in the manufacture of polycarbonate plastics and epoxy resins. BPA is one of the highest volume production chemicals with an annual increase in demand of 6% to 10% per year. For example, in 2003, the global production of BPA was >2 million metric tons (Vandenberg et al., 2007). In a survey [The National Health and Nutrition Examination Survey (NHANES)] conducted by the Centers for Disease Control and Prevention (CDC) in 2003-04, BPA urinary concentrations were determined to range from 0.4 μg/L to 149 μg/L in 93% of Americans who are 6 years of age and older (Calafat et al., 2008). It has been hypothesized that there is a potential relationship between chemical exposure and the increase in incidence of negative

health trends in the population e.g., abnormal penile/urethra development in males and early sexual maturation in females during the period of reproductive tract development (vom Saal et al., 2007). In addition, neurobehavioural problems, e.g., attention deficit hyperactivity disorder and autism, childhood and adult obesity, type 2 diabetes, hormonally mediated cancers (e.g., prostate cancer and breast cancer) are also on the increase in the population (vom Saal et al., 2007). Despite a growing number of laboratory studies showing adverse effects of BPA occurring at doses relevant to human exposures, the possible adverse health effects of BPA on public health is the subject of ongoing debate.

This dissertation describes observations from investigation of the effects of developmental BPA exposure on postnatal Leydig cell development, as reflected by cell proliferation and steroidogenesis. In addition, BPA- and diet-induced changes in adipose tissue function and related effects on Leydig cells were investigated in sexually mature male rats.

2. Bisphenol A (BPA)

BPA, also known as 2,2-bis(4-hydroxyphenyl)propane, exists as a white solid at room temperature with a mild "phenolic" odor (CAS # 80-05-7). The molecular formula of BPA is $C_{15}H_{16}O_2$ (figure 1) and molecular weight is 228.29 g mol^{-1}. BPA is a key monomer in the production of polycarbonate plastic and epoxy resins. Because polycarbonate plastics are clear and nearly shatter-proof, they are used in the manufacture of food and drink storage containers (e.g, water and infant bottles and tableware), personal sports safety equipments (e.g., helmets), automobile parts

(e.g., windshields), electronics (e.g., cell phones, computers, compact discs, photographic films) and medical devices (e.g., tubings). Polycarbonate plastics can be identified by the recycle symbol "7" or by the letters "PC" near the recycle symbol. BPA is also used as a polymerization inhibitor in the manufacture of polyvinyl chloride (PVC) plastics. Therefore, PVC plastics showing the recycle symbol "3" also contains BPA. Epoxy resins are used as a lacquer coat in metallic food and beverage cans, water supply tanks and pipes and as a constituent of dental sealants. In 2004, estimated BPA production in the United States was approximately 2.3 billion pounds (NTP, 2008).

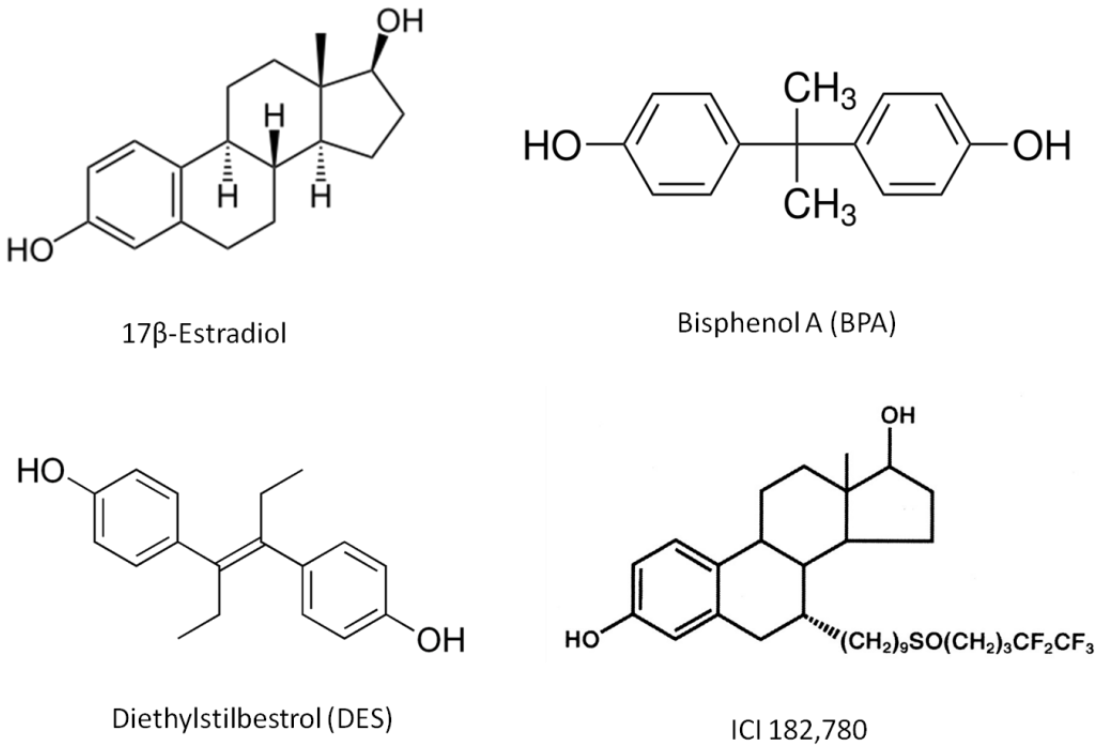

Figure 1. The chemical structure of ESR agonists, 17β-estradiol, BPA, DES and the antiestrogen, ICI 182,780 (Akingbemi et al., 2004).

After enactment of the 'Toxic Chemical Substance Act' in 1976, the first regulatory evaluation of BPA by the National Toxicology Program (NTP) determined that the lowest observed adverse effect level (LOAEL) in laboratory animals is 1,000 parts per million (ppm) in the diet i.e., 50 mg/kg/day. This dose was used to calculate the reference dose by the U.S. NTP and Environmental Protection Agency (EPA, 1993). According to EPA, the reference dose is a maximum acceptable daily oral exposure to a toxic substance by the human population without an appreciable risk of deleterious effects during a lifetime (EPA 1993). After dividing the LOAEL by the uncertainty factor of 1000, the reference dose for BPA was set at 0.05 mg/kg body weight/day (Willhite et al., 2008).

Krishnan and others (1993) were the first to demonstrate the estrogen activity of BPA while working with *Saccharomyces cerevisiae* yeast (Krishnan et al., 1993). The conditioned medium, which was used to grow yeast in autoclaved polycarbonate flasks, showed the presence of substances that competed with [^{3}H] estradiol for binding to ESRs isolated from the rat uterus. Later, this compound was purified by HPLC and determined to be BPA using nuclear magnetic resonance spectroscopy and mass spectrometry. However, the affinity of BPA for ESRs was found to be 2000 fold less compared to estradiol. In other studies, using functional bioassays, it was also determined that BPA was 5000 fold less active in inducing the proliferation or expression of progesterone receptor in human mammary cancer cells (Krishnan et al., 1993).

The adverse effects of BPA *in vivo* at low doses were demonstrated for the first time in 1997. Exposure of male mice to BPA from gestational day (GD) 11 to 17 at the dose of 2 and 20 μg/kg/day was found to increase prostate weights in adult animals (Nagel et al., 1997). In the same year, the estrogenic activity of BPA was demonstrated *in vitro* in pituitary lactotrophs—a well established estrogen responsive cell line—and *in vivo* using ovariectomized Fisher 344 (F344) rats, at doses lower than used in regular toxicological studies (Steinmetz et al., 1997). Although BPA showed estrogenic activity at 1000- to 5000-fold higher concentrations in causing prolactin gene expression, release, and cell proliferation in pituitary cells, it exhibited similar potency as estradiol to induce estrogen response element (ERE) activation (Steinmetz et al., 1997). In other studies, the 2 ng/g body weight dose of BPA increased the size of preputial glands but reduced the size of the epididymides, whereas the 20 ng/g dose decreased daily sperm production by 20% compared to control animals (vom Saal et al., 1998). Another report showed that female mice housed in damaged polycarbonate cages developed sudden, spontaneous increases in meiotic disturbances including aneuploidy in the ovary, which effects were replicated in laboratory studies (Hunt et al., 2003).

Although the initial review by NTP acknowledged there is "credible evidence" that low doses of BPA can cause effects on specific endpoints, it concluded that the effects are not reproducible and that further studies were needed to clarify adverse effects due to BPA (NTP, 2001). Successive review panels convened by the Harvard Center for Risk Analysis and sponsored by American Plastics Council in 2002 and 2006 reached similar conclusions that the weight of evidence does not

support the view that low oral doses of BPA may impair reproductive health (Goodman et al., 2006). In yet another review in 2007, a group of independent researchers from across the globe were convened in Chapel Hill, North Carolina, at the request of the National Institute of Environmental Health Sciences (NIEHS) to review low-dose BPA effects collected from *in vitro*, *in vivo*, wildlife, and human exposure studies (vom Saal et al., 2007). The panel concluded that low-dose exposure of laboratory animals to BPA caused reproductive, neurological, behavioral, and metabolic abnormalities particularly when exposure occurred during critical windows of development (i.e., *in utero* or neonatally) (vom Saal et al., 2007).

2.1 BPA Levels in Human Tissues

BPA is able to leach from containers into foods or liquids when heated or under acidic or basic conditions (Le et al., 2008). BPA may also leach from dental sealants into the saliva of patients. Therefore, BPA has been detected in food, drinking water, air, and dust (Dekant and Volkel, 2008). Human exposure to BPA occurs primarily by ingestion and dental sealants, skin and inhalation of dust are considered non significant sources of exposure (Dekant and Volkel, 2008). However, BPA can transfer across the human placenta and was detected in maternal and fetal plasma, placenta, amniotic fluid, and follicular fluid (Ikezuki et al., 2002; Schonfelder et al., 2002). The concentrations of BPA in tissues are variable and range from 0.3 to 18.9 ng/mL in maternal plasma, 0.2 to 9.2 ng/mL in fetal plasma, and 1.0 to 104.9 ng/g in human placenta. In addition, BPA was measured in the

range of 0.28–0.97 ng/mL in human breast milk (Sun et al., 2004). The recent human estimated daily intake of BPA assessed by NTP is shown in Table 1, all of which are below the current US EPA oral reference dose of 50 μg/kg/day.

Table 1. Estimated daily intake of BPA in humans based on the sources of exposure (NTP, 2008)

Population	**Daily BPA intake (μg/kg bw/day)**
Infant (0 – 6 m) – Formula-fed	1 – 11
Infant (0 – 6 m) – Breast-fed	0.2 - 1
Infant (6 – 12 m)	1.65 – 13
Child (1.5 – 6 years)	0.043-14.7
Adult – general population	0.008 – 1.5
Adult - occupational	0.043-100

2.2 BPA Metabolism

Toxicokinetic studies of BPA in rodents, non-human primates and humans showed absorption of BPA after oral ingestion. BPA undergoes metabolism in the liver mostly by glucuronide conjugation. Other minor BPA metabolites, including sulfated conjugates and BPA-3,4-quinone have also been reported. This is important because conjugated BPA does not bind to ESRs and is known to be biologically inactive compared to the parent or unconjugated compound (Doerge DR and Fisher JW, 2010).

Species differences in the toxicokinetics of BPA have been observed in rodents versus humans. For example, the majority of BPA glucuronide in rodents is

secreted in the bile for elimination through feces and subjected to enterohepatic circulation resulting in a longer half-life (Doerge et al., 2010a). However, non-human primates and humans readily excrete BPA conjugates in urine within six hours, which prevents accumulation in the body. This argument was initially used to argue against extrapolation of BPA effects in laboratory animals to human subjects. However, other studies have shown similar BPA toxicokinetics for rodents and primates (Taylor et al., 2011). Moreover, BPA can readily pass through the placenta to the fetus/embryo *in utero* and through milk to neonates both in laboratory animals and humans (Doerge et al., 2010a; Doerge et al., 2010b).

2.3 Receptor or Signaling Molecules Activated by BPA

The biological actions of BPA are thought to be mostly due to its estrogenic properties. In this regard, the male reproductive tract, including testicular Leydig cells, expresses high levels of estrogen receptors (ESR1, ESR2) along with androgen receptors (ARs) (Zhou et al., 2002). Although BPA is thought to have a higher affinity for ESR2, it is able to induce both ESR1 and ESR2-mediated transcriptional activity with comparable efficacy in several tissues (Matthews et al., 2001; Wetherill et al., 2007). Furthermore, BPA can bind ARs and thereby exhibit antiandrogenic activity (Lee et al., 2003; Wetherill et al., 2002) or activate mutant AR at lower doses (Wetherill et al., 2007). In addition, BPA has been credited with the capacity to elicit biological responses by activation of kinase cascades e.g., MAPK (Song et al., 2002) and antithyroid activity (Zoeller et al., 2005). Other reports have suggested that BPA increased bioavailability of sex steroid hormones by disrupting metabolic

degradation (Cannon et al., 2000; Hanioka et al., 1998; Kester et al., 2002). Several *in vitro* reports have indicated that BPA may interact with estrogen-related receptor γ (ERR γ) and other receptors including membrane bound ESR (NTP, 2008; Ben-Jonathan et al., 2009). BPA promotes seminoma cell proliferation by acting through estrogen membrane receptors, GPCR 30 (Chevalier et al., 2012). Although BPA binds to ESR and other receptors at higher concentrations than endogenous ligands, its potency at low concentration is attributed to low relative binding affinity to serum proteins, which makes more BPA available for binding to receptors (Nagel et al., 1997).

3. Male Reproduction

In mammals, reproduction depends on normal development of reproductive organs along with differentiation of specialized cell types (e.g., androgen secreting Leydig cells). However, development of reproductive organs begins during the prenatal period and is completed in the postnatal period prior to attainment of sexual maturation. The testes or male gonads are the important primary male reproductive organs with twin functions; spermatogenesis and steroidogenesis. However, testis function is under the control of gonadotropic hormones—luteinizing hormone (LH) and follicle stimulating hormone (FSH)—secreted by the anterior pituitary gland. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which controls gonadotropin (LH and FSH) release from the anterior pituitary. The hypothalamus and pituitary are regulated through a classic neuroendocrine feedback loop mediated by gonadal hormones (Figure 2).

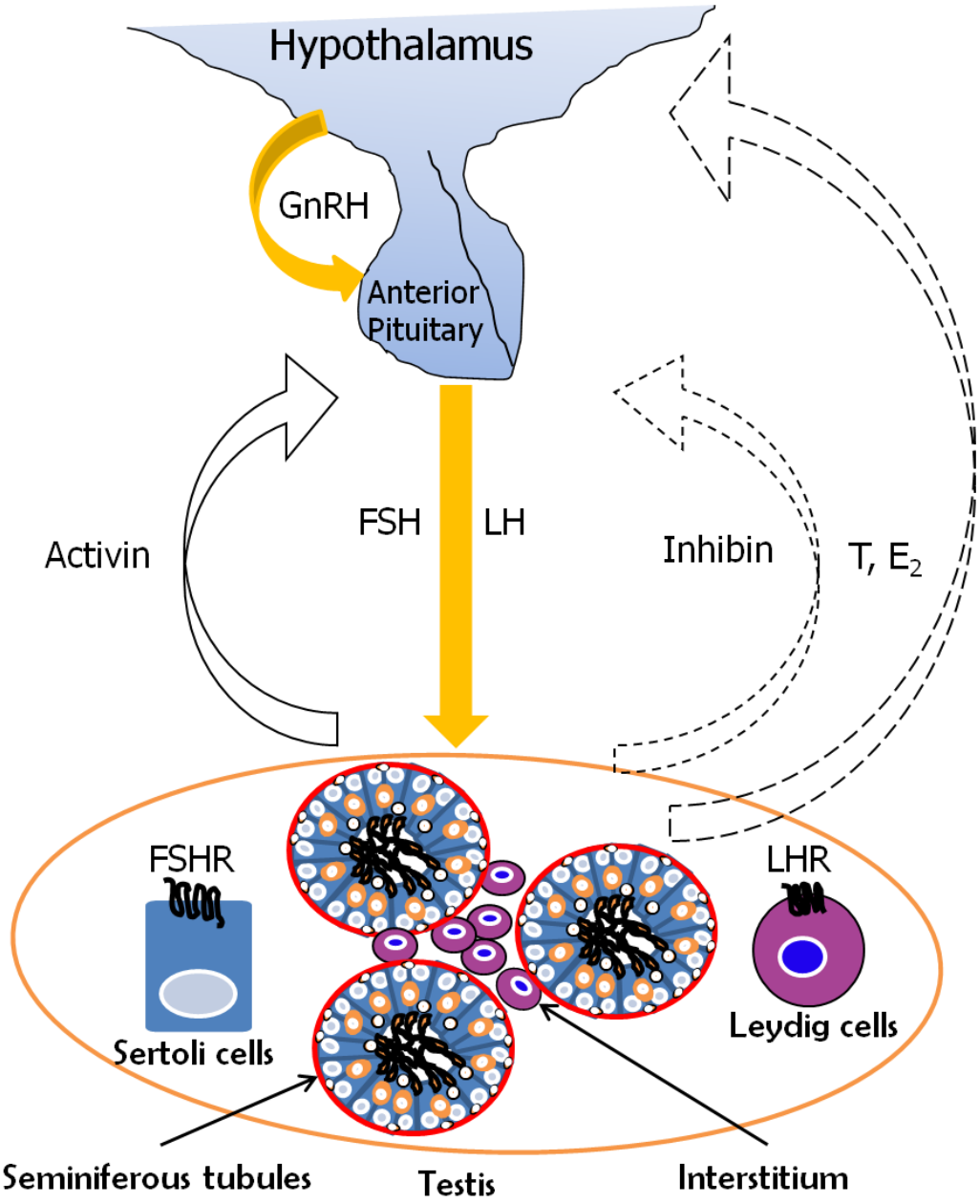

Figure 2. Schematic representation of the hypothalamic-pituitary-gonadal axis. Solid arrows represent positive stimulatory effects and dotted arrows correspond to negative inhibitory actions. GnRH – gonadotropic releasing hormone, LH – luteinizing hormone, LHR – LH receptors, FSH – follicle stimulating hormone, FSHR – FSH receptors, T – testosterone, E_2 – 17β-estradiol.

Pituitary LH binds to G-protein coupled LH receptors (LHR) present in Leydig cells to stimulate testosterone production, whereas FSH acts on its seven trans-membrane FSH receptors (FSHR) present in Sertoli cells to stimulate secretion of products that are required for spermatogenesis. Testosterone (T) promotes secondary sex characteristics and hormonal imprinting of the prostate and hypothalamus during the prepubertal period in males. Sertoli cells not only provide physical support to germ cells but also create an immunologically privileged environment formed by the blood-testis-barrier (BTB) to facilitate the process of spermatogenesis. In addition, during prenatal development, T and anti-Mullerian hormone (AMH), secreted by fetal Leydig cells and Sertoli cells, respectively, are required for regression of the Mullerian duct and development of the Wolffian duct (a precursor of male reproductive tract tissues). In the adult, T is required for normal sexual behavior and function.

3.1 Male Sexual Differentiation

Mammalian sex differentiation starts with genetic determination at the time of fertilization, followed by development of either testis or ovary from the undifferentiated bi-potential genital ridge and subsequent growth of external and internal genital structures. Gonadal sex differentiation depends on expression of the master regulatory gene, *Sex-determining Region Y* (*SRY*), in the undifferentiated gonadal ridge (Hughes, 2001; Wilhelm and Koopman, 2006). In males, a specific subset of genital ridge cells express *SRY* and differentiate into Sertoli cells which

coordinate differentiation of other cell types (e.g., germ cells and Leydig cells) during testis formation.

Following testis determination, differentiation is defined by the phenotypic development of male reproductive tract and is dependent on hormones secreted by testicular cells early in gestation. For example, Sertoli cell derived Anti-Müllerian Hormone (AMH) induces regression of the Mullerian ducts through apoptosis, whereas T, a product of fetal Leydig cells, stimulates development of the Wolffian ducts. These hormones along with insulin-like 3, a product of fetal Leydig cells, control testis descent into the scrotal sac (Yao and Barsoum, 2007). During late gestation and in the neonatal period, T, secreted by fetal Leydig cells, indirectly masculinizes the developing male brain by conversion of T into 17β-estradiol (E_2) by action of the aromatase enzyme (Balthazart, 2011). Masculinization of the brain is necessary for the normal development of male-specific sexual behavior in adults. Thus, disruption of sex steroid actions either by endocrine disruptors (Sharpe, 2006) or due to natural cases of mutations will lead to reproductive dysfunction in adults due to abnormal sexual behavior or phenotypic conditions such as intersex (Imbeaud et al., 1995; Kremer et al., 1995), hypospadias and cryptorchidism and subfertility (Sharpe, 2006).

3.2 Testis Development in Rodents

Around 10 days post coitum (dpc), the gonadal ridge appears as a thickening of the coelomic epithelium along the surface of the mesonephros. Proliferation of coelomic epithelium in the ridge gives rise to the somatic cells of the gonads. The

germ cells migrate from the base of the allantois via the gut mesentery and populate the gonadal ridge between 10.5 and 11.5 dpc. With *SRY* gene expression in Sertoli cell precursors between 10.5 dpc to 12 dpc, sex-specific gonad formation is initiated. Expression of the male-determining gene, *SRY,* induces a cascade of gene expression events resulting in differentiation of the genital ridge into a testis. Along with *SRY* expression, differentiation of Sertoli cells occurs around 11.2 to 12.5 dpc, which then surround germ cells to form testis cords around 12.5 dpc (~48 h after the expression of *SRY*). These events are followed by the differentiation of steroidogenic Leydig cells in the testis interstitium between 12.5 and 13.5 dpc (Ross and Capel, 2005; Wilhelm and Koopman, 2006).

3.3 Testis Structure

Testes are paired organs located in the protective pouch called the scrotum in the inguinal area of most mammals (exception in elephants and cetaceans). Anatomical location of the testes is suitable for maintaining the optimal temperature that is required for the process of spermatogenesis. Scrotum consists of skin supported by tunica dartos and fascia. Below the fascia is the parietal layer of *tunica vaginalis* and vaginal cavity. Furthermore, testis parenchyma is covered by a thick fibrous connective tissue capsule made up of visceral layer of *tunica vaginalis* and *tunica albuginea* through which blood vessels pass to supply nutrients required for testis function. *Tunica albuginea* penetrates the testicular parenchyma to form connective tissue septae that divide the testis into lobules. Each lobule contains convoluted seminiferous tubules active in spermatogenesis (*tubulus contortus*),

which continue as inactive straight tubules (*tubulus rectus*) for transport of spermatozoa into an interconnected network of ducts (*rete testis*) in the mediastinum. Ducts from *rete testis* are connected to form efferent ductules which carry spermatozoa into a single highly convoluted epididymal duct, which continues as ductus deferens and join with the pelvic urethra. Efferent ductules and the epididymal duct absorb the excessive fluid secreted from the Sertoli cells during sperm maturation (figure 3).

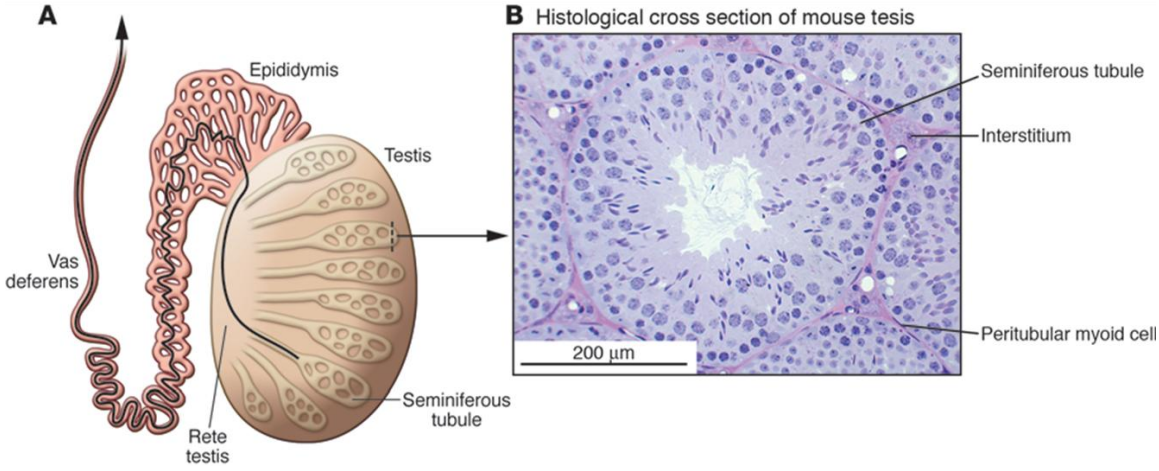

Figure 3. The schematic gross and histological structure of mouse testis. Reproduced with permission of the publisher (Hogarth and Griswold, 2010).

Histologically, testicular parenchyma is divided into tubular and interstitial compartments for spermatogenesis and steroidogenesis, respectively (figure 3). The tubular compartment consists of the seminiferous epithelium which includes Sertoli cells, spermatogonia, developing germ cells, a basement membrane and peritubular myoid cells. The tight junctions between two adjacent Sertoli cells further divide the tubular compartment into adluminal and basal compartments

(Figure 4). From the basal compartment to the tubular lumen, cell types seen are spermatogonia (type A and B), spermatocytes, spermatids, and mature spermatozoa (Figure 4). The interstitial compartment contains Leydig cells, macrophages, capillaries, lymphatic vessels and connective tissue.

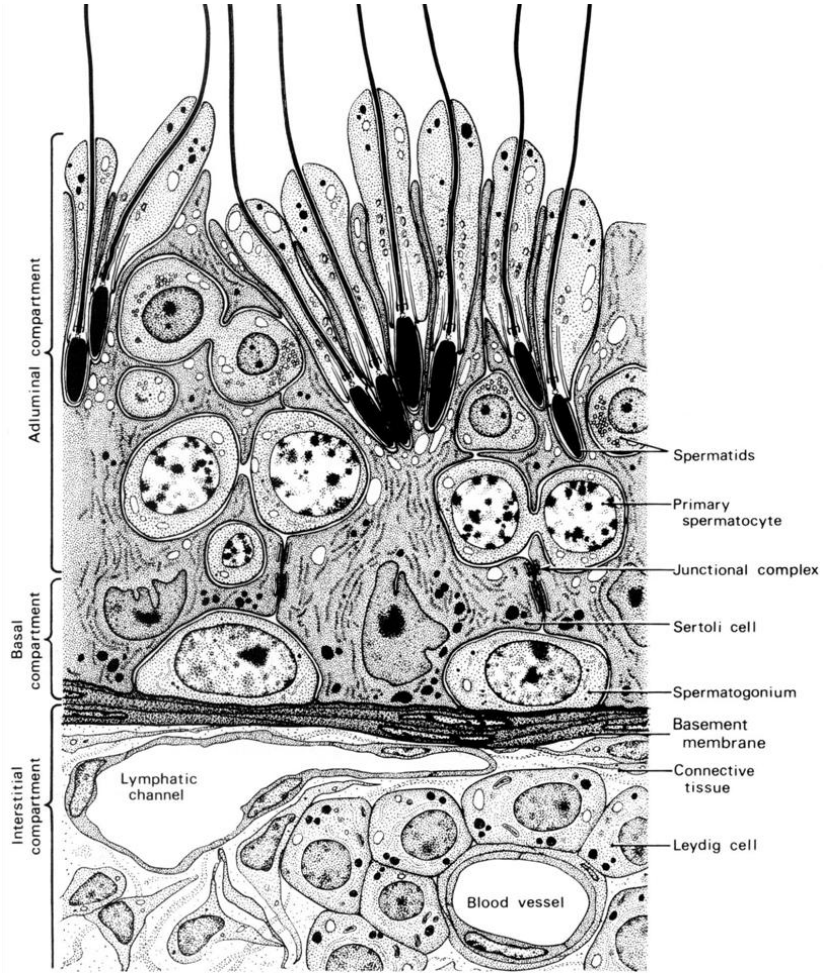

Figure 4. Schematic representation of cell types of the tubular and interstitial compartments of the testis. Reproduced with permission of the publisher (Amann 2010).

3.4 Spermatogenesis

Spermatogenesis is the process of producing mature haploid spermatozoa with fertilizing capacity from immature diploid spermatogonial cells through a series of events that ensure non-interrupted supply of male gametes for reproductive activity. This process provides genetic diversity through meiosis for better evolutionary adaptations and perpetuation of species without transmission of defective genome to the offspring (Manandhar and Sutovsky, 2008). Spermatogenesis occurs in three phases; spermatocytogenesis, meiosis and spermiogenesis (Johnson et al., 1999).

Spermatocytogenesis is the process by which primordial germ cells divide by mitosis to provide a renewing stem cell population and early progenitor spermatogonial cells that then commit to meiosis. Spermatocytogenesis occurs in the basal compartment of the seminiferous tubules. Based on chromatin patterns and nuclear features, two types of spermatogonia (i.e., Type A and B) can be identified in testis of all mammals. Type A spermatogonia which are progenitor spermatognial cells with pale staining and sparse nuclear chromatin, divide and differentiate into Type B with coarse chromatin and a dark staining nucleus. The type B spermatogonia differentiate and enter into the process of meiosis and are then called primary spermatocytes (Johnson et al., 1999; Manandhar and Sutovsky, 2008).

During spermatogenesis, primary spermatocytes migrate within the tubular epithelium to cross Sertoli cell tight junctions (i.e., BTB) into the adluminal

compartment (figure 4). Spermatocytes enter into meiosis, a type of cell division by which diploid (2N) spermatogonial cells become haploid (N) round spermatids (Kopera et al., 2010). Homologous recombination followed by separation of chromosomes occurs in spermatocytes during meiosis I and results in the formation of secondary spermatocytes (Hogarth and Griswold, 2010). Secondary spermatocytes advance towards meiosis II during which sister chromatids are separated into individual cells containing the haploid number of chromosomes (N) and are designated round spermatids. Therefore, round spermatids are genetically different from their parent spermatocytes (Kopera et al., 2010).

The transformation of a round spermatid into spermatozoa through changes in cellular morphology without undergoing cell division is called spermiogenesis. The basic events of spermiogenesis include formation of the acrosome, condensation of chromatin, development of the flagellum, reorganization of the cytoplasm and cellular organelles to shed excessive cytoplasm, and the release of spermatozoa into the lumen of the seminiferous tubules (spermiation). The process of spermatogenesis requires active participation of Sertoli cells, which provide physical support through cell-cell contacts with germ cells, phagocytosis of excessive cytoplasm and critical nutrients (Griswold and Russell, 1999).

Spermatogenesis in the mammalian testis occurs in a temporospatial manner such that different cellular associations (also known as stages) that vary with time and distance are seen along the tubules. For example, there are 14 distinct microscopically recognizable stages in rats and six in humans. Sequential

progression through all stages is called the spermatogenic cycle. The orderly progression of spermatogenic stages along the length of the tubule in a wave-like pattern is described as the spermatogenic wave, which is designed to assure a continuous supply of spermatozoa (Johnson et al., 1999; Manandhar and Sutovsky, 2008).

Spermatogenesis is under the control of somatic cells of the testis (i.e., Sertoli cells and Leydig cells), whose functions are under the control of pituitary gonadotropins (LH and FSH) and locally produced factors. LH controls spermatogenesis indirectly through stimulation of Leydig cells to produce T. Both T and FSH exert their actions on Sertoli cells to control spermatogenesis (Clermont, 1972; Cooke and Saunders, 2002). Also, T can affect Sertoli cell function indirectly via signaling through peritubular myoid cells mediated by ARs (Zhang et al., 2006). Evidence from knockout mouse models and naturally occurring mutations in humans showed that FSH is not absolutely essential for spermatogenesis but is required for quantitative sperm production. On the other hand, T is critically required for post-meiotic development of germ cells (Hogarth and Griswold, 2010).

3.5 Sertoli Cells

Sertoli cells are epithelial supporting cells, which nurse germ cells by providing structural and nutritional support and, hence, are called 'nurse cells' or 'sustentacular cells' of the testis. Sertoli cells reside on a basement membrane and reach to the lumen of the seminiferous tubules (figure 4). Sertoli cells have a complex three-dimensional structure with cytoplasmic crypts, which contain

developing germ cells (Griswold and Russell, 1999). Sertoli cells maintain the integrity of the tubular epithelium by establishing dynamic cell-cell contacts with clones of differentiating germ cells and facilitate movement through the tight junctions (Wang and Cheng, 2007). Tight junctions create an environment, which protects post-meiotic germ cells from attack by the immune system as well as controlling the entry and exit of nutrients, hormones and other chemicals into the tubules (Griswold and Russell, 1999; Wang and Cheng, 2007).

In addition to structural support, Sertoli cells secrete nutrients (e.g., lactate, transferrin) and growth factors (e.g., stem cell factor) required for germ cell development (Griswold and Russell, 1999). Because of their nursing and supportive function, sperm production in the adult testis correlates with Sertoli cell numbers (Orth et al., 1988). Furthermore, Sertoli cells sustain high intra-testicular T levels by producing androgen binding protein (ABP), which serves to sequester T in the testis. High levels of intra-testicular T (~30 times higher than serum) are necessary for normal spermatogenesis (Griswold and Russell, 1999).

In the prenatal testis, Sertoli cells are the first somatic cells to differentiate and play a central role in sex determination (Hogarth and Griswold, 2010; Hughes, 2001). Sertoli cells originate from the coelomic epithelium (Karl and Capel, 1998) and secrete AMH [also known as mullerian inhibiting substance (MIS)], which inhibits the growth of the Mullerian ducts (Behringer et al., 1994). Sertoli cells also secrete Desert Hedgehog (Dhh) protein that regulates male-specific germline fate, spermatogenesis (Bitgood et al., 1996) and differentiation of Leydig cells (Yao et al.,

2002). The number of Sertoli cells in the adult testis is determined by proliferative activity occurring prior to puberty. In the male rat, Sertoli cells stop proliferating at PND 16-18, which coincides with formation of the BTB and tubular lumen. At termination of proliferative activity on PND18, the number of Sertoli cells remains constant throughout the postnatal period. Thus, chemicals, which inhibit Sertoli cell proliferation and differentiation, have permanent effects on sperm production in the adult testis.

Sertoli cell function is under the control of FSH, which is known to act as a mitogen in Sertoli cells *in vitro* and *in vivo* (McLachlan et al., 1994). However, T regulates Sertoli cell proliferation and function through ARs expressed in Sertoli cells. In addition, Sertoli cells express the aromatase enzyme, which converts T to E_2, as well as ESRs. Administration of aromatase inhibitors, e.g., latrozole to boars increased Sertoli cell numbers and testes size (At-Taras et al., 2006; Berger et al., 2008). However, studies in ESR1, ESR2 and aromatase knockout mice showed no changes in Sertoli cell numbers in adult animals (Gould et al., 2007). Interestingly, estrogenic chemicals have the potential to affect Sertoli cell development and function. For example, neonatal exposure of male rats to DES decreased Sertoli cell numbers (Atanassova et al., 1999), whereas *in utero* or neonatal exposure to BPA increased Sertoli cell numbers in the adult (Atanassova et al., 2000; Wistuba et al., 2003). Furthermore, neonatal exposures to BPA affected male fertility in association with aberrant expression of Sertoli cell junctional proteins (Salian et al., 2009). Recently, it was demonstrated that the AMH gene has EREs and is regulated by estrogen and estrogenic chemicals (Chen et al., 2003; Nagai et al., 2003). Also,

several studies have shown that exogenous chemicals can perturb Sertoli cell tight junctions by induction of oxidative stress, and thereby cause reproductive dysfunction (Wong and Cheng, 2011).

The close proximity of testicular cells allows multiple interactions to occur and thereby facilitates synchronization of function in tubular and interstitial compartments (Schlatt et al., 1997). Sertoli cell-derived products such as AMH, stem cell factor, activin, inhibin and Dhh are known to regulate Leydig cell function (Yao and Barsoum, 2007). On the other hand, T produced by Leydig cells controls Sertoli cell function and spermatogenesis (De Gendt et al., 2004). Thus, environmental toxicants can affect testicular function by disrupting cell-cell interactions.

3.6 Leydig Cells

Leydig cells are endocrine cells found in the intertubular space of the testis that produce the male sex steroid hormone T, which is required for gonadal sex differentiation, development of secondary male sexual characteristics, support of spermatogenesis, and maintenance of the male phenotype and libido. T is also required for male brain programming during the neonatal period and development of accessory sex glands in adulthood. Leydig cell function is mainly under the control of the pituitary gonadotropin, LH. In mammals, it is speculated that there are at least two distinct populations of Leydig cells. For example, in rats, two populations of Leydig cells have been identified; fetal Leydig cells and adult Leydig cells. In contrast, three phases of Leydig cell development have been identified in

humans; fetal Leydig cells, infantile Leydig cells and adult Leydig cells (O'Shaughnessy et al., 2006).

3.6.1 Origin and Differentiation of Fetal Leydig Cells

Despite the essential role of fetal Leydig cells (FLCs) in male reproductive development, less is known about the origin and differentiation of the FLC population. In rats, Leydig cells differentiate in the interstitial region around 13.5 dpc following Sertoli cell differentiation. However, Leydig cell differentiation starts in the eighth week of gestation in humans. Although the exact origins of FLCs are not known, they probably arise from coelomic and mesonephric epithelium and/or migrating neural crest cells (Brennan et al., 2003; Burgoyne et al., 1988; Griswold and Behringer, 2009; Karl and Capel, 1998; Lejeune et al., 1998).

Fetal androgen production begins about day 13 dpc in rats and reaches a peak before birth. Although FLCs can respond to LH, FLC development and androgen production is considered to be independent of LH stimulation (Ge and Hardy, 2007; Yao and Barsoum, 2007). FLCs mainly produce androstenedione which is then converted to T by the HSD17B3 enzyme, which is expressed in tubular cells including Sertoli cells. T is required for Wolffian duct development and also causes masculinization of the male brain. Together with insulin-like 3, T stimulates differentiation of the gubernaculum ligament, which facilitates testis descent. Thus, FLC dysfunction causes developmental abnormalities such as pseudo-hermaphroditism, hypospadias and cryptorchidism (Griswold and Behringer, 2009; O'Shaughnessy et al., 2006). FLC development is controlled by factors secreted by

Sertoli cells such as Dhh, platelet-derived growth factor α (PDGFα) and AMH (Barsoum and Yao, 2010). However, AMH appears to be a negative regulator of Leydig cells because AMH or AMH receptor null mice developed Leydig cell hyperplasia and tumors (Behringer et al., 1994; Mishina et al., 1996; Racine et al., 1998). In contrast, mice overexpressing AMH showed feminization and undescended testes with reduced Leydig cell numbers and serum T concentrations (Behringer et al., 1990; Lyet et al., 1995; Racine et al., 1998).

3.6.2 Postnatal / Adult Leydig Cell Development

Adult or postnatal Leydig cell populations are distinctly different from FLCs and produce T in the pubertal and adult period. It has been suggested that adult Leydig cells were derived from interstitial mesenchymal-like cells in the neonatal testis because mesenchymal-like cell numbers diminish as distinct Leydig cells proliferate between postnatal days 14 and 28 (Hardy et al., 1989). Although some FLCs possibly persist into the postnatal period, it is unlikely that FLCs contribute significantly to T production in the adult (Benton et al., 1995).

Postnatal Leydig cell development in the rat occurs in three distinct and well characterized stages; progenitor, immature and adult Leydig cells. However, recent studies have identified undifferentiated stem Leydig cells (SLCs), which are capable of self-renewal and differentiation to Leydig cells (Chen et al., 2009; Ge et al., 2006). SLCs are spindle shaped cells, which are PDGFRα positive but are negative for LHR and HSD3B (Ge et al., 2006). Between postnatal days 10 and 14, some SLCs become committed and give rise to progenitor Leydig cells (PLCs); these are small and

spindle-shaped cells resembling SLCs in appearance. PLCs express LHR and steroidogenic enzymes, including P450 side-chain cleavage enzyme (CYP11A1), HSD3B, and 17α-hydroxylase (CYP17A1) (Shan et al., 1993). PLCs mainly produce androsterone because of decreased expression of androgen biosynthetic enzymes required for T biosynthesis and increased expression of steroid metabolizing enzymes especially 3 alpha-hydroxysteroid dehydrogenase (3α-HSD) (Shan et al., 1993). PLCs initially show high mitotic activity but gradually enlarge, become round and reduce their proliferative capacity to become immature Leydig cells (ILCs) between PND 14 and 28 (Ge and Hardy, 1997).

By postnatal day 28, transformation of PLCs into ILCs is accomplished by changes in morphological appearance from spindle-shaped to spherical with concomitant increases in expression of HSD3B, CYP11A1 and CYP17A1. ILCs have increased steroidogenic capacity and produce five-fold more T compared to PLCs (Benton et al., 1995). Although T production is higher in ILCs than PLCs, it is not the major steroid produced because ILCs express high levels of testosterone-metabolizing enzymes such as 3α-HSD and 5α-reductase, thereby producing mostly the androgen metabolite, 5α-androstane-3α, 17β-diol (ADIOL) (Ge and Hardy, 2007).

ILCs proliferate once to double their numbers to ~25 million cells per testis between PND 28 and 56 and become terminally differentiated into adult Leydig cells (ALCs). The concomitant decline in levels of androgen metabolizing enzymes with increased expression of T biosynthetic enzymes allows ALCs to produce T as a

predominant sex-steroid. The entire process of proliferation and differentiation of adult Leydig cells is under the primary regulation of LH, steroid hormones (androgens, estrogen), and growth factors (Wu et al., 2007).

3.6.3 Luteinizing Hormone (LH) Signaling and Steroidogenesis in Leydig cells

Androgen secretion is primarily regulated by pituitary LH and Leydig cells are the only binding sites for LH in the testis. Binding of LH to its receptor in Leydig cells stimulates activation of adenylyl cyclase (AC) that catalyzes cyclic adenosine-3',5'-monophosphate (cAMP) formation from adenosine triphosphate (ATP). Then, cAMP activates protein kinase A, which in turn activates StAR and the transcription factor, CREB (cAMP response element-binding) by phosphorylation. Furthermore, MAPK activation by LH action is also known to activate StAR by phosphorylation. StAR regulates steroidogenesis by controlling the transport of cholesterol from the outer to the inner mitochondrial membrane, where the cytochrome P450scc (CYP11A1) enzyme is located. Conversion of cholesterol to pregnenolone by CYP11A1 is the first enzymatic step in steroid hormone biosynthesis (Manna et al., 2009). Subsequent to CYP11A1 action, three other enzymes are required for T synthesis: 3β hydroxysteroid dehydrogenase (HSD3B), cytochrome P450 17α hydroxylase (CYP17A1) and 17β hydroxysteroid dehydrogenase 3 (HSD17B3). Although LH is the primary regulator of androgen biosynthesis, several other hormones and local factors, including epidermal growth factor (EGF), insulin-like growth factor (IGF), prolactin, gonadotropin-releasing hormone and macrophage-derived factors, bind to specific membrane receptors in Leydig cells and activate

protein kinase cascades (e.g., Ras/Raf, MAPK/ERK) and StAR expression (Manna et al., 2009). The T biosynthetic pathway in Leydig cells is shown in figure 5.

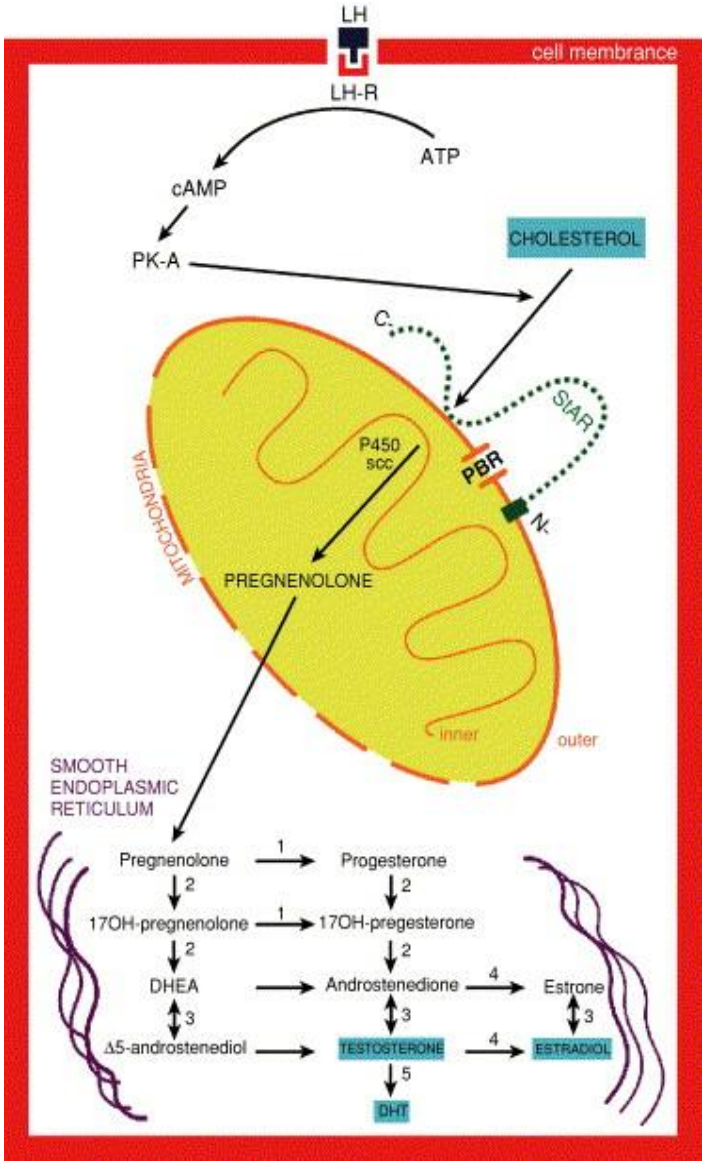

Figure 5. Metabolic steps involved in androgen biosynthesis in Leydig cells. After LH binding to LHR on the cell membrane, cAMPs are generated by action of adenylyle cylase. cAMP activates protein kinase A (PKA), which phosphorylates steroidogenic acute regulatory protein (StAR) and the peripheral benzodiazepine receptor (PBR). Activated StAR and PBR transport cholesterol from outer to inner mitochondrial membrane where cytochrome P450scc (CYP11A1) converts the cholesterol to pregnenolone which diffuses into smooth endoplasmic reticulum and undergo further conversions. Reaction **1**, 3β-Hydroxysteroid dehydrogenase (HSD3B); reaction **2**, cytochrome P450 17α-hydroxylase (CYP17A1); reaction **3**, 17β-hydroxysteroid dehydrogenase (HSD17B3); reaction **4**, cytochrome P450 aromatase (CYP19A1); reaction 5, 5α-reductase. Reproduced with permission of the publisher (Haider, 2004).

3.7 Role of Estrogen in Male Reproduction

Earlier classification of testosterone as a “male hormone” and estradiol as a “female hormone” is being reconsidered after evidence of estrogen function in male reproduction was made available. Although T is the predominant male hormone responsible for maintenance of fertility, it is now evident that small quantities of estrogens are required for normal male reproductive function. The possibility of estrogen function in the male was postulated in 1934 after the finding that T is converted to estrogen in stallions (Rochira et al., 2005). Further, evidence for estrogen function in males was provided by the creation of estrogen receptor 1 knockout (ESR1KO) mouse in 1993 (Lubahn et al., 1993), which showed complete infertility. The underlying mechanism of infertility was identified as lack of fluid absorption from the efferent ductules (Hess et al., 1997). In addition, widespread distribution of ESR1 and ESR2 in the male reproductive tract suggests a role for estrogen in male reproduction (Mowa and Iwanaga, 2001). The serum levels of estrogen measured about 40 pg/mL in male rats (Brewster et al., 1997) and ranges between 20 and 40 pg/mL in men (Nagata et al., 2001). Observations from *in vitro* studies of human germ cells indicate that E_2 may serve as a survival factor for round spermatids and E_2 deficiency promotes apoptosis (Pentikainen et al., 2000). These observations led to the conclusion that estrogens and estrogen receptors (ESRs) are "essential" for fertility.

A role for estrogens in the male was also evident in aromatase knockout (ArKO) male mice which exhibited progressive infertility with advancing age (Fisher

et al., 1998). The aromatase (P450arom or CYP19A1) enzyme converts T into E_2 and its presence in Leydig cells, Sertoli cells, germ cells and spermatozoa supports the high concentration of estrogens in the testis. However, there are species differences in distribution of this enzyme because germ cells in the boar, ram and stallion are aromatase-negative, which is probably compensated for by aromatase expression in Leydig cells of these species (Rochira et al., 2005). Similarly, reports of infertility due to congenital estrogen deficiency in men as a result of naturally occurring mutations of the aromatase gene (Faustini-Fustini et al., 1999) and ESR1 (Smith et al., 1994) support the view that estrogen plays a role in male reproduction.

3.7.1 Estrogen Biosynthesis in Males

Circulating androgens are the precursors for estrogen biosynthesis in males. The aromatase enzyme converts T and androstenedione (androgens) to E_2 and estrone, respectively. Androgens and estrogens in circulation are bound reversibly to a β-globulin, the sex hormone binding globulin (SHBG), and by a lesser degree to albumin. Estrogen synthesized in the male reproductive tract (locally produced) and circulating estrogens may interact with ESRs in an intracrine, paracrine, and/or endocrine fashion to regulate male reproductive activity (Carreau et al., 2003).

3.7.2 Mechanism of Estrogen Receptor Action

Important components of the estrogen signaling system include the ligand, i.e. estrogen, and ESR transcription factors i.e., ESR1, and ESR2. ESRs are members of the super family of nuclear hormone receptors and are considered to be ligand-

inducible transcription factors (Mangelsdorf et al., 1995). The ESR1 and ESR2 receptors are encoded by *ESR1* and *ESR2* genes, respectively, which are located on different chromosomes in the genome (Deroo and Korach, 2006). Both receptors are known to bind E_2 with equal affinity. However, ESR2 has been shown to interact constitutively with EREs in a ligand-independent manner and may attenuate ESR1 activity. Therefore, the relative amounts of ESR1 and ESR2 in a given tissue seem to determine overall responsiveness to estrogen (Akingbemi, 2005; Couse et al., 2001; Hall et al., 2001).

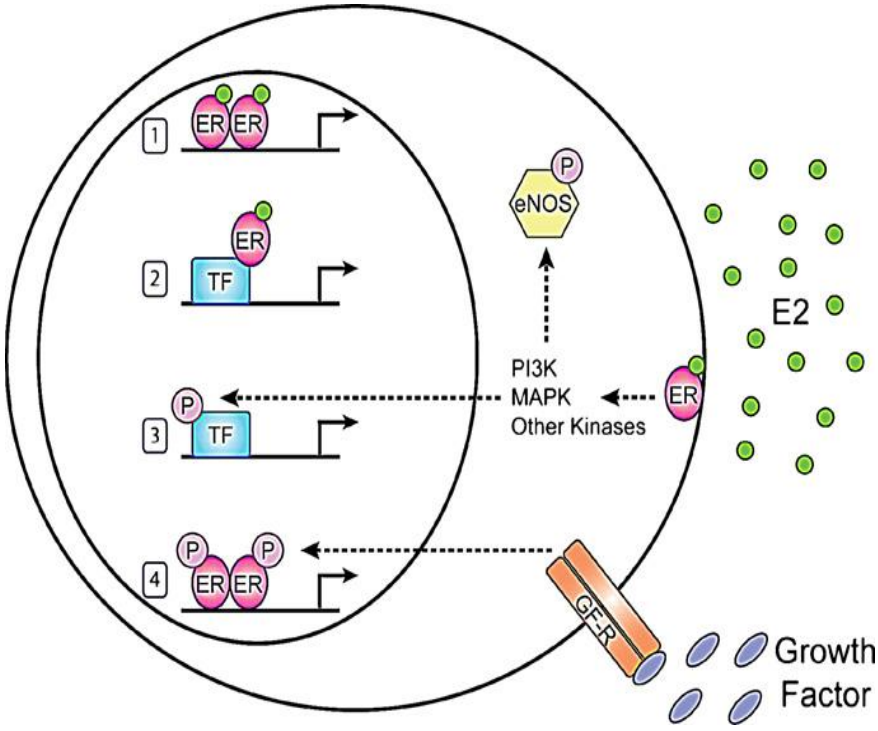

Figure 6. Estrogen receptor-mediated signaling. 1. The classical genotropic ERE-dependent pathway, 2. Genotropic ERE-independent (tethered) pathway, which is mediated by the AP-1 transcription factor, 3. Membrane initiated pathway, activates second messengers, and 4. Ligand-independent pathway which is mediated through growth factor receptors. Reproduced with permission of the publisher (McDevitt et al., 2008).

Four ESR-mediated pathways have been identified (Figure 6). The classical or canonical model for estrogen action is a ligand-dependent pathway. Estrogen binding triggers conformational changes in ESRs after phosphorylation on serine and tyrosine residues. Binding of estrogen to ESRs causes release of the receptors from an inhibitory complex loosely bound to heat shock proteins (hsp90) to form homodimers (ESR1/ESR1 and ESR2/ESR2) or heterodimers (ESR1/ESR2). The receptor dimers along with other transcriptional cofactors (co-repressor and co-activator proteins) bind to specific DNA response elements called estrogen response elements (EREs) in the promoter region of various target genes. This complex then induces or suppresses transcription of estrogen-target genes depending on co-factors recruited, which result in changes in protein expression and affect physiological function (Couse et al., 2001; Hall et al., 2001; Hasbi et al., 2005).

In the ERE-independent pathway, gene activation by estrogen bound ESRs occurs without direct DNA binding but by interaction with other DNA tethered transcription factors such as the activator protein 1 (AP-1) complex and AP-1-responsive elements. This pathway requires both AF-1 and AF-2 domains of the receptor. However, ESR2 lacks the AF-1 domain and is not able to induce transcription of AP-1-regulated genes suggesting differential physiological roles for ESR1 and ESR2 (Hall et al., 2001; McDevitt et al., 2008).

Both classical and ERE-independent pathways involve binding of the ESR complex directly to DNA to elicit biological responses, do not require second messengers, and take several hours to days to cause their effect. However, many

actions of estrogen cannot be explained by these pathways. For example, rapid actions observed in bone, breast, blood vessels, and the nervous system are mediated by ESRs present in the plasma membrane, which activates second messengers (Hall et al., 2001). Also, decreased testicular androgen production was observed after treatment of testicular tissue from the Atlantic croaker with BSA-conjugated estrogen (which cannot pass through the plasma membrane) (Loomis and Thomas, 2000). These effects occur rapidly within seconds to minutes (nongenomic pathway). There is consensus that ESR1 and ESR2 present on the plasma membrane carry out the rapid nongenomic action of estrogen (Wade et al., 2001). Recent studies have also demonstrated the existence of a 7-transmembrane G protein-coupled receptor, GPR30, which mediates rapid signaling by estrogen (Ropero et al., 2006). However, the physiological relevance of non-genomic signaling by estrogen in the male reproductive system remains to be clarified (Couse et al., 2001).

Growth factors such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) may activate ESRs and increase expression of ESR target genes in a ligand-independent manner. The biological role of this pathway is not yet clear but it has been suggested that hormone-independent pathways allow ESR activation in the presence of low estrogen levels (Hall et al., 2001). Studies from the ESR1KO mouse showed unresponsiveness of uteri to EGF despite the presence of EGF receptors. In other studies, cotreatment of the mouse uterus with anti-EGF antibody reduced the effect of estrogen on the uterus. These observations suggest that estrogens and EGF are dependent on each other to exert their actions in

estrogen responsive tissues. The ligand-independent pathway requires N-terminal AF-1 domain of the receptor and phosphorylation by various cellular kinases. Upon activation, the ESR is able to dimerize and bind to EREs and regulate the transcription of estrogen targeted genes as in the classical genomic pathway (Hall et al., 2001).

3.7.3 Physiological Roles of Estrogen in the Male

Altogether, results from the studies of ESRs, aromatase knockout mice (Hess et al., 1997; Lubahn et al., 1993) and naturally occurring mutations in men (Faustini-Fustini et al., 1999) determined that estrogen is involved in the following: 1) negative feedback on the hypothalamus and pituitary; 2) male sexual behavior by masculanization of the brain; 3) development and function of efferent ductules; 4) Leydig cell proliferation and function; 5) Sertoli cell proliferation and maturation; and, 6) germ cell development.

3.7.4 Effects of Excess Estrogen Activity

Although estrogens are necessary for normal fertility in males, exposure to excess environmental estrogens causes adverse effects on male reproduction. For example, exposures of laboratory animals and wildlife to high levels of estrogenic chemicals resulted in a number of abnormalities, including reduced gonad size, feminization of males, and low sperm count and quality (Akingbemi, 2005). A diverse group of chemicals is known to mimic the action of estrogen, including steroidal and non steroidal compounds such as industrial chemicals [(e.g., bisphenol A (BPA) polychlorobiphenyls, alkyphenols], pesticides (e.g., DDT derivatives,

methoxychlor, kepone), pharmaceutical agents (e.g., DES, tamoxifen, raloxifene), phthalates (e.g., di-2-ethylhexylphthalate, di-n-butyl phthalate), and phytoestrogens (e.g., genistein, daidzein) (Akingbemi, 2005; Toppari et al., 1996). In other experiments, overexpression of the aromatase gene induced cryptorchidism, spermatogenic arrest, Leydig cell hyperplasia, and decreased serum FSH and T levels (Li et al., 2001).

Interestingly, there have been reports of deterioration in male reproductive health in the population in the past few decades. The initial review of the literature (Carlsen et al., 1992) suggested a decline in semen quality during the last 50 years. Reanalysis of the data, adding 47 more studies and controlling for abstinence time, age, percent of men with proven fertility, and specimen collection method, showed the same trend but with greater decline in semen quality than what was reported previously (Swan et al., 2000). In addition, the incidence of testicular cancer, hypospadias and cryptorchidism are also increasing in the population (Paulozzi, 1999; Toppari et al., 1996). Males exposed to DES during pregnancy have increased incidence of male reproductive disorders, such as cryptorchidism, testicular cancer, poor semen quality, epididymal cysts, hypospadias and microphallus (Toppari et al., 1996). Clinical and laboratory research findings suggest that exposure to supranormal levels of exogenous estrogens (xenoestrogens) in the fetal period were the underlying cause of reproductive abnormalities (Toppari et al., 1996).

3.7.5 Estrogen Regulation of Leydig Cells

Leydig cells are primary targets for estrogen in the testis (Abney, 1999) and estrogen modulates Leydig cell function through paracrine, autocrine and endocrine action. In the rat, Leydig cells express only ESR1, whereas human Leydig cells express both ESR1 and ESR2 (Cavaco et al., 2009). Several studies have shown that E_2, a natural estrogen, blocks the regeneration of Leydig cells in ethane dimethylsulfonate-treated mature rats. Also, E_2 may block androgen secretion by direct competitive inhibition of steroidogenic enzymes (Abney, 1999). However, the exact mechanisms by which E_2 act to elicit these effects in Leydig cells have not been fully characterized.

4. Relationship between adipose tissue and reproduction

Adipose tissue is a type of connective tissue comprised mostly of adipocytes. Adipose tissue is considered to be an energy storage depot and stores majority of the body fat. Although considered initially as an inert tissue, there is increasing evidence showing that adipose tissue is a dynamic endocrine organ and secretes cytokines that integrate various body functions in response to external stimuli (Kershaw and Flier, 2004). The cytokines (i.e., adipokines) act through a network of autocrine, paracrine and endocrine pathways to regulate several aspects of physiology including glucose and lipid metabolism, neuroendocrine function, reproduction, immunity, and cardiovascular function (Kershaw and Flier, 2004). Interestingly, a growing number of adipokines, involved in the control of energy balance and metabolism, have recently been identified as putative regulators of

pubertal development, gonadotropin function, and/or fertility (Fernandez-Fernandez et al., 2006; Tersigni et al., 2011). Furthermore, there is general consensus that energy imbalance due to energy deprivation or excess impairs fertility in both females and males (Pasquali et al., 2007; Wade and Jones, 2004).

According to the American Obesity Association in a report released in 2009, nearly one-third of the adult American population is obese [body mass index (BMI) of 30 and higher] and about 65% are overweight (BMI of 25 and higher). Obese men suffer from infertility, which is associated with hypotestosteronemia, reduced sperm number and motility, increase in reactive oxygen species (ROS) and sperm DNA damage, and erectile dysfunction (Pasquali et al., 2007). It is estimated that 80% of male partners who seek assisted reproductive technology intervention were either overweight or obese (Bakos et al., 2011a). The cost of treatment of infertility and related conditions is higher among overweight and obese patients than in the general population (Koning et al., 2010).

The exact mechanisms by which obesity or overweight cause infertility are not known. However, expression of receptors for adipose tissue-derived molecules such as adiponectin, leptin, and resistin, has been documented in the hypothalamus, pituitary glands, and gonads. Furthermore, gonadotropins control expression of these receptors in the testis (Caminos et al., 2008; Nogueiras et al., 2004; Tena-Sempere and Barreiro, 2002). Therefore, adipocyte derived factors possibly control reproduction by acting at all levels of the hypothalamo-pituitary-gonadal axis (Barreiro et al., 2004; Caminos et al., 2008; Nogueiras et al., 2004; Tena-Sempere

and Barreiro, 2002; Tena-Sempere et al., 2002). Among adipokines, leptin and adiponectin have been the most investigated.

4.1 Leptin

Leptin is a 16 kDa protein hormone involved in body weight regulation by suppressing food intake and stimulating energy expenditure (Kershaw and Flier, 2004). Leptin and its receptors (ob-R) are products of the *ob* and *db* genes, respectively. Loss of function of leptin (*ob/ob*) and its receptor (*db/db*) cause morbid obesity in rodents and humans (Israel and Chua, 2010). In particular, leptin or leptin-receptor deficient male mice showed hypothalamic hypogonadism due to GnRH deficiency, delayed puberty and infertility. In addition, atrophic testicles and impaired spermatogenesis and altered sexual behavior were observed in these animals (Tena-Sempere and Barreiro, 2002). Supplementation of leptin to *ob* male mice was found to reverse abnormalities due to leptin deficiency (Mounzih et al., 1997).

Leptin has the capacity to cross the blood-brain-barrier and act at the hypothalamus and pituitary. For example, systemic administration of leptin or leptin analogues increased FSH and LH secretion in male rats (Barash et al., 1996; Gonzalez et al., 1999). A recent study also demonstrated that leptin controls reproduction in part by activating kisspeptin neurons and GnRH secretion in the arcuate and paraventricular nuclei of the hypothalamus (Quennell et al., 2011). While physiological levels of leptin act at the hypothalamus and pituitary glands to stimulate GnRH release and gonadotropin (LH and FSH) secretion, supra-

physiological levels, as in obesity, inhibit GnRH and gonadotropin secretion. Furthermore, higher levels of leptin may act through their receptors (ob-R) in Leydig cells (Caprio et al., 2003) to inhibit T production by decreasing StAR and CYP11A1 enzyme protein (Tena-Sempere et al., 2001).

4.2 Adiponectin

Adiponectin, which is present at high levels in circulation and corresponds to 0.01 to 0.05% of total serum proteins, is a 244 amino acid polypeptide with a molecular weight of 30 kDa. Because it was discovered independently from different laboratories, adiponectin is called by different names e.g., adipocyte complement-related protein (Acrp30), adipose most abundant gene transcript 1 (apM1), adipose-specific gene adipoQ (AdipoQ), and gelatin-binding protein of 28 kDa (GBP28). Adiponectin exists mostly in different forms; low-molecular-weight (LMW, trimer), middle-molecular-weight (MMW, hexamer), and high molecular-weight (HMW) (Berg et al., 2002), which correspond to molecular weights of 90, 180, and >250 kDa. However, full-length adiponectin is composed of an N-terminal collagenous domain and a C-terminal globular domain (Fruebis et al., 2001). Adiponectin circulates in the blood either as a trimer or an oligomer. Many studies have shown that post-translational modifications (e.g., glycosylation) facilitate oligomerization of adiponectin monomers to form higher molecular weight forms (Wang et al., 2006).

Adiponectin exerts its action through two receptors; AdipR1 and AdipoR2. The receptors differ structurally and functionally from G-protein coupled receptors

due to a reverse orientation of the C- and N-terminals, which are not coupled to G-proteins (Tang et al., 2005). However, recent reports indicated that a new trans-membrane protein belonging to the cadherin family may act as a receptor for adiponectin (Hug et al., 2004). Adiponectin receptors are present in many reproductive tissues, including the central nervous system, ovaries, oviduct, endometrium, and testes (Michalakis and Segars, 2010). It has been suggested that adiponectin is involved in several physiological functions. For example, adiponectin receptors are located in regulatory centers of the hypothalamus but it is not clear whether adiponectin can pass the blood brain barrier to act on the brain to regulate food intake.

There is evidence showing that adiponectin increases fatty oxidation in muscle and liver to reduce body weight (Fruebis et al., 2001). Furthermore, adiponectin acts as an insulin sensitizing hormone by increasing glucose uptake in liver, muscle and adipose tissue as well as decreasing gluconeogenesis in the liver (Berg et al., 2001; Berg et al., 2002). Also, adiponectin increased lypolysis by activating hormone-sensitive lipase in adipocytes, which supports mobilization of fat for utilization by other tissues. Other studies showed that adiponectin protects the cardiovascular system and suppresses development of atherosclerosis in blood vessels by reducing free radical production and protecting the heart from injury due to myocardial infarction (Ouchi et al., 2006). On the other hand, low adiponectin levels have been associated with various cancers, including breast, endometrial, gastrointestinal and prostate cancers (Michalakis and Segars, 2010).

Unlike leptin, the role of adiponectin in reproduction is yet to be clarified. However, adiponectin decreased GnRH release by GT1-7 cells derived from hypothalamic neurons (Wen et al., 2008) as well as basal and GnRH-stimulated LH secretion from LβT2 immortalized gonadotrope cells (Lu et al., 2008). Transient overexpression of adiponectin decreased serum LH in male mice compared to wild type (Lu et al., 2008).

Studies of AdipoR1 and AdipoR2 knockout mice showed that AdipoR2, but not AdipoR1, is required for testis function. AdipoR2 knockout mice showed infertility, reduced testes weights, atrophy of the seminiferous tubules and aspermia but with no alterations in serum T levels (Bjursell et al., 2007). Although serum T levels were not affected, it is possible that Leydig cell steroidogenesis is compromised in adipoR2 knockout mice and contributes to infertility in these animals. Indeed, adiponectin was found to decrease T production in rat testicular explants (Caminos et al., 2008; Pfaehler et al., 2012).

4.3 Sex Hormones and Adipokines

Sex steroids (T and E_2) are known to regulate adipokine secretion and adipose tissue function. Adiponectin and leptin levels are more abundant in females than in males suggesting that T has an inhibitory (Nishizawa et al., 2002) while E_2 has a stimulatory effect on adipokine secretion (Gui et al., 2004). Studies of transgenic mice and natural cases of E_2 deficiency in humans provided evidence for E_2 regulation of adipose tissue function in males (Heine et al., 2000; Jones et al., 2007; Ohlsson et al., 2000). For example, estrogen resistant (ESR1 mutation) or

deficient (aromatase mutation) men exhibited glucose intolerance, hyperinsulinemia, and lipid abnormalities with elevated triglycerides and low high-density lipoprotein cholesterol, suggesting that estrogen is involved in carbohydrate and lipid metabolism. The BMI in these patients was in the overweight range with accumulation of abdominal adipose tissue and hepatic steatosis (Jones et al., 2007). Similarly, ArKO male mice, with decreased serum E_2 levels, showed a 50% increase in gonadal fat pads (Jones et al., 2007; Ohlsson et al., 2000). In contrast, developmental exposure of laboratory rodents to estrogens increased weight gains in later life (Newbold et al., 2009) and male mice lacking estrogen sulfotransferase (EST), a key estrogen metabolizing enzyme, showed increased adiposity and metabolic disturbances (Khor et al., 2008; Tong et al., 2004). Observation in AR knockout male mice included increased adiposity and altered metabolic homeostasis (Yanase et al., 2008). Together, these findings suggest that estrogens and androgens play pivotal roles in maintaining adipose tissue function in the male (Heine et al., 2000; Jones et al., 2007; Ohlsson et al., 2000).

Adiponectin levels are low in individuals suffering from obesity, diabetes and heart disease. Similarly, longitudinal studies have shown that low serum T (hypogonadism) in men is a risk factor for the later development of metabolic syndrome, type 2 diabetes, and insulin resistance (Kapoor and Jones, 2008; Traish et al., 2009) associated with decreased adiponectin levels. Thus, altered adipokine secretion after developmental exposures to environmental estrogens or as in obesity, metabolic syndrome, and type 2 diabetes have implications for male reproduction. Although recent evidence suggests that GnRH secretion by the

hypothalamus and/or gonadotropin release from the pituitary gland is disrupted in altered metabolic states (Michalakis and Segars, 2010), mechanisms governing the association between energy balance and reproduction remain to be clarified.

4.4 Xenoestrogens and Adipokines

The obesity epidemic in human population is thought to coincide with the marked increase in production of industrial chemicals over the past 40 years (Baillie-Hamilton, 2002). Recent reports suggest that exposures to environmental estrogens (e.g., DES, BPA and genistein) in the fetal and neonatal period can alter developmental programming and predispose laboratory animals to obesity and diabetes later in life (Newbold et al., 2009). Also, exposure of laboratory animals to xenoestrogens cause disturbances in carbohydrate and lipid metabolism leading to development of metabolic syndrome, obesity and related infertility (Alonso-Magdalena et al., 2006; Hugo et al., 2008; Miyawaki et al., 2007; Rubin et al., 2001; Rubin and Soto, 2009; Somm et al., 2009). Therefore, it is possible that xenoestrogens cause reproductive toxicity not only by acting directly in reproductive tissues but also by indirectly modulating adipokine secretion. For example, BPA suppressed adiponectin secretion from human adipose tissue explants (Hugo et al., 2008) and altered leptin mRNA synthesis in cultured adipocytes (Phrakonkham et al., 2008). Furthermore, it has been shown that exposure to BPA during development can increase body weight in adulthood (Rubin and Soto, 2009) and cause aberrant in adipokine secretion in laboratory animals (Ben-Jonathan et al., 2009).

5. Oxidative Stress and Testis Function

Oxidative stress results from an imbalance in production of free radicals and the capacity of the antioxidant system to protect against oxidative damage to cellular protein, lipids and nucleic acids. Oxidative damage causes tissue inflammation and injury resulting in cell death by necrosis or apoptosis (Lennon et al., 1991). The most biologically relevant free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS) derived from oxygen (O2) and nitrogen (N2), respectively (Evans et al., 2002). Examples of ROS are superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$).

Despite poor vascularization and low oxygen tension of the testis (Free et al., 1976), spermatogenesis (Henkel, 2011; Maiorino and Ursini, 2002) as well as steroidogenesis (Peltola et al., 1996) generate ROS and affect antioxidant capacity, meaning that physiological ROS levels are required for testicular function. For example, ROS stimulates sperm capacitation (Aitken and Baker, 2004) and mediates LH-cAMP-induced steroidogenesis in Leydig cells (Tai and Ascoli, 2011). It has been hypothesized that low oxygen tension is one mechanism by which the testis protects itself from free radical-mediated damage (Aitken and Roman, 2008). However, other antioxidant enzymes and non-enzymatic free radical scavengers are present to prevent testicular dysfunction due to oxidative stress.

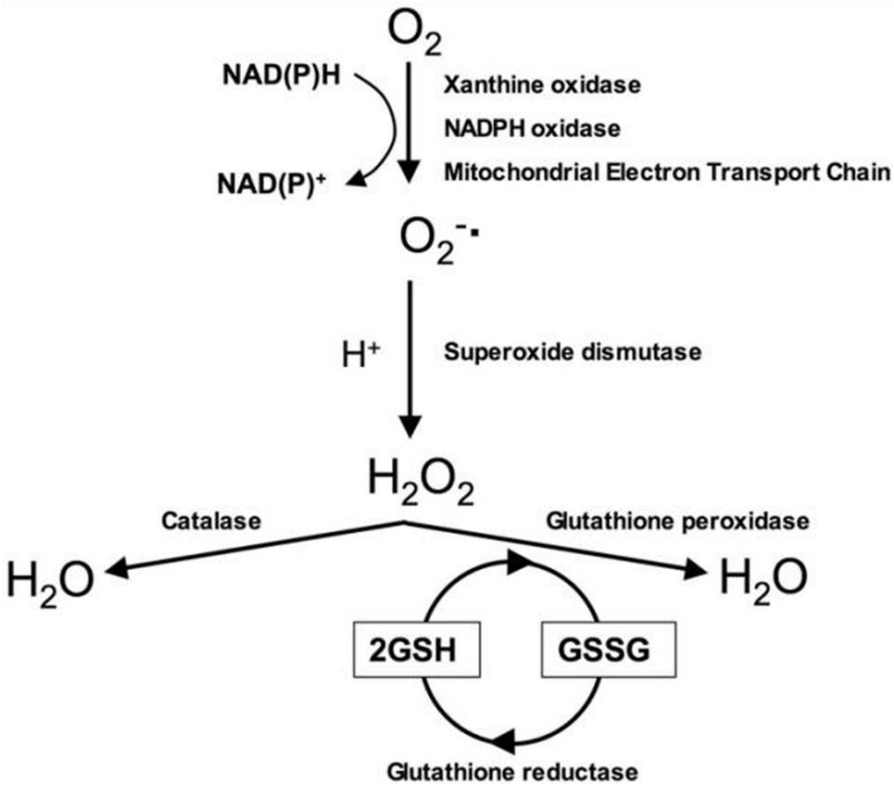

Figure 7. Major pathways of reactive oxygen species (ROS) generation and metabolism. Reproduced with permission of the publisher (Aitken and Roman, 2008).

The major pathways for ROS generation and metabolism in the testis are shown in Figure 7. Intracellular sources of ROS include the electron transport chain in the mitochondria, endoplasmic reticulum, nuclear membrane electron transport systems and cellular plasma membrane (Agarwal et al., 2005). Enzymatic pathways utilizing nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), xanthine oxidase (XO), cyclooxygenases, lipoxygenases, monooxygenase and cytochrome P450 systems are potential extra-mitochondrial ROS sources. The highly reactive ROS, superoxide anion ($\cdot O_2^-$) is converted to less reactive and stable

H_2O_2 and O_2 by the activity of the superoxide dismutase (SOD) enzyme. Thus, SOD prevents the formation of highly pernicious hydroxyl radicals by superoxide anion. Three forms of SOD i.e., cytosolic (SOD1), mitochondrial (SOD2) and extracellular (SOD3) have been reported with copper, manganese and zinc as cofactors.

Hydrogen peroxide (H_2O_2) has the capacity to pass through cell membranes and cause oxidative damage in neighboring cell types. Testicular cells degrade H_2O_2 to O_2 and H_2O by the action of catalase or glutathione peroxidase (GPx). Catalase seems to be the predominant enzyme in the interstitium, while GPx activity depends on the availability of the glutathione substrate which exists in a reduced (GSH) and oxidized form (GSSG). GSH is a tripeptide electron donor and may be oxidized to glutathione disulphide (GSSG). GSH levels in cells are maintained by the activity of the glutathione reductase enzyme utilizing NADPH. Glutathione S transferase (GST) is involved in detoxifying highly reactive peroxides and xenobiotics by conjugating them with glutathione (GSH) and thereby protecting against tissue damage. Unfortunately, antioxidant enzyme expression decreases with age and results in oxidative stress, which is known to be one of the causative factors for testicular dysfunction with advancing age.

5.1 Leydig Cells and Oxidative Stress

The major sources of ROS in testis are Leydig cells, spermatozoa and testicular macrophages. Leydig cells, present in the interstitium in the immediate vicinity of blood vessels, are exposed to an oxygen-rich environment more than any other cell type in the testis (Free et al., 1976). Also, testicular macrophages produce

high levels of ROS in the interstitium (Hales, 2002). Therefore, Leydig cells are at risk of oxidative damage from increased ROS production. In addition, cytochrome P450 enzymes in Leydig cells such as (CYP11A1, CYP17A and CYP19), which are involved in steroidogenesis generate free radicals in the process. LH activation of MAPK3/1, necessary for steroid biosynthesis, is possibly mediated by mitochondria-derived ROS (Tai and Ascoli, 2011) and cAMP, a downstream activator of steroidogenesis due to LH-mediated signaling, was found to stimulate mitochondrial oxygen consumption and electron transport in MA-10 cells (Allen et al., 2006). In contrast, excessive oxidative stress has an inhibitory effect on androgen biosynthesis. For example, H_2O_2 inhibits Leydig cell T production by inhibiting CYP11A1 activity and StAR protein expression (Tsai et al., 2003). Thus, there is a possibility that environmental chemicals may inhibit Leydig cell function by suppressing antioxidant capacity and inducing oxidative stress (Wong and Cheng, 2011).

Excess oxidative stress has been linked to age-related decline in T production in Leydig cells (Cao et al., 2004; Luo et al., 2006) and impaired spermatogenesis (Gosden et al., 1982). Increased lipid peroxidation and decreased mRNA levels and catalytic activities of SOD and GPX, and reduced GSH levels were observed in Leydig cells isolated from aged rats although catalase mRNA and activity were unaffected by the aging process (Cao et al., 2004; Syntin et al., 2001). Persistent stimulation of rat Leydig cells in culture with LH caused apoptosis by inducing oxidative stress (i.e., increased ROS production and lipid peroxidation) and attenuated Leydig cell antioxidant capacity (Aggarwal et al., 2009). On the other hand, it has been

hypothesized that chronic sub-lethal exposure to oxidative stress may attenuate against the aging process presumably by protecting against oxidative stress (Calabrese et al., 2011; Gems and Partridge, 2008).

6. Statement of Research Objectives

Endocrine disruptor chemicals exhibit greater potency during sexual differentiation in rodents and humans (Colborn et al., 1993). Therefore, developmental exposures to chemicals during critical periods of differentiation may cause adverse effects, which may not become apparent until much later in life (Newbold et al., 2007a). There has been an increase in the incidence of reproductive abnormalities, obesity and metabolic syndromes in males in developed countries, including the United States (Carlsen et al., 1992; Rubin and Soto, 2009; Swan et al., 2000). Exposure of human populations to pesticides and industrial pollutants present in foods, beverages, and plastics have raised concerns that these substances can interfere with endogenous sex hormone function and cause a variety of developmental anomalies. Further, these agents are linked to the etiology of metabolic disorders, including diabetes and obesity (Newbold et al., 2007b). These findings have been replicated in laboratory animals exposed to estrogenic compounds in the perinatal period (Pflieger-Bruss et al., 2004; Rubin and Soto, 2009). BPA is a known estrogenic industrial chemical found in more than 90% of the human population in United States (Calafat et al., 2008). Studies to elucidate the adverse effects of early-life exposures to BPA on postnatal Leydig cell development and function are warranted.

Perinatal or developmental exposure to BPA (2.4μg/kg/day) was previously found to decrease intratesticular T concentration by suppressing Leydig cell T production without effect on serum T levels in adult male rats (Akingbemi et al., 2004). BPA is known to affect adipokine secretion, which integrates energy balance and reproduction and inhibits secretion of adiponectin from human adipose tissue explants (Hugo et al., 2008). Adiponectin receptors (adipoR2) are expressed in Leydig cells and have the capacity to regulate androgen secretion (Caminos et al., 2008). Exposure to environmental estrogens (e.g., BPA) may alter estrogen metabolism and oxidative stress which in turn may play a role in the pathogenesis of Leydig cell dysfunction (Cao et al., 2004; Kabuto et al., 2004; Milagro et al., 2006; Murugesan et al., 2007; Wadia et al., 2007). Thus, it was hypothesized that developmental BPA exposure; 1) affects postnatal Leydig cell development by stimulating proliferation but has an inhibitory effect on steroidogenesis; 2) regulates adiponectin secretion by adipose tissue and Leydig cells, which is influenced by high fat diet feeding in adult male rats; and, 3) acts in concert with diet to alter estrogen metabolism and oxidative stress levels in Leydig cells. Specific objectives were to investigate:

1) Direct BPA action in Leydig cells affecting proliferation and steroidogenesis,
2) BPA regulation of adipose tissue-derived molecules (e.g., adiponectin) as regulators of Leydig cell function,
3) Effects of developmental exposure to BPA and diet on estrogen metabolism and antioxidant capacity in Leydig cells.

CHAPTER 2

BPA REGULATION OF LEYDIG CELL PROLIFERATION AND STEROIDOGENESIS

1. Abstract

The presence of bisphenol A (BPA) in consumer products has raised concerns about potential adverse effects on reproductive health. Testicular Leydig cells are the predominant source of the male sex steroid hormone testosterone, which supports the male phenotype. The present report describes the effects of developmental exposure of male rats to BPA by gavage of pregnant and lactating Long Evans dams at 2.5 and 25 μg/kg body weight from gestational day 12 to day 21 postpartum. This exposure paradigm stimulated Leydig cell division in the prepubertal period and increased Leydig cell numbers in the testes of adult male rats at 90 days. Observations from in vitro experiments confirmed that BPA acts directly as a mitogen in Leydig cells. However, BPA-induced proliferative activity in vivo is possibly mediated in part by other factors: e.g., i) protein kinases (e.g., mitogen-activated protein kinases or MAPK); ii) growth factor receptors (e.g., insulin-like growth factor 1 receptor-beta and epidermal growth factor receptors); and, iii) the Sertoli cell-secreted anti-Mullerian hormone (AMH, also called Mullerian Inhibiting Substance [MIS]). On the other hand, BPA suppressed protein expression of the luteinizing hormone receptor (LHR) and the 17beta-hydroxysteroid

dehydrogenase enzyme (HSD17B3) in Leydig cells, thereby decreasing androgen secretion. We interpret these findings to mean that the likely impact of deficits in androgen secretion on serum androgen levels following developmental exposure to BPA is alleviated by increased Leydig cell numbers. The present results reinforce the view that BPA causes biological effects at environmentally relevant exposure levels and its presence in consumer products potentially has implication for public health.

2. Introduction

BPA is used in the manufacture of a variety of consumer products, including polycarbonate plastics, epoxy resins, electronics, medical equipment, and dental sealants (Pflieger-Bruss et al., 2004; Wetherill et al., 2007), and qualifies as one of the world's leading volume–production chemicals with an annual increase in demand of 6%-10% (Burridge, 2003). BPA is able to leach from containers into foods when heated, from dental sealants into the saliva of patients after application and can cross the human placenta into fetal tissues (Ikezuki et al., 2002; Schonfelder et al., 2002). It had been determined that 90% of the population in the United States has BPA in their urine samples (Calafat et al., 2008; Lakind and Naiman, 2008), implying widespread exposure of the population to this compound in the environment. For example, the levels of BPA were determined to be 0.3-18.9 ng/mL in maternal plasma, whereas the levels were 0.2-9.2 ng/mL in fetal plasma and 0.28–0.97 ng/mL in breast milk (Schonfelder et al., 2002; Sun et al., 2004). In the adult US population, the mean urinary BPA level was determined to be about 2.5

ng/mL (Calafat et al., 2008) and the concentration of un-conjugated or free BPA in blood was approximately 1 ng/mL (Vandenberg et al., 2010).

It was observed previously that exposure of male rats to BPA at ~2.5 μg/kg/day from gestational day (GD) 12 to postnatal day (PND) 21 did not affect serum testosterone (T) levels but decreased Leydig cell T production and intratesticular T concentrations in adult animals (Akingbemi et al., 2004). The present study is focused on postnatal differentiation of Leydig cells in male rats perinatally exposed to BPA. In the rat, Leydig cell development progresses through three stages and is defined by two features: proliferation and sex steroid hormone secretion. Early in the neonatal period, mesenchymal Leydig cell precursors differentiate into highly proliferative progenitor Leydig cells (PLCs) from PND 14 to 21 and then into immature Leydig cells (ILCs) between PND 21 and 35. Although ILCs are less able to divide than PLCs, they gradually transform into fully steroidogenic adult Leydig cells (ALCs) by PND 56. ALCs are devoid of any mitotic capacity but have a 150- and 5-fold greater capacity for T production than PLCs and ILCs, respectively (Benton et al., 1995).

Serum androgen levels derive from both steroidogenic capacity and the number of Leydig cells in the testes. Therefore, it is hypothesized that disparities in Leydig cell T production and serum androgen levels in BPA-exposed animals is likely due to changes in proliferative activity early in development, which affect Leydig cell numbers. In this regard, a primary feature of Leydig cell development is the occurrence of active waves of mitosis in the prepubertal period, and it is

conceivable that mitogens utilize a combination of mechanisms to regulate Leydig cell division. For example, cell cycle proteins, which support DNA synthesis (e.g., proliferating cell nuclear antigen, PCNA) or the transition from the G1 to S phase of the cell cycle (e.g., cyclin D3) are expressed at higher levels in highly proliferative PLCs than in ILCs and ALCs (Ge and Hardy, 1997; Sriraman et al., 2000). Similarly, a physiological role for MAPK3/1 in the regulation of Leydig cell division was indicated by measurement of greater p-MAPK3/1 levels in PLCs than in ILCs and ALCs (Sherrill et al., 2010), and observations showing that Leydig cell-specific deletion of MAPK3/1 resulted in reduced Leydig cell numbers in mice (Yamashita et al., 2011). Altogether, these findings imply that the levels of PCNA, cyclin D3 and p-MAPK3/1 protein expression are putative markers of Leydig cell division.

There is evidence that the sex steroid hormones regulate Leydig cell mitosis because Leydig cell numbers were decreased in the testicular feminized mice (*tfm*) compared to the wild-type, implying that ARs mediate mitogenic signals in Leydig cells (Ge and Hardy, 2007). Several reports also indicated that a primary target for estrogen action in the testis is the Leydig cell because the natural estrogen, E_2 inhibited proliferation and regeneration of Leydig cells in male rats treated with the Leydig cell toxin ethane dimethylsulfonate (EDS) (Abney, 1999). Evidence has also been provided showing that Leydig cells of several mammalian species express growth factor receptors, e.g., insulin-like growth factor 1 receptors (IGF1R) and epidermal growth factor receptors (EGFR), which are known to mediate estrogenic activity and regulate proliferative activity in Leydig cells (Chandrashekar and

Bartke, 2007; Ge and Hardy, 1997; Hu et al.; Levine et al., 2000; Musa et al., 2000; Shiraishi and Ascoli, 2007; Smith, 1998).

Given the physiological requirement for cell-cell interactions and the possibility that chemicals with estrogenic activity act simultaneously in multiple testicular cells, it was concluded that paracrine signaling molecules have a role in mediating BPA effects. For example, the AMH is a glycoprotein secreted by Sertoli cells to induce regression of Mullerian ducts during the process of sexual differentiation (Lee and Donahoe, 1993). Interestingly, receptors for AMH are present in Leydig cells (AMHR2) and AMH was found to suppress Leydig cell regeneration in EDS-treated male rats (Lee et al., 1999; Salva et al., 2004). On the other hand, Leydig cell hyperplasia was evident in *Amh* knockout mice compared to the wild-type (Behringer et al., 1994). These observations demonstrate that the AMH acts as a negative regulator of Leydig cell division and it is likely that BPA regulates expression of the *Amh* gene, which possesses estrogen response elements (EREs) in its promoter region (Chen et al., 2003; Nagai et al., 2003).

With the approach of puberty, development of steroidogenesis in Leydig cells is differentially regulated to optimize T production capacity during the transition from PLCs to ALCs. Therefore, LH receptors (LHR) and steroidogenic enzymes, including cytochrome P450 side-chain cleavage enzyme (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B), cytochrome P45017α-hydroxylase/$_{C17/20}$-lyase (CYP17A1), and 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3), are expressed at higher levels in ALCs than in ILCs and PLCs (Benton et al., 1995).

Because ALCs produce T required for spermatogenesis and maintenance of male secondary sexual characteristics, disruption of Leydig cell division and/or T biosynthesis due to activity of exogenous estrogens may be a factor in the causation of reproductive tract anomalies attributed to the action of environmental chemicals (Pflieger-Bruss et al., 2004; Zirkin, 1998). The present study was designed to determine whether exposure of male rats to BPA alters proliferative activity and Leydig cells numbers and/or affect T production capacity. In addition, attempts were made to identify the cellular and molecular mechanisms of BPA action in Leydig cells.

3. Materials and Methods

3.1 Animals

All experimental and euthanasia procedures were performed in accordance with a protocol approved by Auburn University Institutional Animal Care and Use Committee and recommendations of the panel on Euthanasia of the American Veterinary Medical Association. Time-bred pregnant Long-Evans dams at gestational day (GD) six were obtained from Harlan-Heklad (Indianapolis, IN) and were allowed to acclimatize for five to six days at the College of Veterinary Medicine Division of Laboratory Animal Health Housing Facility. Pregnant and nursing dams were housed one per cage, whereas weanling rats were kept in groups of two to four depending on age and size. Animals were kept on a 12 h light, 12-h dark cycle, with ambient temperature of 68°F to 74°F and were provided feed and water *ad libitum*. Standard rodent diets typically contain significant amounts of phytoestrogens

(genistin and daidzin) (Thigpen et al., 1999), which have the potential to modulate endocrine responses in toxicological studies (Brown and Setchell, 2001). Also, use of polycarbonate cages may result in the leaching of BPA into the environment and alter experimental outcomes (Howdeshell et al., 2003; Hunt et al., 2003). In order to minimize background exposure of animals to estrogenic compounds, rats were fed on Teklad Global Soy Protein-Free Extruded Rodent Diet (#2020X, Harlan-Heklad, Indianapolis, IN) and housed in polypropylene cages with glass water bottles. Assignment of rats to groups was done by body weight randomization to ensure equal weight distribution.

3.2 Experimental Protocol

Pregnant dams were gavaged once a day with the olive oil vehicle or BPA (catalog # 14939 Fluka, Sigma-Aldrich) in the oil vehicle at 2.5 or 25 μg/kg body weight (bw) from GD 12 through weaning at PND 21, i.e., during the perinatal period (n=14). Pregnant and nursing animals were weighed at 48 h-intervals and average body weights in each group were used to calculate BPA dosage. All dams carried pregnancy to full term and pregnancy outcome, including litter size and pup weight, were assessed on the date of birth, which was designated as PND 1, and up to PND 7. Subsequently, male pups were distributed equally among dams within the same experimental group to randomize maternal exposure (Hilakivi-Clarke et al., 2002). Randomly selected animals from each group were sacrificed and processed for assessment of Leydig cell differentiation at 21, 35 and 90 days of age. In addition,

primary Leydig cell and Sertoli cell cultures were used in experiments designed to determine that BPA acts directly in testicular cells.

3.3 Analysis of Proliferative Activity

Previous studies of Leydig cell development demonstrated that the initial phase of Leydig cell differentiation is primarily a mitotic phase, which achieves huge increases in the number of Leydig cells in the testis. This prepubertal increase in population is critical to attaining the normal number of Leydig cells and maintaining optimal androgen secretion in the adult testis (Benton et al., 1995). For these reasons, PLCs (PND 21) are routinely used in proliferation assays (Sherrill et al., 2010). Therefore, proliferative activity was assessed by [^{3}H] thymidine uptake using PLCs isolated from male rats at the end of BPA exposure (i.e., in vivo exposure) and by BPA-free PLCs incubated in culture media containing BPA (0, 0.01, 10 nM; 18 h) and ovine LH (10 ng/mL) (NIDDK, USA), i.e., in vitro treatment. On the other hand, Leydig cell numbers were enumerated in testes of control and EDS-treated 60 day-old male rats after exposure to BPA in the perinatal period as well as in 90 day-old male rats perinatally exposed to BPA without EDS treatment using stereological techniques.

Assays of [^{3}H] thymidine uptake involved incubation of Leydig cells in triplicate in culture media containing 100 ng/mL LH (NIDDK, USA) and 1 μCi/mL of [^{3}H] thymidine (specific activity, 80 Ci/mmol; lot no. 3106516; DuPont-NEN Life Science Products). After radiolabeling (3 h), Leydig cells were rinsed in Dulbecco PBS containing ethylenediaminetetra-acetic acid (EDTA; catalog # E-5134, Sigma)

and were thereafter divided into 0.3-0.5 × 10^6 aliquots and lysed in microcentrifuge tubes containing 0.5 mL of hyamine hydroxide (catalog # 802387; MP Biomedicals). Cellular [^{3}H] thymidine uptake was quantified by liquid scintillation counting (Ge and Hardy, 1997).

Given that all levels of the hypothalamic-pituitary-gonadal axis are targets for estrogen action, it was important to ask whether BPA acts directly as a mitogen in Leydig cells. This determination helped to clarify that BPA-induced changes in Leydig cell proliferation is not entirely due to action in the hypothalamus and pituitary gland. Therefore, PLCs, free of prior BPA exposure, were incubated in culture media containing BPA at 0.01 and 10 nM in the presence of ovine LH (10 ng/mL) for 18 h. After treatment, Leydig cells were harvested and processed for [^{3}H] thymidine uptake and scintillation counting.

The EDS-treated rat model was used to assess Leydig cell proliferation *in vivo*. EDS (courtesy of Dr. Earl Gray, Jr., Reproductive Toxicology Branch, US Environmental Protection Agency, Research Triangle Park, NC) was administered to 60 day-old control and BPA exposed male rats (n=5) at 80 mg/kg bw in dimethyl sulfoxide/water (1:3, v/v)] (Sherrill et al., 2010). The EDS model has been routinely used to recapitulate development in vivo (Myers and Abney, 1991) because EDS-induced elimination of the original Leydig cell population is followed by resurgent waves of PLCs appearing in the testis. Thus, the EDS model provides data, which complement results of [^{3}H] thymidine uptake assays. Following EDS administration, Leydig cell regeneration was allowed until 88 days of age, i.e., 28 days post-EDS

treatment, when Leydig cell numbers were enumerated using stereological methods. Also, it was asked whether BPA induction of proliferative activity early in development affected Leydig cell numbers in the adult testis. Accordingly, the numbers of Leydig cells were enumerated in testes of 90 day-old control and male rats that were exposed to BPA from GD 12 to PND 21.

3.4 Identification of Molecular Targets of BPA Action Affecting Cell Division

In order to gain some insight into the mechanisms of BPA action, assays were performed to identify putative molecular targets affecting Leydig cell division. Four different categories were considered: i) cell cycle proteins, e.g., PCNA, cyclin D3; ii) MAP kinases, e.g., extracellular regulated kinase (MAPK3/1); iii) hormone transcription factors, e.g., LHR, ESR1, AR; and, iv) growth factor receptors, e.g., IGF1Rβ, EGFR. Therefore, PLCs isolated from control and BPA-exposed male and not labeled with [^{3}H] thymidine were processed to obtain whole cell lysates for Western blot analyses of proteins of interest. IGF1Rβ and EGFR protein were also measured in BPA-free PLCs after incubation in culture media containing BPA (0, 10 nM) and ovine LH (10 ng/mL) for 3 h. In other experiments, BPA-free PLCs were incubated in culture media containing IGF1 and EGF (10 ng/mL) for 3 h and subsequently processed for [^{3}H] thymidine uptake and liquid scintillation counting. Furthermore, Leydig cells were isolated from groups of 30, 18 and eight animals at 21, 35 and 90 days of age, respectively, for analysis of IGF1Rβ protein levels for comparison to EGFR (Hancock et al., 2009).

Because the *Amh* gene has EREs in its promoter region (Chen et al., 2003; Nagai et al., 2003), BPA disruption of Sertoli cell secretion of the AMH ligand will likely contribute to mitogenic effects in Leydig cells, which express AMH receptors (AMHR2). Consequently, AMHR2 protein levels were measured in western blots of PLCs at 21 days of age following perinatal BPA exposure. To determine whether expression of the AMHR2 protein correlates with proliferative capacity during development, AMHR2 protein levels were analyzed in western blots of Leydig cells obtained at 21, 35, and 90 days of age. Finally, it was asked whether BPA acts directly on Sertoli cells to regulate secretion of AMH using neonatal rat Sertoli cells. This approach was based on focus on the effects of perinatal (i.e., developmental) exposures and is in agreement with exposure paradigms in vivo. Therefore, neonatal Sertoli cells were isolated from 15 six day-old male pups that were not previously exposed to BPA and incubated first in serum-free culture media for 48 h and then in fresh media containing BPA (0, 0.01 and 10 nM) for 24 h. Sertoli cells were harvested at the end of BPA treatment and processed to obtain lysates for measurement of AMH protein by Western blot analysis.

3.5 Measurement of Steroidogenic Capacity

Although it was observed previously that perinatal exposure of male rats to BPA caused a decrease in Leydig cell T production capacity, the sites of BPA-induced lesions in the androgen biosynthetic pathway were not identified (Akingbemi et al., 2004). Because differentiation is characterized by unique biochemical and morphological features at different stages, Leydig cells were isolated and pooled

from 21, 35 and 90 day-old control and male rats exposed to BPA. Although BPA exposure was terminated at day 21 postpartum, this approach helped to define BPA regulation of steroidogenic capacity in Leydig cells through the entire period of development (neonatal, pubertal and adult). The amounts of T production were assayed after aliquots of 0.5 to 2 × 10^5 Leydig cells were incubated in microcentrifuge tubes containing DMEM/F12 culture media and LH (100 ng/mL) for 3 h at 34°C. In addition, the relationship between T production and peripheral androgen levels was described by measuring serum T concentrations in the same animals that were processed for Leydig cell isolation. In all cases, T concentrations were assayed in aliquots of spent media and serum samples by a previously described tritium-based radioimmunoassay (RIA) with an inter-assay variation of 7-8% (Cochran et al., 1979). In order to localize BPA-induced lesions affecting androgen biosynthesis, the levels of LHR, steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes (CYP11A1, HSD3B, CYP17A1, and HSD17B3) were analyzed in western blots of ALCs isolated at 90 days of age from control and BPA-exposed animals.

3.6 Isolation of Testicular Cells

Leydig cells were isolated from Long-Evans male rats after they were sacrificed by CO_2 asphyxiation and involved collagenase digestion of testis followed by Percoll density centrifugation according to a procedure described previously (Klinefelter et al., 1993), but excluding the elutriation step. After testis digestion but before Percoll density centrifugation, seminiferous tubules were removed by

passage of testicular fractions through nylon mesh (pore size, 0.2 μm; Spectrum Laboratories Inc., Rancho Dominguez, CA). Cell fractions were loaded on to a Percoll gradient centrifugation for 60 min to isolate bands of Leydig cells. Yields of Leydig cells were estimated with a hemocytometer, whereas purity was assessed by histochemical staining for HSD3B using 0.4 mM etiocholan-3β-ol-17-one enzyme substrate (catalog # E-5251, Sigma) (Payne et al., 1980). In all cases, the culture media consisted of DMEM/F-12 buffered with 14 mM $NaHCO_3$, containing 0.1% BSA and 0.5 mg/ml bovine lipoprotein (Klinefelter and Ewing, 1988). PLCs and ILCs were cultured at a density of 1 to 2.0 × 10^6 cells per well in 6-well plates and ALCs at 0.1 to 0.2 × 10^6 cells per well in 12-well plates (Corning-Costar Company, New York, NY) in an atmosphere containing 5% O_2 and 5% CO_2 and at a temperature of 37°C.

Sertoli cells were isolated according by the usual protocol from 5 or 6 day old male Long Evans rats after they were sacrificed by CO_2 asphyxiation (Orth, 1982). Briefly, testes were decapsulated and subjected to sequential enzymatic digestion using a buffer containing 0.1% collagenase, 0.1% hyaluronidase, and trypsin inhibitor in 0.5% BSA to eliminate peritubular and Leydig cells. The resulting mixed cell suspension, was trypsinized to eliminate clumps, resuspended in serum-free DMEM/F-12 media (minimal media) before culture at 37°C in matrigel-coated 12 well plates (Collaborative Research, Waltham, MA). This method of isolation yields Sertoli cell populations with purity of > 95% and preserves the functionality of Sertoli cells for at least 7 days (Cooke et al., 1994; Karl and Griswold, 1990). The media was changed after 4 h to remove spermatogonia followed by culture of Sertoli cells for 48 h prior to treatment with BPA (0, 0.01, 10 nM; 24 h).

3.7 SDS-PAGE and Western Blot Analysis

Cells were homogenized in T-PER lysis buffer (Pierce Chemical Co., Rockford, IL) freshly supplemented with protease inhibitor cocktail (catalog # 78410, Pierce Biotechnology, Inc.). Tubes were centrifuged at 12000 rpm for 10 min at 4°C to remove cellular debris. Protein concentration was measured by Biorad protein assay (Bio-Rad) using BSA as standard. Protein aliquots of 50-µl whole-cell lysate was dissolved in 50-µl of Laemmli buffer containing 5% β-mercaptoethanol and boiled for 5 min at 95°C. Equal amount of reduced protein (10-20 µg) from different groups were resolved on varying percentages of Tris-HCl acrylamide gels for SDS-PAGE and were transferred to nitrocellulose membranes (catalog # 1620116, Bio-Rad), which were subsequently incubated with blocking buffer (5% whole milk in 0.1% Tween-20 PBS) for 1 h at room temperature (RT) to reduce nonspecific binding by antibody. Membranes were then incubated with primary antibodies in blocking buffer overnight at 4°C (Table 2). On the next day, blots were washed three times in 0.1% tween-20 PBS (TPBS) to remove unbound antibodies before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Membranes were washed four times with 0.1% TPBS and incubated with chemiluminescent developing reagent (catalog # E2400; Denville Scientific, Metuchen, NJ) for 1 min before exposure to X-ray films (catalog # E-3012; Denville). The presence of the appropriate proteins was visualized by developing the film, which was scanned using Epson 4180 Perfection scanner (Epson-America). Relative protein amounts in identified immunoblots were measured as optical density of the bands on exposed autoradiographic films using Doc-lt LS software (Ultra-Violet

Products Ltd.). Phosphorylated proteins were normalized to corresponding total protein levels, whereas other proteins were normalized to β-actin levels (ACTB).

Table 2. Primary antibodies used in Western blotting procedures

Antibody	Dilutions	Host species	Catalog No.	Company
PCNA	250	mouse monoclonal	sc-53409	Santa Cruz Biotechnologies
Cyclin D3	250	rabbit polyclonal	sc-182	Santa Cruz Biotechnologies
ESR1	1000	mouse monoclonal	ab 2746	Abcam Inc
AR	1000	rabbit polyclonal	sc-185	Santa Cruz Biotechnologies
LHR	1000	rabbit polyclonal	sc-25828	Santa Cruz Biotechnologies
StAR	2000	rabbit polyclonal	Sc-25806	Santa Cruz Biotechnologies
CYP11A1	250	goat polyclonal	sc-18043	Santa Cruz Biotechnologies
CYP17A1	10000	goat polyclonal	sc-46081	Santa Cruz Biotechnologies
HSD17B3	500	goat polyclonal	sc-66415	Santa Cruz Biotechnologies
HSD3B	1000	rabbit polyclonal	sc-28206	Santa Cruz Biotechnologies
b-actin	2000	goat polyclonal	sc-1616	Santa Cruz Biotechnologies
EGFR	500	rabbit polyclonal	sc-03	Santa Cruz Biotechnologies
IGF-1Rβ	500	rabbit polyclonal	Sc-713	Santa Cruz Biotechnologies
p-MAPK3/1	500	mouse monoclonal	Sc-7383	Santa Cruz Biotechnologies
MAPK3/1	2000	rabbit polyclonal	Sc-93	Santa Cruz Biotechnologies
AMHR2	1000	Rabbit polyclonal	Sc-67287	Santa Cruz Biotechnologies
AMH	500	goat polyclonal	Sc-6886	Santa Cruz Biotechnologies

3.8 Stereological Method

Testes from three to five animals per group were analyzed to enumerate Leydig cell numbers. Testes were obtained after whole-body perfusion with 4% paraformaldehyde PBS (pH 7.2) and stored until embedded in paraffin. Sampling of testicular tissue was done according to the fractionator method described previously (Petersen et al., 1999; Sherrill et al., 2010). Briefly, sections were de-waxed and hydrated before treatment with 0.9% hydrogen peroxide in methanol.

Sections were then incubated with 2.5% BSA in a humidified chamber, washed in PBS and incubated overnight with CYP17A1 antibody (sc-46081; Santa Cruz Biotechnology) at RT. On the following day, sections were washed and applied with biotinylated anti-goat secondary antibody (BA-5000; Vector Laboratory, Burlingame, CA) for 1 h before washing in PBS. Subsequently, sections were incubated in ABC reagent for 30 minutes followed by DAB reagent for 2 to 5 minutes. Slides were mounted with VectaShield (Vector) and sealed with clear nail polish. Immunostained sections were digitally captured at 20X by Nikon Eclipse E600 microscope (Nikon Instruments, Lewisville, TX) equipped with epifluoresence, bright-field, and differential interference optics. Nine to six sections were sampled for 3 to 5 fields at 20X magnification from each testis. Images were recorded with a Spot RT Slider digital camera and Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI). Leydig cell density was determined based on the number of cells present in the defined areas of 6 μM-thick sections. The density of the testis is considered to be approximately 1 g/ml (Mori and Christensen, 1980). Therefore, testis weight was used as an estimate of testis volume (mm^3), and the total number of Leydig cells per testis was determined by multiplying cell density by testis volume.

3.9 Statistical Analysis

Data are presented as the mean ± SEM. Data describing pregnancy outcome and reproductive parameters up to PND 7 were based on the litter as a unit of measurement, whereas parameters obtained thereafter were collected from

randomly selected animals within each treatment group. Leydig cells isolated from control and BPA-exposed animals at 21, 35 and 90 days were pooled from 28-30, 20 and eight animals per group, respectively. When required, culture of isolated Leydig cells were performed in triplicate and subsequently assayed in duplicate or greater in order to facilitate statistical analysis. Experiments performed in vitro were repeated at least three times using Leydig cells isolated from groups of 35 prepubertal rats at 21 days of age on each occasion. Data were analyzed by one-way ANOVA with nonparametric statistical analysis. Dunnett's post test was used for multiple group comparison (Appendix I). When there were only two groups, unpaired t-test was used (GraphPad, Inc., San Diego, CA). Differences of $P \leq 0.05$ were considered to be significant.

4. Results

4.1 General Observations

Perinatal exposure to BPA did not affect pregnancy outcome, including litter size, birth weights of pups and pup sex ratio. Body weights, measured at 21, 35 and 90 days of age, were equivalent in BPA-exposed and control animals ($P > 0.05$). Similarly, paired and relative testes weights (proportion to body weights) were not affected by BPA. These parameters are shown in Table 3.

4.2 BPA stimulated proliferative activity

As determined by [^{3}H] thymidine uptake, exposure of male rats to BPA by maternal gavage in the perinatal period, i.e., from GD 12 to PND 21, induced

proliferative activity in PLCs at termination of BPA exposure compared to control (Fig. 8A) ($P<0.01$). The mitogenic effects of BPA result in part from direct BPA action because incubation of PLCs with BPA at 0.01 nM decreased, whereas the 10 nM concentration increased [^{3}H] thymidine uptake compared to control (Fig. 8B) ($P<0.01$). As expected, EDS administration to 60 day-old control and male rats that were perinatally exposed to BPA eliminated Leydig cells from the testes as indicated by low serum T levels 10 days post-EDS treatment (8C) ($P<0.001$). However, 28 days after EDS administration, i.e., PND 88, animals from 2.5-μg BPA dose group showed greater serum T levels compared to control animals (8D) ($P<0.05$). The results of stereological analysis were similar to serum T and showed that compared to control, Leydig cell numbers were approximately 23% greater in the 2.5-μg BPA dose group after 28 days of EDS administration (Fig. 8E) ($P<0.001$). Also, perinatal exposure of male rats to BPA increased Leydig cell numbers at 90 days of age by approximately 16% and 11% in the 2.5- and 25-μg BPA dose groups compared to control (Fig. 8F) ($P<0.05$).

4.3 BPA Increased Expression of Mitogenic Regulatory Proteins

Perinatal exposure of male rats to BPA from GD 12 to PND 21 increased PCNA and cyclin D3 protein levels in PLCs compared to control (Fig. 9A, B) ($P<0.05$), which was related to MAPK3/1 activation as evidenced by increased phosphorylation (p-MAPK3/1) (Fig. 9C) ($P<0.01$). The results of immunoblot analysis showed that perinatal exposure to BPA caused greater LHR (Fig. 10A, B), ESR1 (Fig. 10A, C) and AR (Fig. 10A, D) protein levels than in control PLCs ($P<0.05$).

Exposure of male rats to BPA from GD 12 to PND 21 increased IGF1Rβ and EGFR protein expression in PLCs compared to control (Fig. 11A, B) ($P<0.05$). The results were replicated *in vitro* because incubation with BPA (10 nM, 24 h) increased IGF1Rβ and EGFR protein in PLCs compared to control (Fig. 11C, D) ($P<0.05$). Incubation with IGF1 and EGF (10 ng/mL, 3 h) also induced proliferative activity in PLCs evidenced by increased [^{3}H] thymidine uptake compared to control (Fig. 11E, F) ($P<0.01$). Greater levels of IGF1Rβ (present study) (Fig. 11G) and EGFR protein (Fig. 11H) (Hancock et al., 2009) were measured in PLCs than in ILCs and ALCs ($P<0.01$).

Expression of the AMHR2 protein in PLCs was increased by exposure to BPA in the perinatal period (i.e., GD 12 to PND 21) compared to control (Fig. 12A) ($P<0.01$). Of interest, greater AMHR2 protein was present in highly proliferative PLCs than in ILCs and ALCs (Fig. 12B) ($P<0.05$), indicating there is developmental regulation of the AMHR2 protein in Leydig cells. In other experiments, BPA treatment (10 nM, 24 h) decreased AMH protein expression in neonatal rat Sertoli cells (Fig. 12C) ($P<0.01$), implying that BPA regulates secretion of the AMH ligand.

4.4 Exposure to BPA Decreased Leydig Cell T Production Associated With Reduced LHR and Steroidogenic Enzyme Protein

The results of RIAs showed that Leydig cell T production was decreased immediately following perinatal exposure to BPA (PND 21) and later in the postnatal period (PND 35 and PND 90) ($P<0.05$). However, decreased Leydig cell T production did not translate into lower serum T levels (Fig. 13) ($P>0.05$).

Nevertheless, the sites of BPA-induced lesion in the androgen biosynthetic pathway were identified as LHR and the HSD17B3 enzyme because LHR and HSD17B3 protein were decreased in BPA-exposed ALCs compared to control at 90 days of age (Fig. 14) ($P<0.05$).

Table 3. Pregnancy outcome and reproductive parameters in male rats exposed to BPA.*

	Dose of BPA (µg per kg /day)		
Parameters	0	2.5	25
Litter size (number of pups) [a]	12.35 ± 0.57	11.28 ± 0.59	10.92 ± 0.43
Pup sex ratio (male:female) [a]	1.38 ± 0.24	1.2 ± 0.13	1.08 ± 0.19
Body weights (g, PND 1) [a]	6.15 ± 0.16	6.41 ± 0.11	6.42 ± 0.08
Body weights (g, PND 7) [a]	12.18 ± 0.43	13.3 ± 0.41	13.08 ± 0.21
Body weights (g, PND 21) [b]	50.28 ± 0.70	51.46 ± 0.82	52.59 ± 0.62
Paired testis weights (g, PND 21)[b]	0.21 ± 0.00	0.22 ± 0.00	0.23 ± 0.00
	(0.0044 ± 0.00)	(0.0045 ± 0.00)	(0.0045 ± 0.00)
Body weights (g, PND 35) [c]	123.64 ± 2.57	131.76 ± 1.99	123.42 ± 2.62
Paired testis weights (g, PND 35) [c]	1.09 ± 0.03	1.11 ± 0.07	1.07 ± 0.04
	(0.0088 ± 0.00)	(0.009 ± 0.00)	(0.0087 ± 0.00)
Body weights (g, PND 90) [d]	437.75 ± 13.94	446 ± 11.13	430.42 ± 10.4
Paired testis weights (g, PND 90) [d]	3.24 ± 0.32	3.34 ± 0.11	3.65 ± 0.09
	(0.0074 ± 0.00)	(0.0075 ± 0.00)	(0.0085 ± 0.00)

* BPA exposure was achieved by maternal gavage from GD 12 to PND 21.
[a] Values were based on the litter as a unit of measurement (n=14).
[b–d] Values were based on [b] 28–30, [c] 14, and [d] 8–10 from each group.
Relative testis weights (as proportion of body weights) are in parentheses.

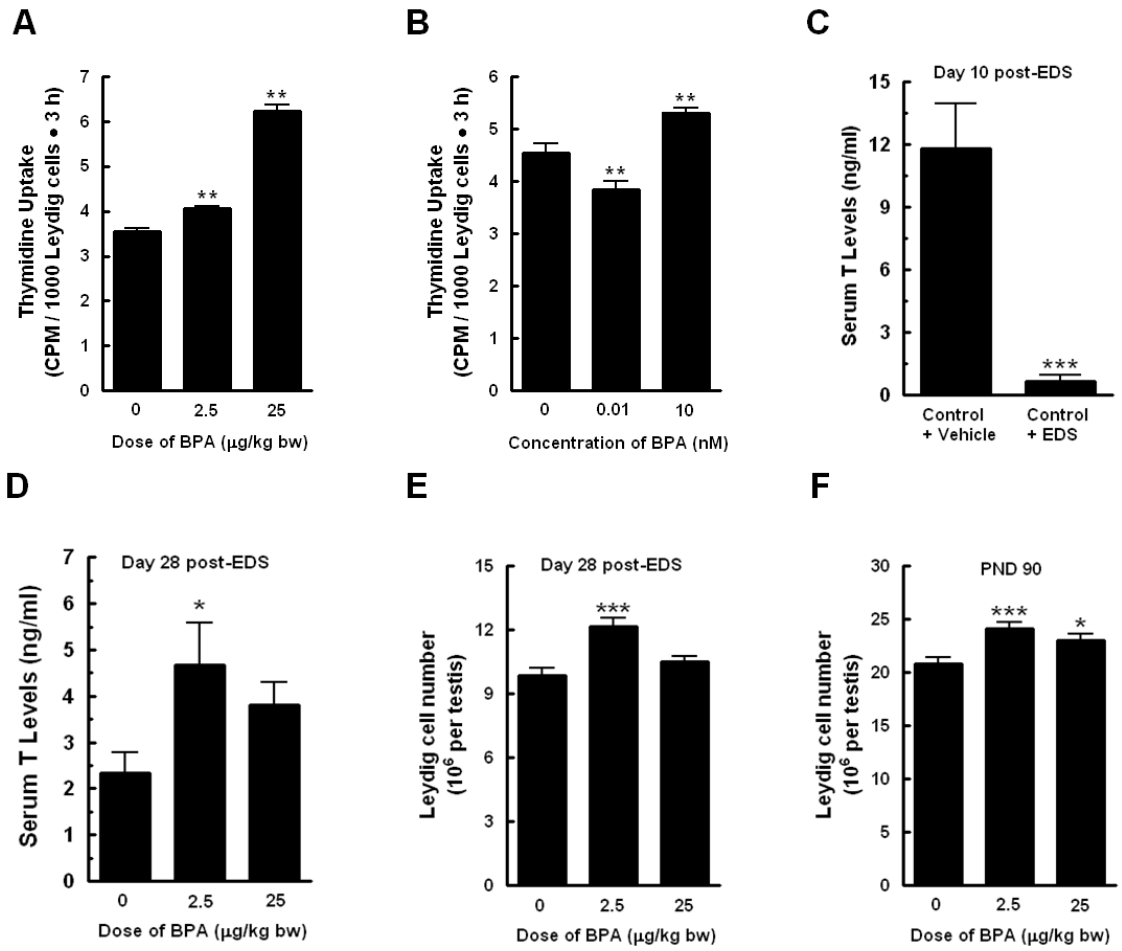

Figure 8. Effect of BPA on Leydig cell proliferation. Proliferative activity was assessed in vivo and in vitro using PLCs isolated and pooled from 28–30 male rats per treatment group at the end of perinatal BPA exposure on Day 21 postpartum (A) and BPA-free PLCs that were incubated in culture media containing BPA and ovine LH (10 ng/mL) for 18 h (B). Proliferative activity was determined by [3H] thymidine uptake assays performed in quadruplicate or greater followed by scintillation counting. Serum testosterone (T) concentrations were measured as a marker for Leydig cell repopulation of testis following administration of the EDS to 60-day-old rats exposed to BPA in the perinatal period. Serum T levels were measured 10 days (C) and 28 days (D) after EDS administration. Leydig cell numbers in the testes were enumerated by stereology 28 days after EDS administration to 60-day-old male rats exposed to BPA in the perinatal period (five animals per treatment group, PND 88; E). Leydig cells were also enumerated in testes of control and 90-day-old BPA-exposed male rats without EDS treatment (three animals per group; F). Perinatal BPA exposure was by maternal gavage from GD 12 to PND 21. PLCs, progenitor Leydig cells at 21 days of age; EDS, ethane dimethyl sulfonate; *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.

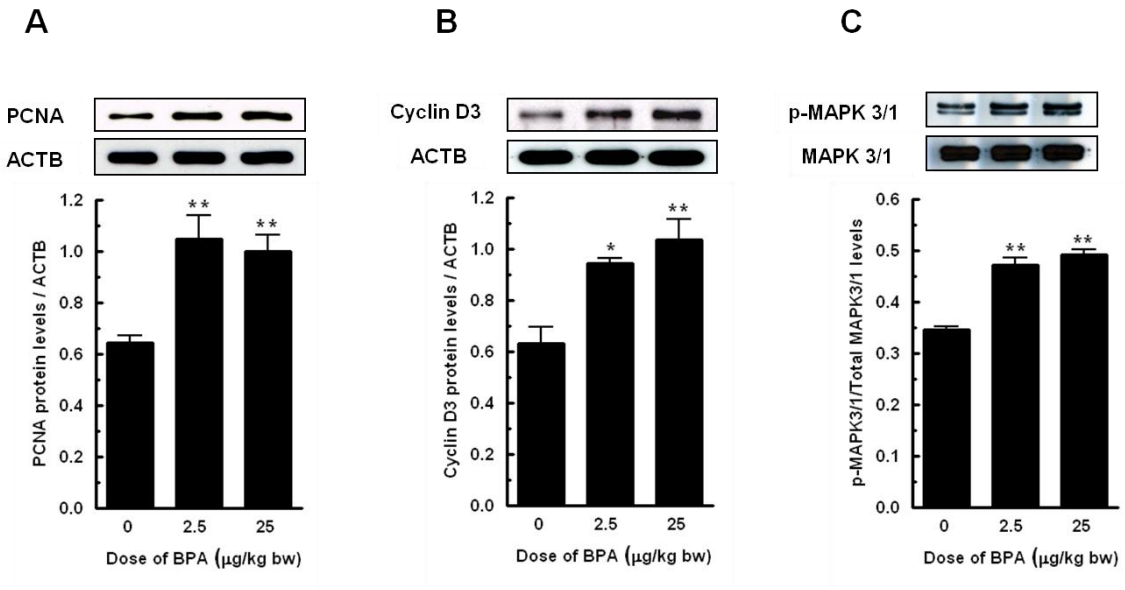

Figure 9. Effect of BPA on cell cycle protein expression in progenitor Leydig cells. At termination of perinatal BPA exposure on day 21 postpartum, PLCs were isolated and pooled from 28-30 male rats per treatment group and subsequently processed to obtain lysates for Western blot analysis of proliferating cell nuclear antigen (PCNA; **A**), cyclin D3 (**B**) and activated (i.e., phosphorylated) extracellular regulated kinase (p-MAPK3/1; **C**). PCNA and cyclin D3 levels were normalized to β-actin (ACTB) while p-MAPK3/1 levels were normalized to total MAPK3/1 levels. Data represent results from densitometric analysis of at least four western blots. PCNA, 36 kDa; cyclin D3, 35 kDa; p-MAPK3/1 and p-MAPK3/1, 42/44 kDa; ACTB, 42 kDa. Perinatal BPA exposure was by maternal gavage from gestational day 12 to postnatal day 21. PLCs: progenitor Leydig cells at 21 days of age. *P < 0.05, **P < 0.01 versus control.

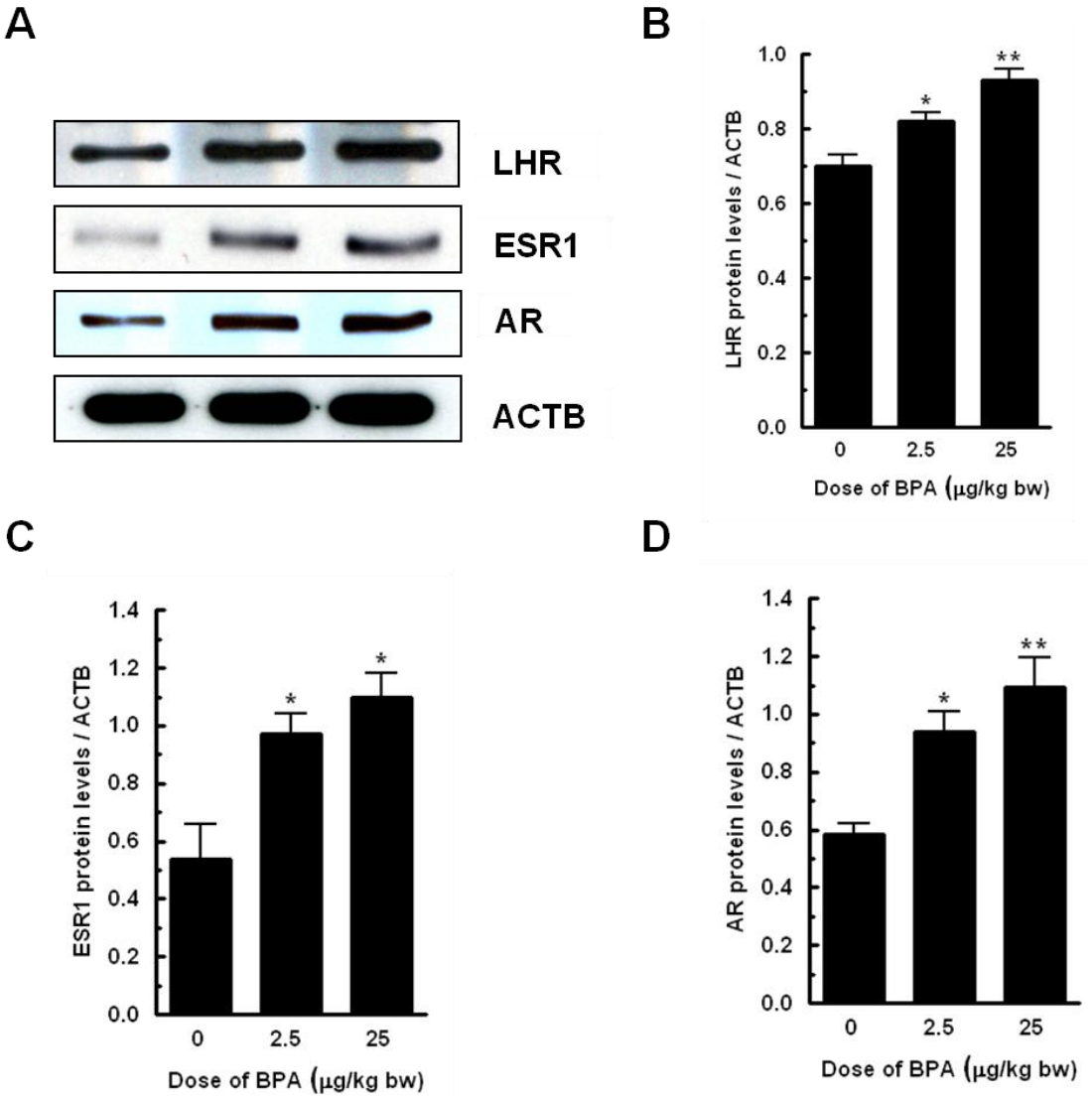

Figure 10. Effect of BPA on hormone receptor expression in progenitor Leydig cells. After perinatal BPA exposure was terminated on day 21 postpartum, PLCs were isolated and pooled from 28-30 male rats per treatment group and subsequently processed to obtain lysates for Western blot analysis of LH receptor (LHCGR), estrogen receptor 1 (ESR1) and androgen receptor (AR) using specific anti-LHCGR (**A**,**B**), anti-ESR1 (**A**,**C**), and anti-AR (**A**,**D**) antibodies and appropriate secondary antibodies. Western blotting procedures were repeated at least four times and protein levels were normalized to β-actin (ACTB). LHCGR, 80 kDa; ESR1, 68 kDa; AR, 110 kDa; ACTB: 42 kDa. Perinatal BPA exposure was by maternal gavage from gestational day 12 to postnatal day 21. PLCs: progenitor Leydig cells at 21 days of age. *P < 0.05, **P < 0.01 versus control.

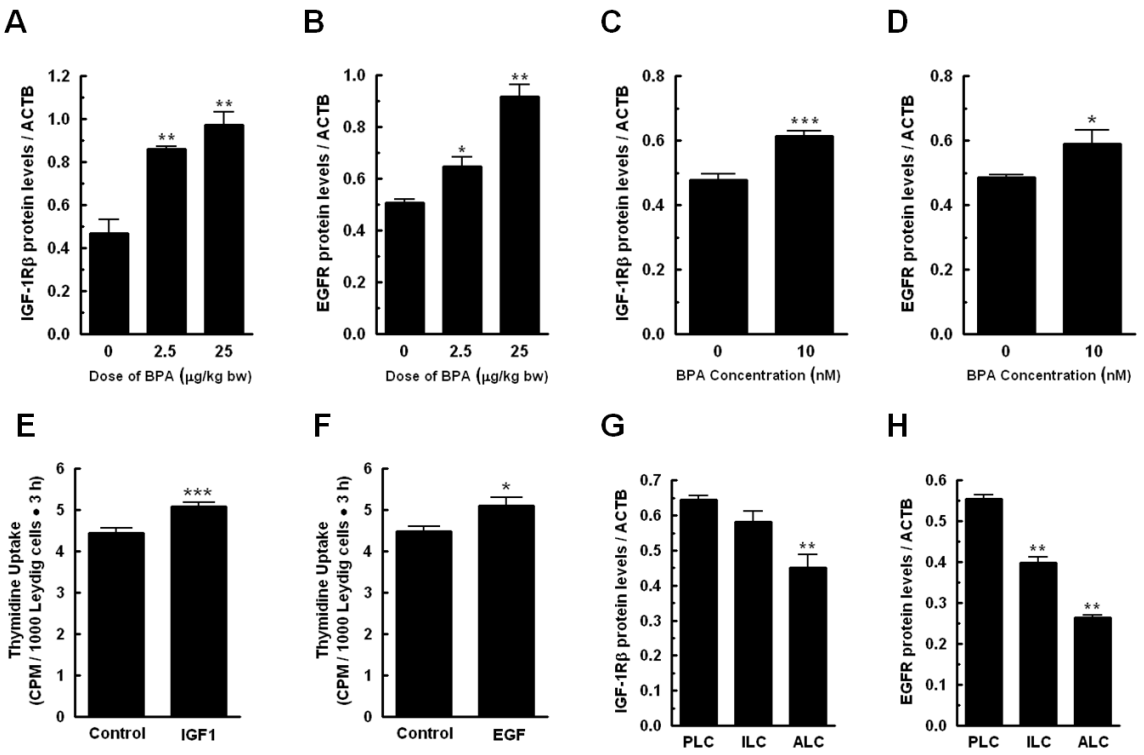

Figure 11. Effect of BPA on growth factor receptor expression. At termination of perinatal BPA exposure (day 21 postpartum), PLCs were isolated and pooled from 28-30 male rats per treatment group (**A**, **B**) or were isolated from thirty five 21 day-old male rats not previously exposed to BPA for incubation in culture media containing BPA and ovine LH (10 ng/mL) for 18 h (**C**, **D, E, F**). IGF1Rβ and EGFR protein levels were analyzed in western blots using antisera specific to IGF1Rβ and EGFR. In addition, BPA-free PLCs were incubated in culture media containing IGF1 (**E**) or EGF (**F**) (10 ng/mL) and ovine LH (10 ng/mL) for 3 h and subsequently processed for [^{3}H] thymidine uptake and scintillation counting (**E**, **F**). Moreover, expression of the IGF1Rβ (**G**) and EGFR protein (**H**) were analyzed in BPA-free PLCs, ILCs and ALCs obtained from 35, 20 and eight male rats at 21, 35 and 90 days of age. EGFR protein levels were reported previously (Hancock et al., 2009). Data from in vitro experiments represent results from three separate and independent experiments (**C**, **D**, **E**, **F**). Western blotting procedures were repeated at least four times and protein levels were normalized to β-actin (ACTB). IGF1Rβ, 97kDa; EGFR, 170 kDa; ACTB, 42 kDa. Perinatal BPA exposure was by maternal gavage from gestational day 12 to postnatal day 21. PLC, progenitor Leydig cells at 21 days; ILC, immature Leydig cells at 35 days, ALC, adult Leydig cells at 90 days. *P < 0.05, **P < 0.01, ***P < 0.001 versus control.

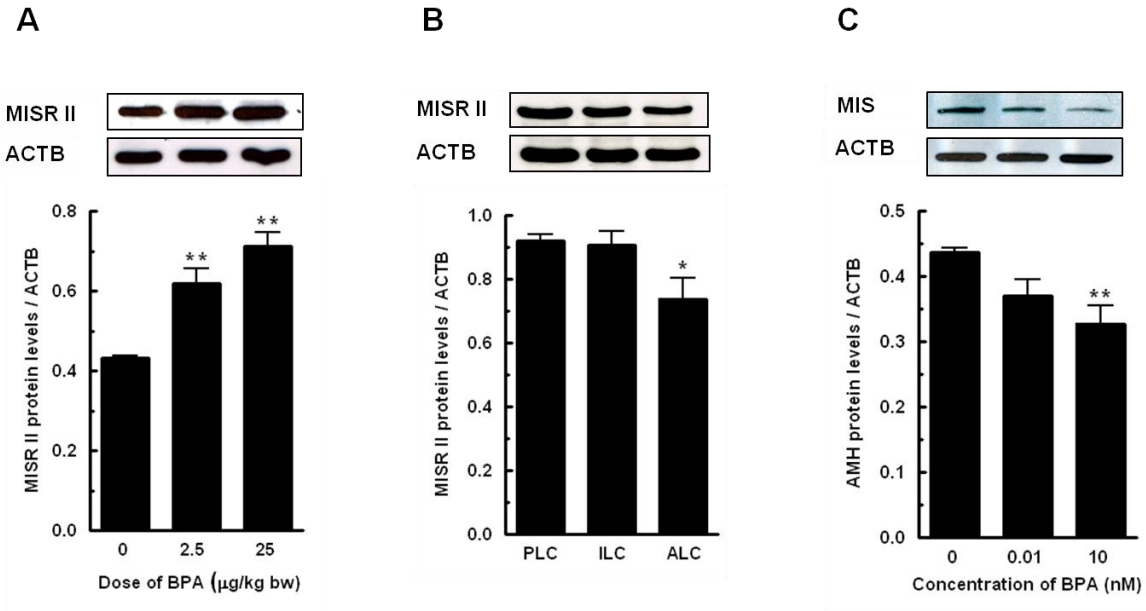

Figure 12. Effect of BPA on AMHR2 and AMH protein expression. Following perinatal BPA exposure, which was terminated on day 21 postpartum, PLCs were isolated and pooled from 28-30 male rats per treatment group and subsequently processed to obtain lysates for Western blot analysis of MISRII protein (**A**). Also, developmental expression of the MISRII protein was analyzed in BPA-free PLCs, ILCs and ALCs obtained from 35, 18 and eight male rats at 21, 35 and 90 days of age (**B**). Finally, MIS protein levels were analyzed in western blots of neonatal Sertoli cells isolated from 15 six day-old male rats after incubation in culture media containing BPA for 24 h (**C**). Western blotting procedures were performed using antisera specific to MISRII and MIS and appropriate secondary antibodies and were repeated at least four times (**A**, **B**). Protein levels were normalized to β-actin (ACTB). Data from in vitro experiments represent results from three separate and independent experiments (**C**). MISRII, 63 kDa; MIS, 65 kDa; ACTB, 42 kDa. Perinatal BPA exposure was by maternal gavage from gestational day 12 to postnatal day 21. PLC, progenitor Leydig cells at 21 days of age; ILC, immature Leydig cells at 35 days; ALC, adult Leydig cells at 90 days. *P < 0.05, **P < 0.01 versus control.

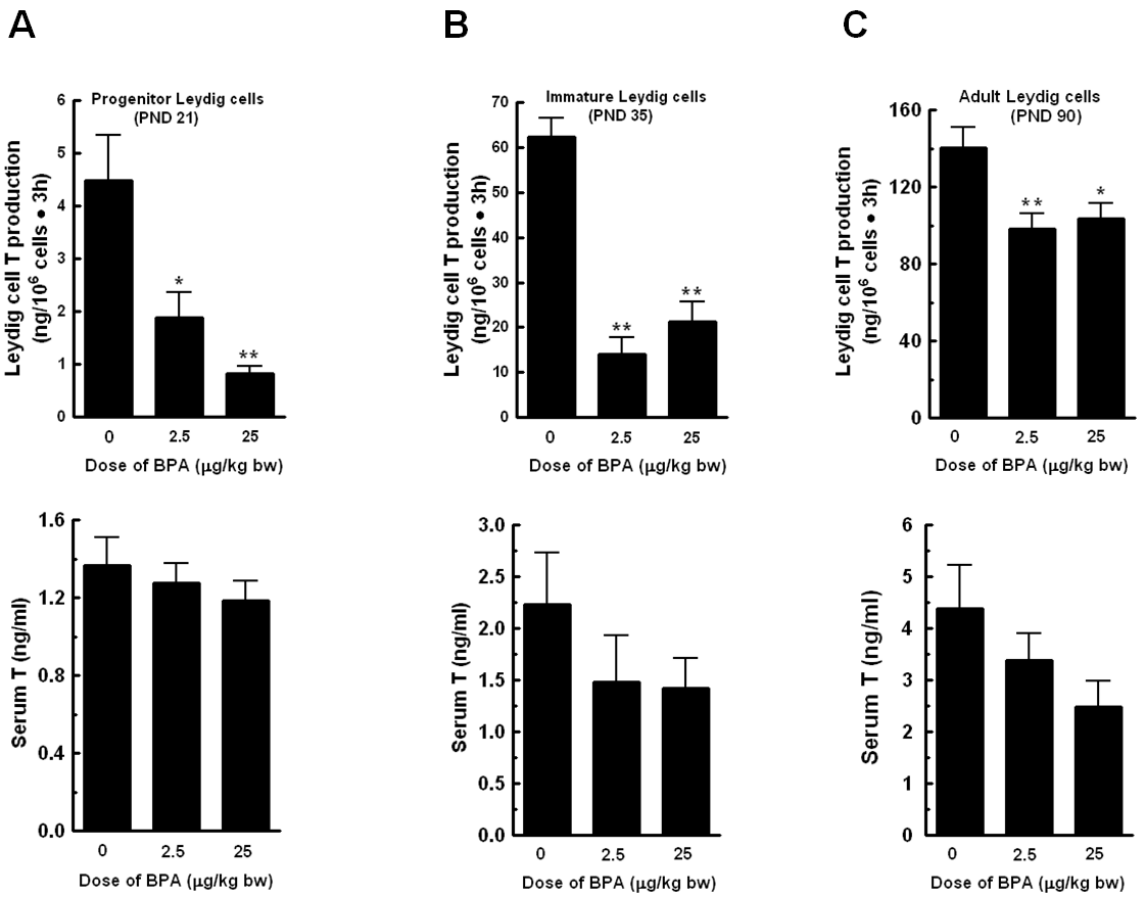

Figure 13. Effect of BPA on steroid hormone secretion. After perinatal BPA exposure was terminated on day 21 postpartum, Leydig cells were isolated and pooled from 28-30, 14, and eight male rats per treatment group at 21, 35 and 90 days and were then incubated in DMEM/F12 culture media containing LH (100 ng/ml) in triplicate. Serum was obtained from blood collected from animals at the time of sacrifice. Leydig cell testosterone (T) production and serum T concentrations were analyzed by RIA, which were performed in duplicate. Perinatal BPA exposure was by maternal gavage from gestational day 12 to postnatal day 21. PND: postnatal day. *P < 0.05, **P < 0.01 versus control.

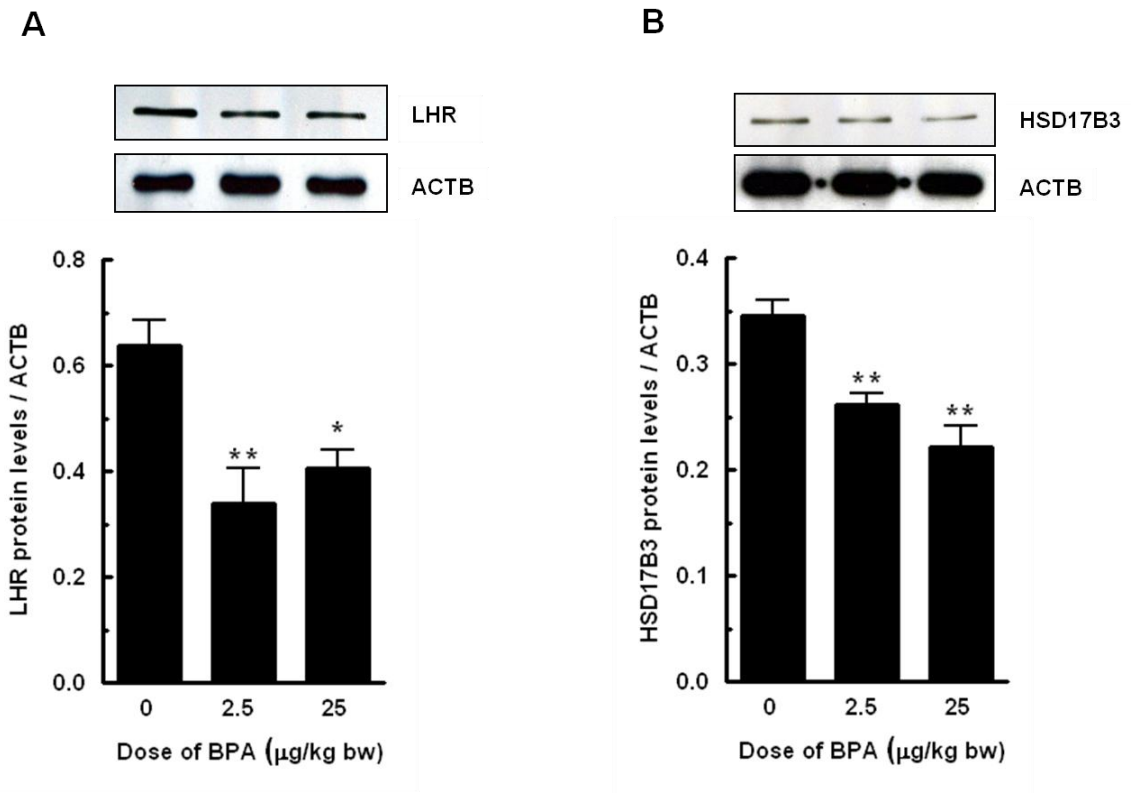

Figure 14. Effect of BPA on LH receptor and HSD17B3 protein expression. Adult Leydig cells were isolated and pooled from eight male rats per treatment group at 90 days of age after perinatal exposure to BPA. The androgen biosynthetic pathway was analyzed by Western blotting using anti-LHR (A) and anti-HSD17B3 (B) antibodies and appropriate secondary antibodies. Data represent results from at least four Western blot procedures and protein levels were normalized to β-actin (ACTB). LHR, 85 kDa; HSD17B3, 35 kDa; ACTB, 42 kDa. Perinatal BPA exposure was by maternal gavage from gestational day 12 to postnatal day 21. *P < 0.05, **P < 0.01 versus control.

5. Discussion

Although serum BPA levels were not measured in the present study, it was previously determined that exposure of nursing dams to BPA at 100 μg/kg bw delivered BPA to the offspring at levels that are decreased by a factor of 300, i.e., 0.32 μg/kg bw (Doerge et al., 2010b). Therefore, maternal exposures to BPA at 2.5 and 25 μg/kg bw represent BPA doses to the offspring of about 8 and 80 pg/kg bw. These dosage regimens approximate BPA levels in the environment (Vandenberg et al., 2010). In the present study, gavage of pregnant dams with BPA appeared to not affect pregnancy outcome, including litter size, birth weights of pups, pup sex ratio, as well as body and testes weights in adult animals. A previous study reached a similar conclusion because perinatal exposure of male rats to BPA at 70 μg/kg/day increased body weights at birth compared to control animals but differences were no longer apparent in adulthood (Somm et al., 2009). It is likely that low dose BPA exposure paradigms do not result in sustainable changes in body weight. Exposure to BPA in the perinatal period as in this study affected the two features of Leydig cell differentiation: proliferation and steroid hormone secretion. Importantly, the results indicated that the developmental effects of BPA were present into adulthood. For example, the greater number of Leydig cells in the testis of adult rats at 90 days of age after exposure to BPA was terminated on day 21 postpartum is the result of increased Leydig cell proliferative activity early in development (21 days). On the other hand, BPA suppressed development of steroidogenic capacity in Leydig cells, which was evident at the end of the exposure period (21 days) and into adulthood at

90 days. Overall, these findings showed that developmental exposures to BPA impact testis development.

BPA-induced proliferative activity in Leydig cells was linked to cell cycle progression as indicated by PCNA and cyclin D3 protein expression (Sriraman et al., 2000) and involved MAPK3/1 activation (Kim et al.; Ropero et al., 2006; Song et al., 2002). This is perhaps not surprising because MAPK signaling pathways are known to regulate the actions of growth factors and estrogenic agents in reproductive tract tissues (Hall et al., 2001; Smith, 1998), including Leydig cells (Yamashita et al., 2011). Also, BPA increased expression of LHR, ESR1 and AR in prepubertal Leydig cells, which has the effect of amplifying hormone-mediated activity supporting tissue differentiation (Oliveira et al., 2004; Woodham et al., 2003). BPA effects were due, at least in part, to direct action in Leydig cells as confirmed by results of in vitro experiments showing that 10 nM BPA increased, whereas the 0.01 nM concentration decreased [^{3}H] thymidine uptake. The opposite effects seen at 0.01 and 10 nM concentrations (a 1000-fold difference) are possibly related to the capacity of estrogenic chemicals to cause mostly non-monotonic (non-linear) effects in estrogen sensitive tissues. The non-monotonic effect (U-shaped or inverted U-shaped) is typical of receptor-mediated responses and is thought to vary with molecular events underlying observed changes (Bouskine et al., 2008; Welshons et al., 2003; Wetherill et al., 2007).

The possibility that induction of proliferation by BPA involved growth factor receptors was indicated by the presence of greater IGF1Rβ and EGFR protein levels

in BPA-exposed Leydig cells than in control. This interpretation is supported by the results of in vitro assays showing that incubation with IGF1 and EGF increased [^{3}H] thymidine uptake by Leydig cells. Indeed, IGF1Rβ- and EGFR-mediated activities were previously determined to increase the rates of Leydig cell mitosis (Ge and Hardy, 1997; Musa et al., 2000; Shiraishi and Ascoli, 2007) while protein expression of both IGF1Rβ (this study) and EGFR declined during the transition from prepubertal (PLCs, ILCs) into adult Leydig cells (ALCs) (Hancock et al., 2009). Specific involvement of IGF1Rβ and EGFR in mediating BPA effects required their activation, which was not confirmed in the present study. Nevertheless, the results support the view that growth factor receptors are putative mediators of mitogenic signals in Leydig cells.

A paracrine relationship is known to exist between Leydig cells and Sertoli cells (Lee et al., 1999; Rouiller-Fabre et al., 1998; Salva et al., 2004). Recent reports suggest that Sertoli cell-secreted AMH is a target for endocrine disruptors with estrogenic activity (Chen et al., 2003). In this regard, perinatal exposure of male rats to BPA was found to increase AMHR2 protein in Leydig cells, whereas the results of in vitro assays showed that BPA decreased expression of the AMH ligand in Sertoli cells. Therefore, it is proposed that deficits in AMH potentially impact paracrine interactions between Sertoli cells and Leydig cells and likely contribute to the mitogenic effects of BPA in Leydig cells. It is also likely that BPA downregulation of the AMH protein in Sertoli cells may be part of a larger effect on Sertoli cell differentiation. If that were the case, BPA effects in neonatal Sertoli cells may persist into adulthood with potential adverse effects on sperm production.

Additional studies are required to determine effects on Sertoli cell development and function after BPA exposures occurring during development.

In contrast to stimulatory effects on cell division, perinatal exposure of male rats to BPA suppressed development of steroidogenic capacity in the postnatal period as evidenced by decreased LHR and HSD17B3 protein in adult Leydig cells. This finding is relevant because the pituitary gonadotropin LH is the primary factor regulating cholesterol availability and steroidogenic enzyme activity required for androgen biosynthesis. On the other hand, the HSD17B3 enzyme catalyzes the final enzymatic step in androgen biosynthesis, forming T from androstenedione (Benton et al., 1995). Although androstenedione accumulation was not measured in the present study, BPA-induced inhibition of androgen secretion likely resulted from diminished LH stimulation and decreased HSD17B3 protein and activity in Leydig cells.

In conclusion, the results of this study demonstrated that perinatal exposure of male rats to BPA impaired postnatal Leydig cell differentiation. Although BPA suppressed T production, serum T levels were unaffected presumably due to greater Leydig cell numbers, which resulted from increased proliferative activity in the prepubertal period. The present results are similar to reports showing that *in utero* exposure of male rats to BPA increased Leydig cell numbers and did not affect serum T levels at 60 days of age (Thuillier et al., 2009). Interestingly, short-term exposure of prepubertal male rats to BPA at 2.4 μg/kg b.w. from day 21 to day 35 postpartum, but not at higher doses, was found to decrease serum T levels

(Akingbemi et al., 2004). It is likely that BPA effects in Leydig cells are affected by the dose, time and duration of exposure. Exposure of male Long-Evans rats to 2.4 μg/kg b.w. from GD12 to PND 21 was previously found to decrease intratesticular T levels in adult male rats (Akingbemi et al., 2004). Although levels vary with the strain of rats and assay methods used, intratesticular T levels in the adult rat are about 30-fold greater (e.g., 70 ng/ml) and are not subject to circadian and diurnal fluctations as serum T levels (e.g., 2 ng/ml). Therefore, intratesticular T more accurately reflects Leydig cell T production capacity than serum T levels (Jarow and Zirkin, 2005). Although perinatal exposure to BPA decreased intratesticular T by as much as 40% in adult male rats (Akingbemi et al., 2004), it has been suggested that much less than 50% is required to support sperm production (Zirkin et al., 1989). Because the rat is an efficient sperm producer (McLachlan et al., 1994), it is likely that a 40% decrease in intratesticular T levels have no impact on quantitative indices of sperm production. Moreover, the present data showed that BPA interferes with secretion of the AMH by Sertoli cells. This finding has relevance to paracrine regulation of testicular cells and their function. The long-term effects of BPA on the capacity of somatic cells of the testis (i.e., Sertoli cells) to support qualitative sperm production into advancing age require further investigation. Interestingly, recent observations indicate that BPA has the capacity to affect metabolic activity in human adipose tissue and may be a factor in the etiology of metabolic syndromes (Ben-Jonathan et al., 2009). Thus, an accumulating body of evidence supports the view that BPA causes biological effects in several body tissues.

CHAPTER 3

EFFECT OF BPA AND DIET ON ADIPONECTIN REGULATION OF LEYDIG CELLS

1. Abstract

The possibility that early-life exposure to bisphenol A (BPA) alters developmental programming and predispose to obesity in later life has been proposed. Several factors of adipose tissue origin (e.g., adiponectin) that regulate energy balance potentially affect male reproduction. In this regard, the present study describes reproductive and metabolic parameters after developmental exposure of Long-Evans male rats to BPA at 2.5 and 25 μg/kg body weight (bw) from gestational day 12 to day 21 postpartum followed by maintenance on normal or high fat diet (NFD or HFD) from postnatal day 71-98. Although amount of calories consumed per gram body weight gain over a period of 28 days were similar, HFD feeding increased body weights in BPA-treated animals (25 μg/kg bw) compared to NFD age-matched animals ($P<0.05$). However, HFD animals consumed less food (g) per unit gain in body weight compared to the NFD group ($P<0.01$), ostensibly due to differences in energy content of diets (NFD=3.1 Kcal/g vs HFD=4.5Kcal/g). Serum T levels were greater in male rats that were fed HFD and exposed to the 25 μg/kg bw dose of BPA compared to control HFD animals ($P<0.05$). Although developmental BPA exposure inhibited androgen secretion, HFD feeding

alone had the effect of increasing T production by up-regulating HSD17B3 enzyme expression in Leydig cells. In addition, BPA exposure and/or HFD feeding decreased serum adiponectin levels and expression of adiponectin and its receptors (adipoR2) in Leydig cells. Altogether, these results suggest that early-life exposures to BPA alone and or with HFD feeding may induce a metabolic syndrome with implication for endocrine function of the testis.

2. Introduction

A growing body of evidence indicates that exposures to environmental chemicals during the critical period of development can disrupt programming of endocrine signaling pathways and cause adverse health effects in adulthood (Heindel, 2003). There is experimental evidence showing that early-life exposures to estrogens (e.g. DES) cause obesity later in life (Newbold et al., 2007b). The recent increase in obesity rates in the population has been attributed to chronic low-level exposure to industrial chemicals, which coincided with the marked increase in their levels in the environment (i.e., food, water and air) over the past 40 years (Baillie-Hamilton, 2002). There is general agreement that several factors (e.g. leptin and adiponectin) that regulate energy balance have the potential to modulate pubertal development and fertility in the male (Fernandez-Fernandez et al., 2006), and may have a role in reproductive dysfunction associated with metabolic syndromes (Sullivan et al., 2011; Wade and Jones, 2004). Therefore, endocrine disruptor chemicals that affect endocrine signaling pathways potentially may cause reproductive abnormalities and impact metabolic homeostasis.

Early-life exposures to BPA have been associated with increased body weights in adult rodents (Akingbemi et al., 2004; Miyawaki et al., 2007; Rubin et al., 2001; Wei et al., 2011). Observations from several studies have shown that BPA has the capacity to act directly on adipose tissue. For example, BPA was found to promote proliferation and differentiation of preadipocytes (Phrakonkham et al., 2008). BPA also inhibited adiponectin secretion from human adipose tissue explants and 3T3-L1 adipocytes (Hugo et al., 2008; Kidani et al., 2010). Hence, BPA has been classified as an obesogen. Obesogens are environmental agents, which have the capacity to disrupt metabolic signaling and cause obesity (Grün and Blumberg, 2006). Adiponectin and its receptor (AdipoR2) are present in testicular Leydig cells and are known to affect androgen biosynthesis (Caminos et al., 2008). Therefore, it is likely that adiponectin and its receptors provide a link between energy homeostasis and male reproductive activity.

There is evidence that HFD consumption increases adipose tissue mass and cause infertility in the male (Bakos et al., 2011b; Fernandez et al., 2011; Ghanayem et al., 2010; Olivares et al., 2010). However, the mechanisms involved in HFD-induced infertility are not clear. Nevertheless, it has been suggested that HFD-induced obesity affects chemical-induced reproductive toxicity (Ghanayem et al., 2010). In particular, recent studies demonstrated that perinatal BPA exposure may predispose to HFD-induced obesity and metabolic syndrome in adulthood (Rubin and Soto, 2009).

The present study was designed to determine whether regulation of Leydig cell function by developmental BPA exposure is associated with changes in adiponectin secretion and if this effect is influenced by a HFD in sexually mature male rats.

3. Materials and Methods

3.1 Animal studies

The experimental protocol regarding BPA exposure has been described in Chapter 2. Briefly, timed-pregnant Long-Evans dams (N=14) were gavaged daily with olive oil vehicle or BPA (2.5 or 25 μg/kg bw/ day) from gestational day 12 through postnatal day 21. During this period, pregnant dams and male weanlings were fed a soy-free diet (#2020X, Harlan Teklad). For the present study, weanling male offspring from BPA-exposed dams were randomly selected and maintained on a soy- and BPA-free diet until PND 70, after which animals were reconstituted randomly into two subgroups (n=8-10) and fed either a normal (NFD, #2020X, Harlan) or a high fat diet (HFD, # 88137, Harlan) from PND 71-98 i.e., for 28 days. NFD and HFD were formulated to have fat levels that provide 16% and 42% of their energy contents, respectively (Table 4). Animals were kept in pairs and were fed *ad libitum*, and food intake and body weight gains were recorded weekly during this period. At day 98 postpartum, HFD animals were fasted overnight prior to sacrifice by CO_2 asphyxiation and at PND 100 for the NFD group. At sacrifice, truncal blood was collected from a cohort of 8–10 animals per group to analyze serum biochemistry. In addition, gonadal fat and Leydig cells were isolated from the same

animals and stored at −80° C until processed for Western blot analysis. The levels of adiponectin were analyzed in gonadal fat, whereas adiponectin and AdipoR2 protein and T production were measured in Leydig cells.

Table 4. Composition of normal (NFD) and high fat (HFD) diets

Parameters	**2020X (NFD)**	**TD88137 (HFD)**
Protein (%)	19.1	17.3
Carbohydrate (%)	47	48.5
Fat (%)	6.5	21.2
Total Saturated fatty acids (%)	0.8	13.3
Total Monounsaturated fatty acids (%)	1.1	5.9
Total Polyunsaturated fatty acids (%)	2.9	0.9
Cholesterol (%)	-	0.2
Calories from fat (%)	16	42
Calories from protein (%)	24	15.2
Calories from carbohydrates (%)	60	42.7
Energy (Kcal/g)	3.1	4.5

3.3 Serum Adiponectin and Testosterone Measurement

Adiponectin concentrations were measured by ELISA using rat adiponectin as the standard (Linco Research Immunoassay, St. Charles, MO, USA). For the adiponectin assay, inter-assay and intra-assay coefficients of variation were 6.54% and 1.59%, respectively, while sensitivity was 0.155 ng/mL. Serum T levels were measured in duplicate by the RIA procedure described in Chapter 2.

3.4 Adult Leydig Cell Isolation

The procedure for isolation of Leydig cells is as described in Chapter 2.

3.5 Western Blot Analysis

Protein sample preparation and Western blotting procedures were performed according to standard procedures and as described in Chapter 2. AdipoR2 and 17β-HSD3 (Santa Cruz Biotech. Inc. CA, USA) and adiponectin primary antibodies (# 5903-50, Biovision, Mountain View, CA, USA) were used with appropriate secondary antibodies (Santa Cruz Biotech. Inc. CA, USA).

3.6 Statistics

Measurements are described as mean ± SEM. Adult male rats between 98 and 100 days of age that were perinatally exposed to BPA and maintained on NFD or HFD were randomly selected for analysis (n = 8–10). Data from animal studies were analyzed by two-way ANOVA with nonparametric statistical analysis. Both BPA- and diet-groups were treated as fixed factors (Fixed model). Differences between groups were tested for BPA or diet effects using the Bonferroni post test for multiple group comparison (GraphPad, Inc., San Diego, CA) (Appendix I). Differences of $P \leq 0.05$ were considered significant.

4. Results

4.1 General observations

Over a period of 28 days, i.e., PND 70-98, food consumption was similar in all groups (Table 5 and 6). Due to higher energy content of the HFD, however, energy intake and body weight gains were greater in HFD than in NFD animals ($P<0.05$). For example, HFD rats were about 15% heavier than corresponding NFD animals

(Table 6). Overall, food consumed was less in HFD compared to NFD diet groups but energy intake per unit body weight gain (g) was similar in all groups (P>0.05). Body and testes weights are as shown in Table 6.

4.2 Effect of BPA and Diet on Testosterone Production

Perinatal BPA exposure did not affect serum T levels in adulthood but the levels were increased in HFD animals exposed to BPA at the 25 µg/kg bw dose compared to HFD control animals (Fig. 15A) (P<0.05). Although developmental exposure to BPA decreased androgen secretion, maintenance on the HFD had the effect of increasing Leydig cell androgen biosynthesis (Fig. 15B) (P<0.01). However, the increase in androgen secretion due to the HFD was smallest in animals previously exposed to BPA at the 25 µg/kg bw dose (Fig. 15B) possibly due to BPA-diet interaction (P=0.0147,) (Figure 18, Table 7). Differences in androgen secretion by Leydig cells (increase or decrease) were related to changes in expression of HSD17B3 enzyme protein in all groups (Fig. 15C) (P<0.01).

4.3 Effect of BPA and Diet on Adiponectin

Perinatal BPA exposure decreased serum adiponectin levels only in male rats exposed to the 25 µg/kg bw dose compared to control (P<0.01) (Fig. 16A). Although HFD feeding caused a decrease in serum adiponectin levels (P<0.05), this effect was absent in animals previously exposed to 25 µg/kg bw BPA in maternal diet (Fig. 16A) (P>0.05). The decrease in serum adiponectin as a result of perinatal exposure to BPA at 25 µg/kg bw dose was not related to adiponectin protein expression in gonadal adipose tissue, which was similar in NFD animals (Fig. 16B) (P>0.05).

However, HFD feeding alone caused a decrease in adiponectin protein levels in gonadal adipose tissue (Fig. 16B) ($P<0.05$) and to the greatest extent in animals exposed to 25 μg/kg bw BPA in the perinatal period ($P<0.01$) presumably due to interaction between BPA treatment and diet ($p=0.045$) (Figure 18, Table 7).

Expression of the adiponectin protein and its receptors (AdipoR2) was decreased in Leydig cells from adult animals exposed to 25 μg/kg bw BPA in the perinatal period and in the HFD control group (Fig. 17A, B) ($P<0.05$).

Table 5. Food intake and body weight change in adult male rats maintained on NFD or HFD from PND70 to 98 after perinatal BPA exposure

Parameters [a]	Normal fat diet (NFD)			High fat diet (HFD)		
	Dose of BPA (µg/kg bw/day)			Dose of BPA (µg/kg bw/day)		
	0	2.5	25	0	2.5	25
0 week						
Body weights at PND 70	353.1 ± 34.4	338.8 ± 31.4	326.6 ± 33	345.8 ± 41.4	339.6 ± 30.1	338.5 ± 24.9
1st week						
Food consumed per rat (g)/week	165.4 ± 13.7	158.8 ± 11.2	157.9 ± 15	170.1 ± 20	171 ± 16	170.3 ± 4
Energy intake (Kcal)/week	512.7 ± 43	492 ± 35	489 ± 46	765 ± 88*	769.5 ± 71†	766 ± 17‡
Body weight gain (g)	32.9 ± 6.2	31.2 ± 7.9	35.1 ± 7.4	44.6 ± 7.7*	47.6 ± 9.6†	47.7 ± 7.4‡
Food consumed (g)/g bw gain	5.1 ± 0.58	5.2 ± 0.57	4.5 ± 0.44	3.8 ± 0.23*	3.67 ± 0.57†	3.6 ± 0.36‡
Energy intake (Kcal)/g bw gain	15.8 ± 1.8	15.97 ± 1.77	14.1 ± 1.4	17.3 ± 1.04	16.5 ± 2.56	16.3 ± 1.6
Body weights	386 ± 37.3	370 ± 37.6	361.7 ± 37.5	390.4 ± 46.6	387.2 ± 37.1	386.2 ± 23.1
2nd week						
Food consumed per rat (g)/week	168.4 ± 11	161.9 ± 7	158.6 ± 19	170.3 ± 22	167.3 ± 15	166 ± 6
Energy intake (Kcal)/week	522 ± 35	502 ± 22	492 ± 60	766 ± 99*	753 ± 66†	746 ± 28‡
Body weight gain (g)	22.6 ± 4.6	26.5 ± 3.8	21.9 ± 8.8	38.4 ± 9.9*	36.9 ± 6.7†	38.4 ± 4.3‡
Food consumed (g)/g bw gain	7.5 ± 0.57	8.5 ± 1.05	7.6 ± 1.6	4.6 ± 0.7*	4.6 ± 0.3†	4.4 ± 0.34‡
Energy intake (Kcal)/g bw gain	23.3 ± 1.76	26.4 ± 3.3	23.6 ± 5	20.5 ± 3.1	20.5 ± 1.3†	19.6 ± 1.5
Body weights	408.6 ± 41.5	396.5 ± 37.6	383.6 ± 39.5	428.8 ± 52.7	424.1 ± 42.8	424.7 ± 25.2
3rd week						
Food consumed per rat (g)/week	173 ± 11	167.3 ± 6.1	161.5 ±16	166.4 ± 24	166.4 ± 19	162 ± 8.5
Energy intake (Kcal)/week	536 ± 34	519 ± 19	501 ± 48	749 ± 109*	749 ± 84†	727 ± 38‡
Body weight gain (g)	25.6 ± 5.34	24 ± 2.8	24.4 ± 9.1	33.7 ± 8.2*	33.4 ± 7.8†	33.3 ± 7‡
Food consumed (g)/g bw gain	6.9 ± 0.9	7 ± 0.7	6.7 ± 0.76	5 ± 0.36*	5.1 ± 0.7†	4.96 ± 0.9‡
Energy intake (Kcal)/g bw gain	21.3 ± 2.8	21.7 ± 2.2	20.8 ± 2.4	22.5 ± 1.6	22.9 ± 3.1	22.4 ± 3.9
Body weights	434.2 ± 44.7	420.5 ± 37.6	408 ± 42.8	462.5 ± 60.5	457.5 ± 48.7	458 ±23.1
4th week						
Food consumed per rat (g)/week	171.8 ± 11	164.6 ± 5	157.25 ± 12	158.8 ± 24	159.9 ± 17	159.2 ± 15
Energy intake (Kcal)/week	533 ± 33	510.3 ± 17	487 ± 36	714 ± 105*	720 ± 74†	716 ± 65‡
Body weight gain (g)	23.5 ± 7.1	22 ± 3.7	18.1 ± 4.5	28.6 ± 6.1	28.2 ± 7.7	28.9 ± 5.1‡
Food consumed (g)/g bw gain	7.4 ± 0.7	7.6 ± 0.9	11.5 ± 2	5.6 ± 0.5*	5.9 ± 1	5.8 ± 0.8‡
Energy intake (Kcal)/g bw gain	22.9 ± 2.1	23.5 ± 2.8	27.8 ± 6.3	25.2 ± 2.4	26.3 ± 4.6	26.2 ± 3.7
Body weights at PND 98	457.7 ± 50	442.5 ± 40	426.1 ± 43	491.1 ± 65	485.7 ± 54	487 ± 26‡

[a] data collected from 8-10 animals per group

* $P < 0.05$ compared to age-matched control animals maintained on NFD

† $P < 0.05$ compared to age-matched BPA-treated animals (2.5 µg) maintained on NFD

‡ $P < 0.05$ compared to age-matched BPA-treated animals (25 µg) maintained on NFD

BPA exposure = gestational day 12 to postnatal day 21 (perinatal period) and animals were maintained on NFD or HFD from PND 71 to PND 98

Table 6. Cumulative increase in body weight gains, food consumption and energy intake, and final body weights and testes weights of male rats maintained on normal and high fat diet

Parameters [a]	Normal fat diet (NFD)			High fat diet (HFD)		
	Dose of BPA (µg/kg bw/day)			Dose of BPA (µg/kg bw/day)		
	0	2.5	25	0	2.5	25
Cumulative (4 weeks)						
Food consumed (g) per rat	678.6 ± 45	652.6 ± 30	635.3 ± 65	665.6 ±93	664.6 ± 68	656.7 ± 32.3
Energy Intake (Kcal) per rat	2103 ± 140	2023 ± 92	1969 ± 202	2995 ± 418*	2990 ± 305†	2955 ± 145‡
Body weight gain per rat	104.6 ± 18.5	103.7 ± 13.3	99.5 ± 14.4	145.3 ± 29*	146.1 ± 29†	150.1 ± 14‡
Food consumed (g)/g bw gain	6.53 ± 0.5	6.31 ± 0.5	6.42 ± 0.5	4.6 ± 0.4*	4.6 ± 0.51†	4.4 ± 0.3‡
Energy intake (Kcal)/g bw gain	10.13 ± 0.72	9.79 ± 0.74	9.96 ± 0.72	10.42 ± 0.82	10.4 ± 1.14	12.14 ± 4.7
At the time of sacrifice						
Body weights (g)	448.9 ± 46	446.4 ± 20	419 ± 4	479.9 ± 65	477.8 ± 51	480.3 ± 26‡
Paired testes weight (g)	3.44 ± 0.31	3.42 ± 0.13	3.56 ± 0.22	3.45 ± 0.23	3.53 ± 0.39	3.46 ± 0.31
Relative testes weight ($\times 10^{-3}$)	7.7 ± 0.56	7.7 ± 0.47	8.6 ± 0.75	7.3 ± 0.84	7.5 ± 0.10	7.2 ± 0.53

[a] data collected from 8-10 animals per group

* $P < 0.05$ compared to age-matched control animals maintained on NFD

† $P < 0.05$ compared to age-matched BPA-treated animals (2.5 µg) maintained on NFD

‡ $P < 0.05$ compared to age-matched BPA-treated animals (25 µg) maintained on NFD

BPA exposure = gestational day 12 to postnatal day 21 (perinatal period) and animals were maintained on NFD or HFD from PND 71 to PND 98

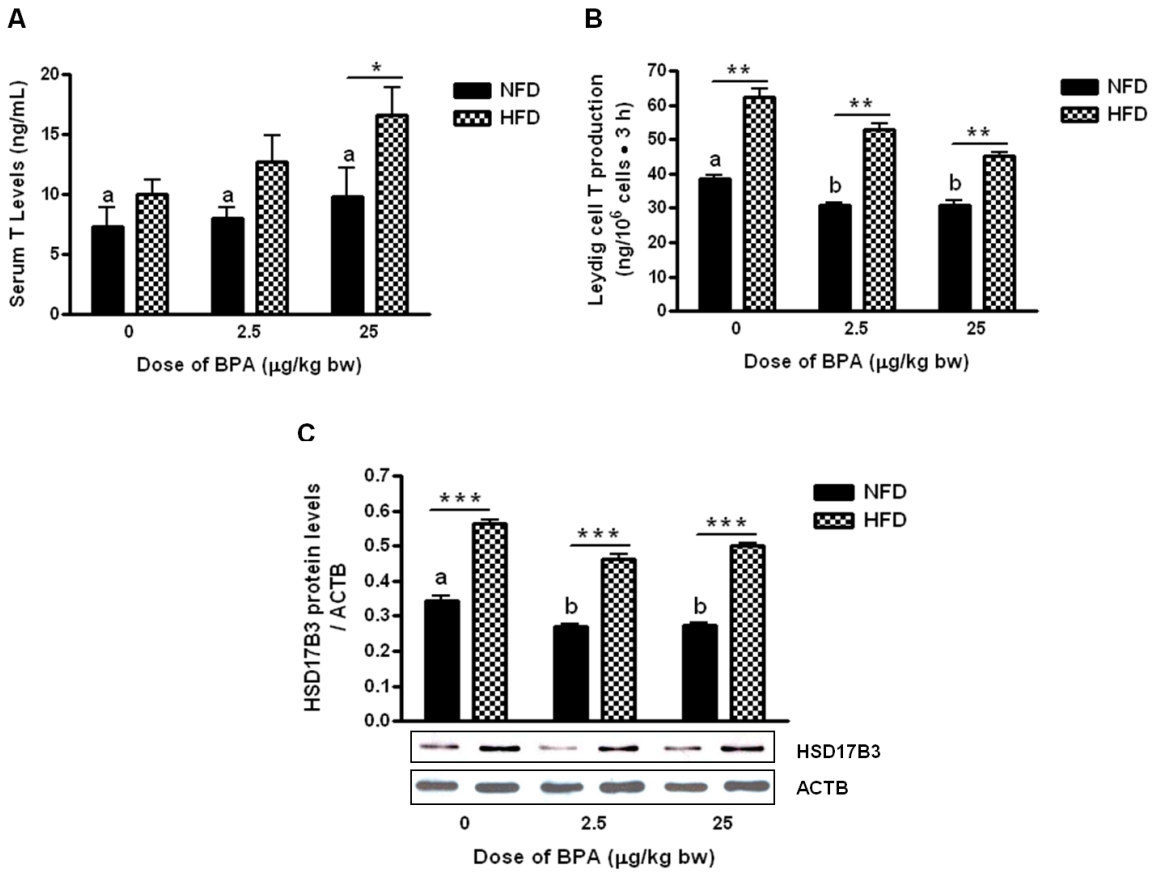

Figure 15. Effect of BPA and diet on steroid hormone secretion. Serum was obtained from blood collected at sacrifice (A), whereas Leydig cells were isolated from testes (B and C) (n=8-10). Leydig cells were incubated in triplicate in DMEM/F12 culture medium for 3 h for analysis of testosterone (T) secretion capacity (B). The concentrations of T in serum and aliquots of spent media were assayed by RIA (B). Protein levels for 17β-HSD3 were analyzed in Western blots of Leydig cells using specific anti-17β-HSD3 antibody and the appropriate secondary antibody (C). The procedures were repeated at least four times, and protein levels were normalized to β-actin (ACTB). BPA exposure = gestational day 12 to postnatal day 21 (perinatal period). Animals were maintained on normal or high fat diet (NFD, HFD) from PND 71 to PND 98. 17beta-hydroxysteroid dehydrogenase 3 (HSD17B3) = 35 kDa, ACTB = 42 kDa. *P < 0.05, **P < 0.01, ***P < 0.001, b (P < 0.05). a, b = BPA effect, * = diet effect.

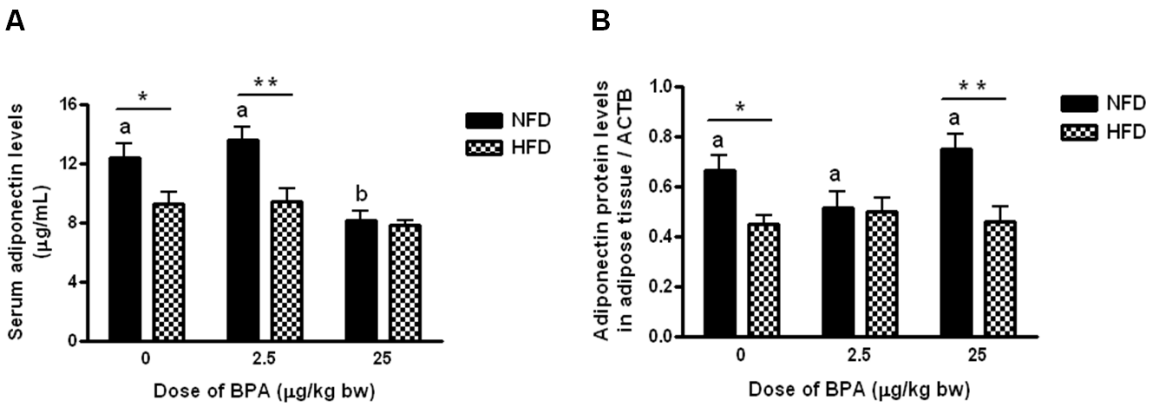

Figure 16. Effect of BPA and diet on adiponectin secretion. Serum and gonadal fat tissue were obtained at the time of sacrifice. Serum adiponectin levels were measured using an adiponectin ELISA kit. Adiponectin proteins levels were analyzed in western blots of gonadal fat tissue using anti-adiponectin antibody and the appropriate secondary antibody. Protein levels were normalized to β-actin (ACTB). BPA exposure = gestational day 12 to postnatal day 21 (perinatal period). Animals were maintained on normal or high fat diet (NFD, HFD) from PND 71 to PND 98. Adiponectin = 30 kDa, ACTB = 42 kDa. *P < 0.05, **P < 0.01, b (P < 0.05). a, b = BPA effect, * = diet effect.

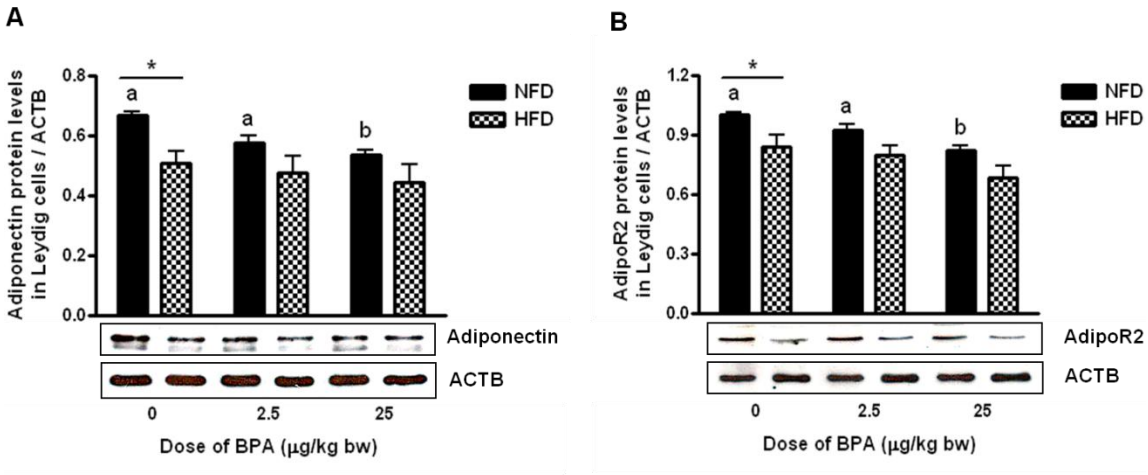

Figure 17. Effect of BPA and diet on adiponectin and adipoR2 protein expression in Leydig cells. Protein levels for adiponectin (A) and adipoR2 (B) were analyzed in Western blots of Leydig cells using specific anti-adiponectin and anti-adipoR2 antibodies and the appropriate secondary antibodies. The procedures were repeated at least four times, and protein levels were normalized to β-actin (ACTB). BPA exposure = gestational day 12 to postnatal day 21 (perinatal period). Animals were maintained on normal or high fat diet (NFD, HFD) from PND 71 to PND 98. Adiponectin = 30 kDa, AdipoR2 = 42 kDa, ACTB = 42 kDa. *P < 0.05, b (P < 0.05). a, b = BPA effect, * = diet effect.

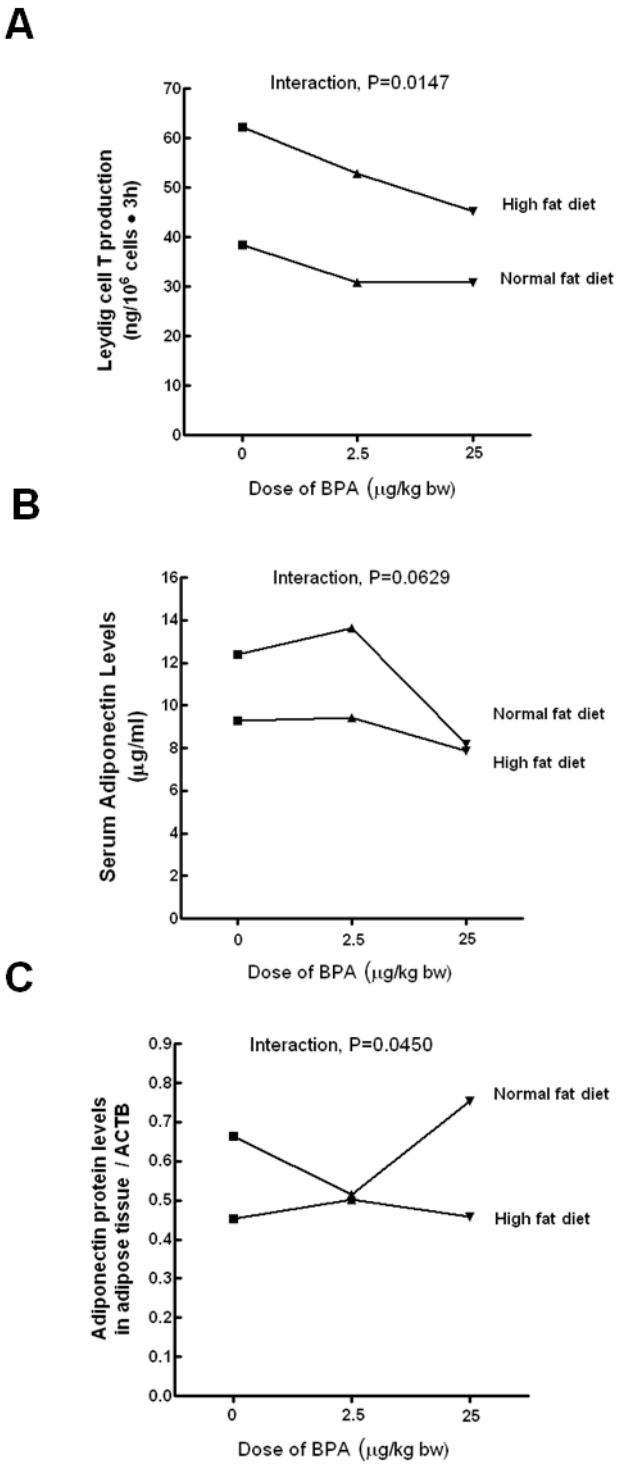

Figure 18. Plots representing interactive effect of BPA and diet on Leydig cell T production, serum adiponectin levels and adiponectin protein in adipose tissue.

Table 7. Interactive effect of BPA exposure and diet on testosterone and adiponectin section

Parameters	Interaction (BPA and Diet)	p-value
Serum testosterone (T) levels	No	0.5290
Leydig cell testosterone (T) production	Yes	0.0147
Leydig cell HSD17B3 protein levels	No	0.1985
Serum adiponectin levels	No	0.0629
Adipose tissue adiponectin protein levels	Yes	0.0450
Leydig cell adiponectin protein levels	No	0.6357
Leydig cell adipoR2 protein levels	No	0.9129

5. Discussion

The present study demonstrated that exposures to BPA during development may cause metabolic changes affecting adiponectin secretion by adipose tissue. Although BPA did not affect body weight, exposure of male rats to BPA decreased serum adiponectin levels and Leydig cell T production. In addition, BPA-exposure in the perinatal period decreased adiponectin and adipoR2 protein levels in Leydig cells. Paradoxically, maintenance on HFD enhanced androgen secretion and decreased adiponectin and adipoR2 expression in Leydig cells.

A previous study showed that perinatal exposure of male rats to BPA at 50 μg/kg/day increased body weights at 18 weeks of age (Wei et al., 2011). Animals fed on HFD gained more body weight than animal fed NFD due to greater energy intake (table 6). Although animals on HFD consumed same of amount of food, energy intake was greater due to high energy content of the HFD (4.5 Kcal/g)

compared to NFD (3.1 Kcal/g), which decreased food consumption (g) per unit body weight gain in HFD animals (table 6).

The higher levels of serum T in HFD-fed animals compared to the NFD group were associated with increased steroidogenic enzyme protein, HSD17B3, in Leydig cells. Previous observations have shown that consumption of diets containing fatty acids and cholesterol stimulate Leydig cell androgen biosynthesis. For example, decreased serum T levels were observed in men after consumption of a low-fat, high-fiber diet (Hämäläinen et al., 1983), whereas a high-fat, low-fiber diet increased serum T levels (Dorgan et al., 1996). In other reports, feeding of saturated fatty acids or a cholesterol-rich diet to weanling male rats for 35 days increased Leydig cell T secretion (Sebokova et al., 1988; Sebokova et al., 1990). It has been suggested that HFDs modulate Leydig cell sensitivity to LH stimulation by changing plasma membrane lipid content, which enhances LH/LHR binding (Sebokova et al., 1988; Sebokova et al., 1990). Furthermore, HFDs may increase steroid hormone secretion by providing more cholesterol, the steroid precursor required for steroidogenesis (Sebokova et al., 1988; Sebokova et al., 1990). On the other hand, non-esterified fatty acids were found to inhibit T secretion from Leydig cells (Meikle et al., 1989), and polyunsaturated fatty acids such as arachidoninic acid decrease HSD17B enzyme activity (Marinero et al., 1998). It appears that the HSD17B enzyme is the prime target of fatty acids for up-regulation of steroidogenesis. Thus, it is possible that enhanced Leydig cell androgen secretion is due to differences in the polyunsaturated fatty acids (PUFAs) content of the diets used in the present study (Table 4).

Exposure to BPA decreased serum adiponectin levels without affecting its protein expression in gonadal adipose tissue. The discrepancy in serum and gonadal adiponectin protein levels might be the result of defective secretion mechanisms (Wang et al., 2007). Before secretion from adipose tissue, adiponectin is organized to form oligomers. For example, formation of inter- and intra-molecular disulfide bonds in endoplasmic reticulum (ER) is essential for adiponectin secretion from adipocytes. Several oxidoreductases inside the ER catalyze disulfide bond formation between adiponectin molecules e.g., protein disulfide isomerase (PDI). However, PDI function depends on the proteins ERp44 and Ero1-Lα, which regulate adiponectin secretion (Wang et al., 2007). Interestingly, BPA is known to bind and inhibit PDI enzyme activity (Hiroi et al., 2006). Overexpression of ERp44 or down-regulation of Ero1-Lα was found to cause increased retention of adiponectin in the cell (Wang et al., 2007; Wolf, 2008). Thus, it is possible that dysregulation of these proteins by perinatal BPA exposure contributes to decreased adiponectin secretion. However, HFD feeding abolished BPA effects on adiponectin by decreasing its levels in both serum and gonadal adipose tissue probably due to changes in adiponectin gene transcription and translation. Decreased adiponectin levels in HFD-fed animals might be due to higher levels of serum T levels because T suppressed adiponectin secretion from white adipose tissue (Lanfranco et al., 2004; Nishizawa et al., 2002; Xu et al., 2005), and AR knockout male mice show increased adiponectin levels (Yanase et al., 2008).

The present data are interesting because adiponectin levels are decreased in obese human subjects in comparison with lean controls and are normalized after

weight loss (Lara-Castro et al., 2006). Also, adiponectin has insulin sensitizing property and is thought to reduce body weight by increasing energy expenditure rather than decreasing food intake (Brooks et al., 2007). Therefore, the present finding is aligned with the view that exposure to BPA along with HFD consumption may contribute to the pathogenesis of metabolic syndromes (Ben-Jonathan et al., 2009). Furthermore, adiponectin circulates in three forms, LMW, MMW, and HMW in the blood at high concentrations (microgram levels). Of the three, HMW adiponectin is more metabolically active and is down-regulated in individuals with obesity and metabolic syndromes (Lara-Castro et al., 2006). However, in this study, we measured total adiponectin and it remains to be determined which form of adiponectin is decreased in the serum of both BPA and HFD-fed animals.

Previous reports have demonstrated that adipoR1 is localized to the seminiferous epithelium, whereas AdipoR2 and adiponectin are expressed in interstitial Leydig cells providing a link between metabolic homeostasis and testis function (Bjursell et al., 2007; Caminos et al., 2008). Indeed, AdipoR2 knockout mice showed infertility, reduced testes weights, atrophy of the seminiferous tubules and aspermia suggesting that AdipoR2 regulates male reproduction (Bjursell et al., 2007). In the present study, BPA exposure and HFD feeding decreased Leydig cell adiponectin and AdipoR2 expression, which may contribute to testis dysfunction observed in metabolic syndromes. However, previous reports have indicated that adiponectin inhibits both basal and LH-stimulated T secretion by Leydig cells (Caminos et al., 2008; Pfaehler et al., 2012). Altogether, these findings are relevant

to public health because both AdipoR1 and AdipoR2 are expressed in the human testis (Civitarese et al., 2004).

In conclusion, the present results indicate that perinatal BPA exposure may affect adiponectin secretion and endocrine function of the testis. In addition, the data demonstrated that HFD consumption in concert with BPA exposure can alter metabolic homeostasis. Furthermore, adiponectin possibly mediate the effects of energy balance on male reproduction.

CHAPTER 4

EFFECTS OF BPA AND DIET ON ESTROGEN METABOLISM AND ANTIOXIDANT CAPACITY IN LEYDIG CELLS

1. Abstract

There is concern that exposure to environmental chemicals may adversely affect reproductive health. Objectives of the present study were to determine if exposure to the industrial chemical BPA and/or consumption of high energy diets increase tissue estrogen burden and induce oxidative stress in Leydig cells. Long-Evans male rats were exposed to BPA by maternal gavage at 0, 2.5 and 25 μg/kg body weight (bw) from gestational day 12 to postnatal day (PND) 21 and were fed a BPA-free diet from weaning to PND 70. Subsequently, animals from control and BPA-treated groups were maintained for a period of 28 days (PND 71 to 98) on a normal or high fat diet (NFD or HFD, Harlan-Teklad, Indianapolis, IN) with 16% or 42% of energy content derived from fat. Animals were sacrificed between days 98 and 100 postpartum when serum and Leydig cells were obtained to assess estrogen metabolism and oxidative stress. Results showed that all HFD male rats exhibited a decrease in serum 17β-estradiol levels ($P<0.05$). However, expression of the estrogen sulfotransferase (EST) enzyme protein in gonadal adipose tissue and Leydig cells was increased by exposure to 25 μg/kg bw BPA, whereas the effect of

BPA on EST protein was reversed in adipose tissue by HFD feeding but was enhanced in Leydig cells. The serum levels of thiobarbituric acid reactive substance (TBARS), a marker of lipid peroxidation and oxidative stress, were greater only in male rats exposed to BPA and maintained on HFD compared to control animals ($P<0.01$). Furthermore, exposure to BPA at 25 μg/kg bw or maintenance on HFD induced oxidative stress in Leydig cells as indicated by increased generation of reactive oxygen species (ROS) compared to the NFD diet group ($P<0.05$). Interestingly, antioxidant enzymes were differentially affected by exposure to BPA and HFD feeding. For example, the superoxide dismutase (SOD) activity was decreased in Leydig cells after HFD feeding, whereas catalase activity was up-regulated both by BPA exposure and the HFD and BPA exposure (25 μg/kg bw) or HFD feeding increased glutathione peroxidase (GPx) activity. The results of *in vitro* experiments showed that incubation of Leydig cells with BPA (10 nM, 18 h) increased ROS production, whereas 0.01 nM BPA, but not the 10 nM concentration, decreased antioxidant enzyme (SOD and GPx) activity and GSH content compared to control ($P<0.05$). Collectively, results indicate that BPA exposure and/or maintenance on HFD induce oxidative stress in Leydig cells. Therefore, BPA disruption of Leydig cell T secretion may be due, in part, to increased ROS levels. Increased ROS production plays a role in the process of Leydig cell aging.

2. Introduction

BPA is used extensively in the manufacture of many consumer products such as polycarbonate plastic bottles and in epoxy resin coating for canned foods and

beverages. BPA has been measured in urine of > 90% of a reference population in the United States. Children between the ages of 6-12 have higher plasma levels of BPA compared to adults (Calafat et al., 2008; Crinnion, 2010). Recent epidemiological studies have shown that higher urinary BPA levels are associated with diabetes mellitus (Lang et al., 2008; Shankar and Teppala, 2011), cardiovascular disease (Lang et al., 2008) and hyperlipidemia (Miyawaki et al., 2007). BPA exposure has also been linked to increased serum markers of inflammation and oxidative stress (Franco and Panayiotidis, 2009; Yang et al., 2009).

The natural estrogen 17β-estradiol (E_2) regulates anti-oxidant capacity in several tissues (Manolagas, 2010). For example, E_2 is known to protect brain neurons and pancreatic β-cells against oxidative stress and apoptosis (Green and Simpkins, 2000; Liu and Mauvais-Jarvis, 2010). The bioactivity of estrogens is regulated in part by EST, which deactivates estrogens by sulfoconjugation (Leiter and Chapman, 1994). EST is highly expressed in white adipose tissue and in Leydig cells (Khor et al., 2008; Tong and Song, 2002). Interestingly, male mice lacking the EST enzyme showed increased epididymal fat accumulation and hypertrophy of adipocytes suggesting that EST has a role in adipose tissue function (Khor et al., 2008). Also, over-expression of EST was found to suppress 3T3-L1 adipocyte differentiation, whereas EST deficiency enhances differentiation of pre-adipocytes (Wada et al., 2011). There is evidence that BPA exerts an inhibitory effect on EST enzyme activity (Kester et al., 2002). Thus, it is possible that by inhibiting the EST enzyme, perinatal BPA exposure affects adipogenesis and adipose tissue function

(Kester et al., 2002). Because mice lacking the EST enzyme exhibit Leydig cell hyperplasia and hypertrophy, disrupted steroidogenesis and reduced fertility (Qian et al., 2001; Tong et al., 2004), it is likely that EST protects Leydig cells from undue exposure to estrogens and provides protection against oxidative stress.

Oxidative stress plays a role in the pathogenesis of many diseases, including diabetes and obesity (Evans et al., 2002; Simmons et al., 2005; Spector, 2000), potentiates effects of chemical toxicity (Ghanayem et al., 2010) and accelerates cellular senescence and aging (Barnes and Ozanne, 2011). Previous studies demonstrated that perinatal exposures to BPA caused oxidative stress in testes and brain of prepubertal male mice (Kabuto et al., 2004) and BPA may act as a mitochondrial toxin by interfering with mitochondrial respiration (Nakagawa and Tayama, 2000). In addition, a BPA metabolite, bisphenol A 3,4-quinone, was found to have greater capacity to induce ROS production than the parent compound by conversion of xanthine dehydrogenase to its highly ROS producing form, xanthine oxidase (Sakuma et al., 2010). These observations suggest that BPA has the capacity to induce ROS generation in the testis, which may impact Leydig cell steroidogenesis.

Related to the present study, high fat diet consumption is known to induce oxidative stress in the body (Milagro et al., 2006), and has been shown to affect fertility in laboratory species (Bakos et al., 2011b) and humans (Bakos et al., 2011a). In fact, oxidative damage due to ROS is considered to be a major contributing factor to infertility associated with diet-induced obesity (Pasquali et al., 2007). Moreover,

male rats exposed to BPA in the perinatal period showed decreased serum adiponectin levels and expression levels in Leydig cells (Chapter 3). Adiponectin has been shown to possess antioxidant activity and protects against oxidative damage due to free radicals in endothelial cells and in hepatic and cardiac tissues (Fukushima et al., 2009; Kamada et al., 2007; Motoshima et al., 2004; Ouedraogo et al., 2006; Tao et al., 2007). Thus, deficiency in adiponectin signaling after perinatal BPA exposure may contribute to increased ROS generation. Both hyperestrogenemia and increased oxidative stress levels have been reported in obese patients (Stefanović et al., 2008).

The present study was designed to investigate whether developmental BPA exposure alters estrogen metabolism and oxidative stress levels in Leydig cells and if this effect is influenced by HFD feeding in sexually mature male rats.

3. Material and Methods

3.1 Animal Studies

BPA exposure and diets are as described in Chapter 3. Briefly, Long Evans male rats were exposed to BPA by maternal gavage at 0, 2.5 and 25 μg/kg body weight from gestational day 12 to postnatal day (PND) 21 and were fed a BPA-free diet from weaning to PND 70. Subsequently, animals from each group were maintained for a period of 28 days (PND 71 to 98) on a normal fat diet (NFD, 2020X) or high fat diet (HFD, TD.88137) obtained from Harlan-Teklad (Indianapolis, IN). The energy derived from fat in the NFD and HFD was 16% and 42%, respectively (Table 4). Animals were sacrificed within 48 h of day 98 postpartum when serum,

gonadal fat tissue and Leydig cells were obtained to assess estrogen metabolism, oxidative stress and antioxidant enzyme capacity.

3.2 Western Blotting Procedures

Leydig cells from *in vivo* experiments were lysed in protein extraction buffer containing protease inhibitors and EDTA. Supernatant was collected after centrifugation at 12,000 RPM and protein concentrations were determined using the Bio-Rad Bradford assay. Samples were kept at -80^0 C until they were used for analysis of oxidative stress parameters and protein expression by Western blotting. Protein lysates from gonadal fat tissue and Leydig cells were analyzed for EST protein expression by immunoblotting using a specific primary antibody (Abcam, Cambridge, MA.) and the appropriate secondary antibody (Santa Cruz Biotech. Inc. CA.).

3.3 *In vitro* studies

The isolation and culture of adult Leydig cells has been described in Chapter 2. Adult Leydig cells were isolated from BPA-free 90 day-old adult Long-Evans male rats and cultured in DMEM/F12 media containing ovine LH (10 ng/mL) and BPA (0, 0.01 and 10 nM) or DES (10 nM) for 18 h. Spent media was collected to measure T production by RIA. In addition, Leydig cells were lysed in protein extraction buffer containing protease inhibitors and EDTA. The supernatant was collected after centrifugation and was used for analysis of oxidative stress parameters.

3.4 Lipid Peroxidation Assay

Lipid peroxidation was measured using the thiobarbituric acid reactive substances (TBARS) assay. The assay measures the levels of malondialdehyde (MDA), a byproduct of lipid peroxidation, which reacts with thiobarbituric acid (TBA) and produces a pink color that can be measured spectrophotometrically. Briefly, 100 µL of serum was mixed with 100 µL of trichloroacetic acid (TCA, 10g/50mL H_2O) and incubated for 15 min on ice. The mixture was then mixed with 400 µL of TBA (50 mg/10 mL H_2O) and heated at 80°C for 30 minutes. The supernatant was collected after centrifugation at 14,000 RPM and the absorbance was read in triplicate at the 532 nm wavelength in a microplate reader (Synergy™ HT, BioTek Instruments, Incorporated, VT, USA). The relative levels of TBARS are expressed as percentage of control.

3.5 Estimation of Reactive Oxygen Species (ROS)

The dichlorodihydrofluorescein diacetate (DCF-DA) assay is a reliable and efficient method to quantify oxidative stress because this compound is able to react with several free radicals (Wang and Joseph, 1999). After reacting with ROS, DCF-DA is oxidized to form a fluorescent product, which can be measured spectrophotometrically (Synergy™ HT, BioTek Instruments, Incorporated, VT, USA). Leydig cell lysates (30 µL) were dissolved in 160 µL of 0.01 mol phosphate buffer (pH 7.4) and 10 µL of DCF-DA (5 µg/mL in ethanol). The mixture was then incubated at room temperature for 1 h prior to measuring the fluorescence product in luminescence spectrometer at an excitation wavelength of 460 nm and an

emission wavelength of 528 nm. PBS was used as blank and values were subtracted from test samples. DCF-DA fluorescence was normalized to mg of protein and expressed as percentage of control.

3.6 Measurement of Total Superoxide Dismutase (SOD) Activity

Total SOD enzyme activity in Leydig cells was assessed by the method described by Marklund and Marklund, 1974. Leydig cell lysates (20 µL) were mixed in 780 µL of tris buffer (50 mM, pH 8.2) followed by measurement of absorbance in a spectrophotometer. Then, 200 µL of freshly prepared pyrogallol solution (2 mM, 2.5 mg/10 mL 0.01N HCl) was added and its oxidation was immediately measured at 420 nm absorbance every 30 seconds for 3 minutes in a Shimadzu UV/visible spectrophotometer (Ramsey, MN, USA). Total SOD activity was expressed as percentage inhibition of pyrogallol auto-oxidation and normalized to unit (mg) protein.

3.7 Measurement of Catalase Activity

Catalase enzyme activity in Leydig cells was measured according to the protocol described previously by Beers and Sizer, 1952. Briefly, Leydig cell lysates (40 µL) were dissolved in a solution containing 760 µL of 0.01 mol phosphate buffer (pH 7.4) and 260 µL of 30 mM H_2O_2. The degradation of H_2O_2 was monitored by Shimadzu UV/visible spectrophotometer (Ramsey, MN, USA) reading of the mixture taken at 240 nm for every 30 seconds for 2 minutes. The relative enzyme activity/mg protein was expressed as percentage of control.

3.8 Measurement of Glutathione Peroxidase (GPx) Activity

To measure GPx activity, Leydig cell lysates (50 µL) were added to a mixture of 650 µL of 0.01 mol phosphate buffer (pH 7.4), 10 µL of glutathione reductase (2.4 U/mL), 100µL of GSH (10 mM) and 100 µL of t-butyl peroxide (1.5 mM). NADPH (100 µL, 1.5 mM) was added to the solution and consumption of NADPH was monitored at 340 nm in a Shimadzu UV/visible spectrophotometer (Ramsey, MN, USA) for 3 minutes at 30-second intervals. GPx activity was normalized to mg of protein concentration and expressed as percentage of control.

3.9 Estimation of Reduced Glutathione (GSH) Levels

GSH levels were measured in both serum and Leydig cells. Protein samples (100 µL) were precipitated by mixing in a solution of 0.1M phosphoric acid (10 µL) followed by centrifugation at 14,000 RPM. The supernatant was collected in new tubes and were processed immediately or stored at -20° C until required. GSH levels in the supernatant were measured after incubation of 10 µL of the processed sample in 180 µL of 0.01 M PBS (pH 7.4) and 10 µL of o-phthalaldehyde (0.1% in methanol) solution for 20 minutes at room temperature. Fluorescence was read in a fluorescence spectrometer (Synergy™ HT, BioTek Instruments, Incorporated, VT, USA) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The GSH content was calculated using a concurrently run GSH standard curve.

3.10 Statistical Analysis

Parameters are described as mean ± SEM. Adult male rats at 98 and 100 days of age that were perinatally exposed to BPA were randomly selected from each group for analysis (n = 8–10). Experiments *in vitro* were performed using cell preparations obtained on three different occasions. Each isolation procedure used testes from eight 90 day-old BPA-free male Long–Evans rats. Data from animal studies were analyzed by a two-way ANOVA with nonparametric statistical analysis (Fixed model). Both BPA- and diet-groups were treated as fixed factors. Differences between groups were tested for BPA or diet effects using the Bonferroni post test for multiple group comparison. When there were only two groups, unpaired t-test was used for data analysis (GraphPad, Inc., San Diego, CA) (Appendix I). Differences of $P \leq 0.05$ were considered significant.

4. RESULTS

4.1 General Observations

Food intake and body weight changes were as described in Chapter 3. Briefly, the amounts of food consumed over a period of 28 days (PND 70-98) were similar in all groups (Table 6). However, energy intake was greater in HFD than in NFD groups, which was translated to greater body weight gains ($P<0.05$). Therefore, food consumed by HFD animals was decreased but energy intake per unit body weight gain (g) was similar to NFD groups ($P>0.05$) (Tables 5 and 6).

4.2 Effect of BPA and Diet on Estrogen Metabolism

Developmental exposure to BPA had no effect (P>0.05) but HFD feeding decreased serum E_2 concentrations in all groups (Fig. 19A; P<0.05). Whereas ESR1 and ESR2 protein levels were not affected by BPA exposure or HFD feeding (Fig. 19B, C; P>0.05), expression of the EST enzyme protein in gonadal adipose tissue was increased after perinatal exposure to 25 μg/kg bw BPA compared to control (Fig. 19D; P<0.01). BPA-induced increase in EST protein was, however, reversed by HFD feeding (p=0.005) (figure 24, table 8). Expression of the ESR1 protein was increased only in Leydig cells from adult male rats exposed to 25 μg/kg bw BPA in the perinatal period compared to control (Fig. 20A; P<0.01), whereas both BPA doses (2.5 and 25 μg/kg bw) had a similar effect on EST protein expression (Fig. 20B; P<0.05). HFD feeding was found to up-regulate ESR1 protein levels in Leydig cells (Fig. 20A; P<0.001) but attenuated BPA-induced increases in EST protein (Fig. 20B; P < 0.01) (P=0.0002) (figure 24, table 8). Changes in ESR1-mediated activity and endogenous estrogen levels affect Leydig cells because treatment of freshly isolated Leydig cells from BPA-free adult male rats with the prototype estrogen DES (10 nM, 18h) decreased androgen secretion (Fig. 20C; P<0.05).

4.3 Effect of BPA and Diet on Oxidative Stress and Antioxidant Capacity *In Vivo*

Developmental exposure to BPA had no effect on lipid peroxidation as indicated by serum TBARS and GSH concentrations (Fig. 21A, B; P>0.05). Similarly, HFD feeding did not affect serum TBARS and GSH levels except in HFD animals exposed to 25 μg/kg bw BPA in the perinatal period (Fig. 21A; P=0.0042) (figure 24,

table 8). Perinatal exposure to 25 μg/kg bw ($P<0.05$), but not the 2.5 μg/kg bw dose ($P<0.05$), and HFD feeding ($P<0.01$) increased ROS production in adult Leydig cells compared to control (Fig. 22A). Moreover, HFD feeding, but not BPA treatment, caused a decrease in total SOD enzyme activity (Fig. 22B; $P<0.01$). Compared to control, catalase activity in Leydig cells was increased by perinatal BPA exposure (Fig. 22C; $P<0.01$), whereas GPx activity was increased by BPA only at the 25 μg/kg bw dose (Fig. 22D; $P<0.05$). However, HFD feeding had the effect of increasing both enzyme (catalase and GPx) activities although the effect of HFD feeding on catalase activity was attenuated in BPA-exposed animals compared to HFD controls ($P=0.0045$) (figure 24, table 8). Compared to control, perinatal exposure to 25 μg/kg bw BPA ($P<0.05$) or HFD feeding ($P<0.01$) increased GSH levels in adult Leydig cells (Fig. 22E).

4.4 Effect of BPA and Diet on Oxidative Stress and Antioxidant Capacity in Leydig Cells *In Vitro*

BPA treatment of Leydig cells for 18 h at 10 nM, but not 0.01 nM, increased ROS production compared to control (Fig. 23A, $P<0.01$). In contrast, 0.01 nM BPA, but not the 10 nM concentration, decreased SOD and GPx activities (Fig. 23B; 23C; $P<0.05$) while reducing GSH levels in Leydig cells (Fig. 23D; $P<0.01$).

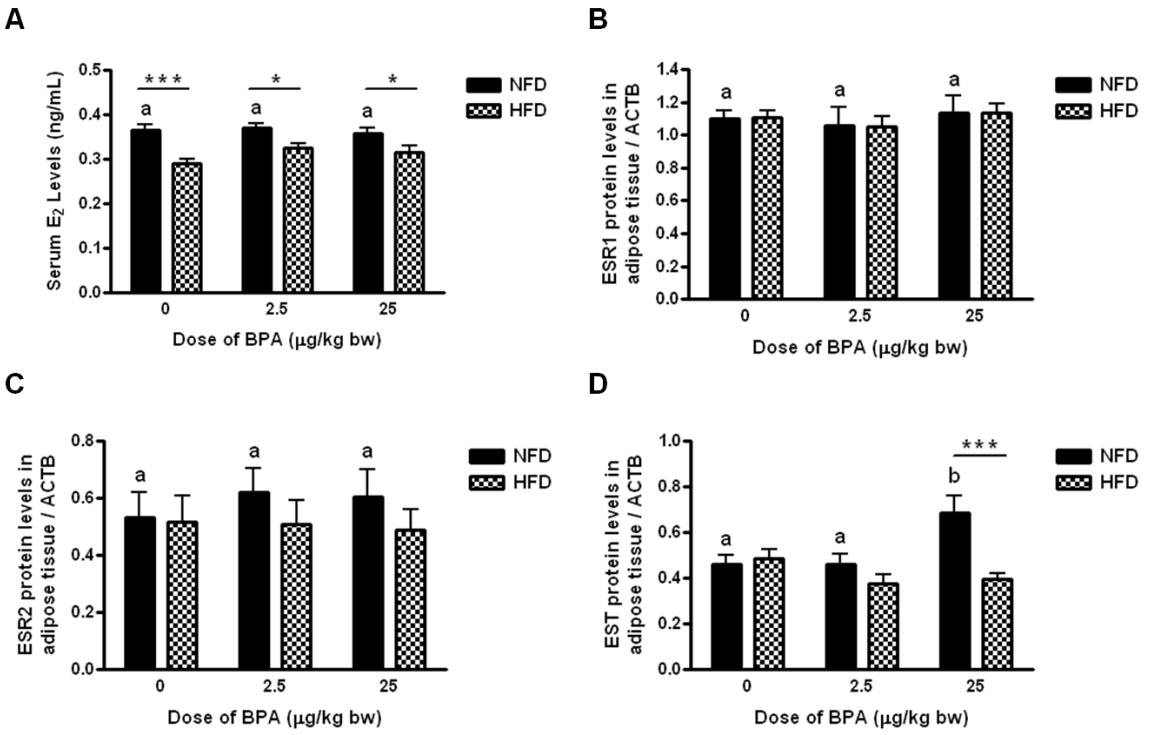

Figure 19. Effect of BPA and diet on serum E_2 and gonadal adipose tissue ESR and EST protein expression. 17β-Estradiol (E_2) levels were measured in duplicate by RIA after obtaining serum from blood collected at the time of sacrifice (A). Protein levels of ESR1 (B), ESR2 (C) and the EST (D) enzyme were analyzed in Western blots of gonadal fat tissue using specific anti-ESR1, -ESR2 and -EST antibodies and the appropriate secondary antibodies. The procedures were repeated at least four times, and protein levels were normalized to β-actin (ACTB). BPA exposure = gestational day 12 to postnatal day 21 (perinatal period). Animals were maintained on normal or high fat diet (NFD, HFD) from PND 71 to PND 98. Estrogen receptor 1 (ESR1) = 68 kDa, Estrogen receptor 2 (ESR2) = 56 kDa, Estrogen sulfotransferase (EST) = 31 kDa, ACTB = 42 kDa. *P < 0.05, **P < 0.01, ***P < 0.001, b (P < 0.01). a, b = BPA effect, * = diet effect.

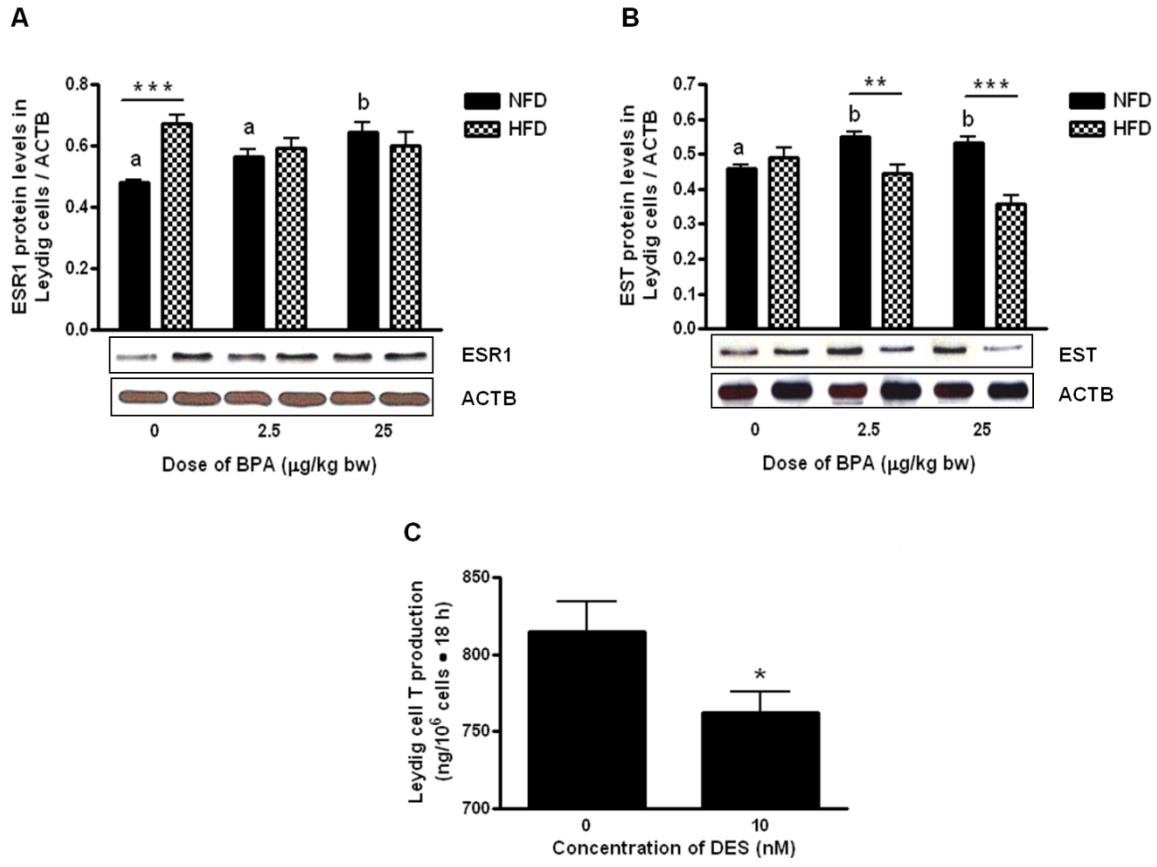

Figure 20. Effect of BPA and diet on ESR1 protein expression and estrogen metabolism in Leydig cells. ESR1 (A) and EST (B) enzyme protein were analyzed in Western blots of Leydig cells using specific anti-ESR1 and anti-EST antibodies and the appropriate secondary antibodies. The procedures were repeated at least four times, and protein levels were normalized to β-actin (ACTB). In separate experiments, Leydig cells, isolated from BPA-free adult male rats, were incubated with diethylstilbestrol (DES, 10 nM) for 18 h and testosterone (T) secretion was analyzed in aliquots of spent media by RIA (C). BPA exposure = gestational day 12 to postnatal day 21 (perinatal period). Animals were maintained on normal or high fat diet (NFD, HFD) from PND 71 to PND 98. Estrogen receptor 1 (ESR1) = 68 kDa, Estrogen sulfotransferase (EST) = 31 kDa, ACTB = 42 kDa. *P < 0.05, **P < 0.01, ***P < 0.001, b (P < 0.05). a, b = BPA effect, * = diet effect.

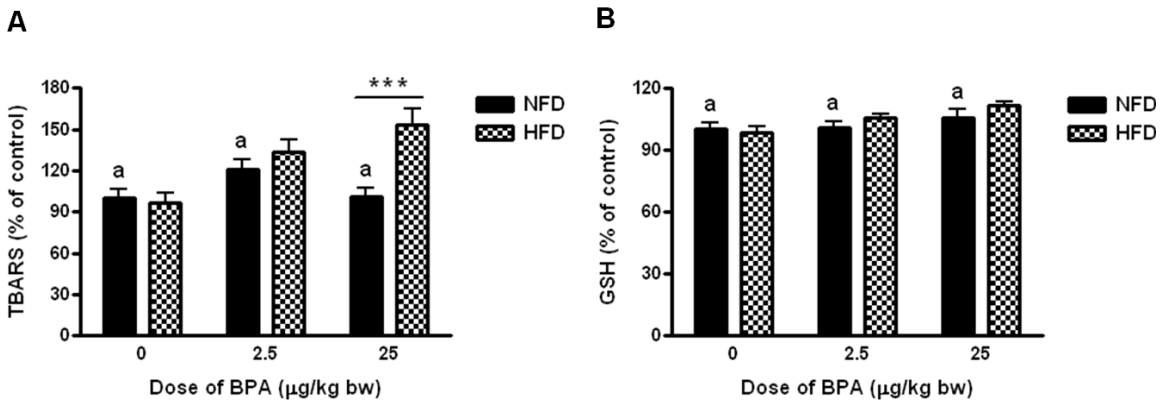

Figure 21. Effect of BPA and diet on oxidative stress body burden. Serum was obtained from blood collected at sacrifice. Lipid peroxidation was assessed in triplicate by measuring levels of thiobarbituric acid reactive substances (TBARS) (A), whereas GSH levels were determined in duplicate using the O-Phthalaldehyde method (B). BPA exposure = gestational day 12 to postnatal day 21 (perinatal period). Animals were maintained on normal or high fat diet (NFD, HFD) from PND 71 to PND 98. ***P < 0.001. a, b = BPA effect, * = diet effect.

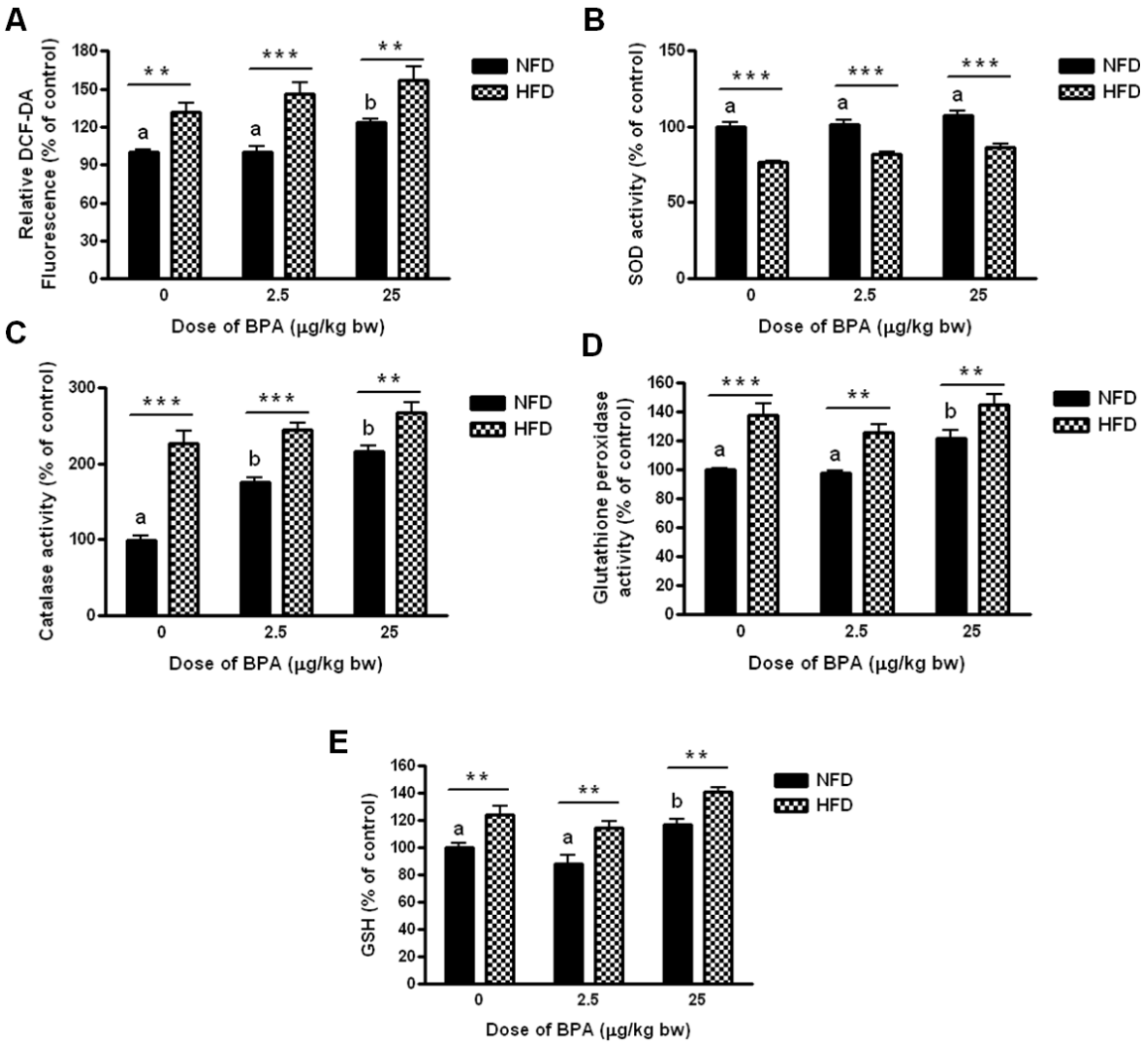

Figure 22. Effect of BPA and diet on oxidative stress and antioxidant capacity in Leydig cells *in vivo*. Leydig cells were isolated at the time of sacrifice. Reactive oxygen species (ROS) were measured as relative fluorescence of dichlorodihydrofluorescein diacetate (DCF-DA) per mg of protein (A). Leydig cell superoxide dismutase (SOD) activity was calculated as percentage inhibition of pyrogallol autoxidation per mg of protein (B), whereas catalase enzyme activity was measured as nmoles of hydrogen peroxide (H_2O_2) degradation in Leydig cell lysates (C). Glutathione peroxidase activity was determined as utilization of NADPH in presence of GSH, t-butyl peroxide and glutathione reductase enzyme (D) while GSH levels were estimated using the o-phthalaldehyde method (E). BPA exposure = gestational day 12 to postnatal day 21 (perinatal period). Animals were maintained on normal or high fat diet (NFD, HFD) from PND 71 to PND 98. **P < 0.01, ***P < 0.001, b (P < 0.05). a, b = BPA effect, * = diet effect.

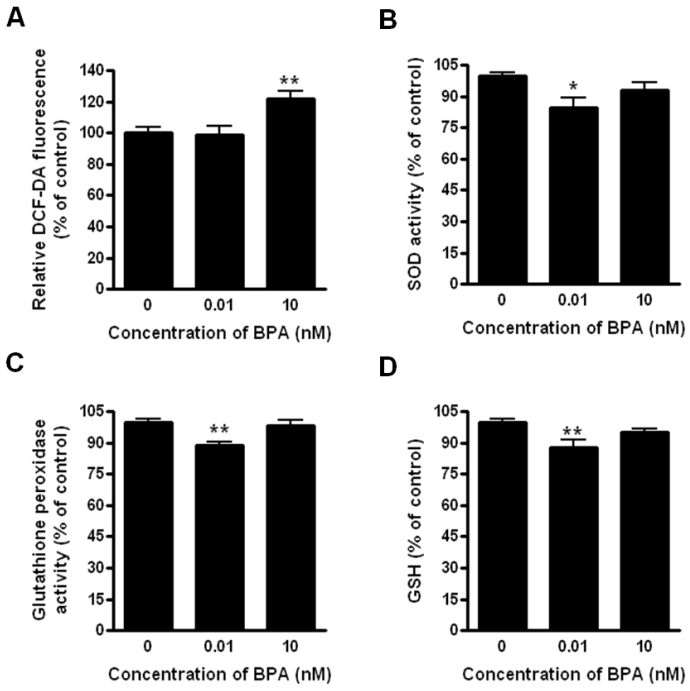

Figure 23. Effect of BPA on oxidative stress and antioxidant capacity in Leydig cells *in vitro*. Leydig cells were isolated from 90 day-old BPA-free Long-Evans male rats. Reactive oxygen species (ROS) production was measured as relative dichlorodihydrofluorescein diacetate (DCF-DA) fluorescence in cell lysates (A). Total SOD enzyme activity was determined as percentage inhibition of pyrogallol autoxidation (B). Glutathione peroxidase activity was measured as utilization of NADPH in presence of GSH, t-butyl peroxide and glutathione reductase enzyme (C) while GSH levels were measured using the O-Phthalaldehyde method (D). Total SOD and glutathione peroxidase enzyme activities and GSH content were measured in BPA-treated Leydig cell lysates and normalized to mg of protein. *P < 0.05, **P < 0.01, versus control.

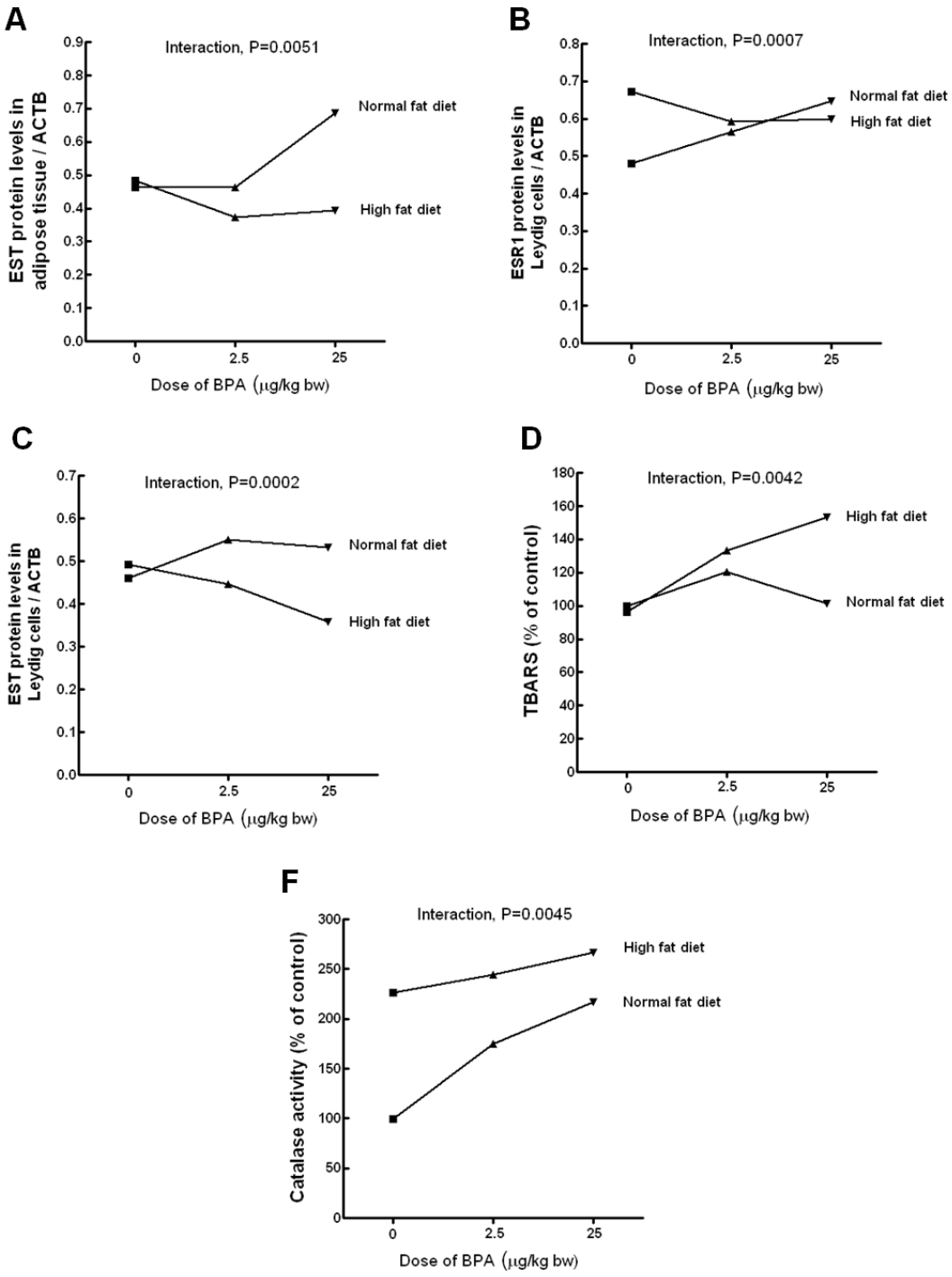

Figure 24. Plots representing interactive effect of BPA and diet on adipose tissue EST protein levels, Leydig cell ESR1 protein levels, Leydig cell EST protein levels, serum TBARS and Leydig cell catalase activity.

Table 8. Interactive effects of BPA exposure and diet on estrogen metabolism and antioxidant capacity in Leydig cells

Parameters	Interaction (BPA and Diet)	p-value
Serum E_2 levels	No	0.3179
Adipose tissue ESR1 protein levels	No	0.9968
Adipose tissue ESR2 protein levels	No	0.8179
Adipose tissue EST protein levels	Yes	0.0051
Leydig cell ESR1 Protein levels	Yes	0.0007
Leydig cell EST protein levels	Yes	0.0002
Serum TBARS	Yes	0.0042
Serum GSH levels	No	0.4525
Leydig cell ROS	No	0.5461
Leydig cell SOD activity	No	0.7121
Leydig cell catalase activity	Yes	0.0045
Leydig cell GPx activity	No	0.4203
Leydig cell GSH activity	No	0.9472

5. Discussion

Developmental exposure to BPA increased protein expression of the EST enzyme in Leydig cells and in adipose tissue, suggesting that BPA may affect estrogen homeostasis. In addition, BPA exposure predisposed male rats to HFD-induced oxidative stress as indicated by higher levels of serum TBARS. Altogether, the present study demonstrated that developmental exposure to BPA causes oxidative stress in Leydig cells in adult animals. The possibility that BPA can act directly in Leydig cells to induce oxidative stress was confirmed by observations *in vitro*.

Previous reports have indicated that HFD consumption increased serum E_2 levels and increased body fat accumulation (Fernandez et al., 2011; Olivares et al.,

2010). Increased E_2 is due mostly to enhanced peripheral aromatization of T by the aromatase enzyme (Ribeiro et al., 2012; Subbaramaiah et al., 2011). However, HFD-fed animals showed decreased serum E_2 levels compared to NFD animals in the present study. Although peripheral aromatase enzyme activity was not analyzed in the present study, it is possible that HFD feeding decreased aromatase enzyme expression and/or activity.

Leydig cells exposed to BPA showed increased levels of ESR1, which may increase sensitivity to estrogen stimulation as observed previously (Bromer et al., 2010; Wadia et al., 2007). In the present study, DES, a synthetic estrogen, was used to demonstrate that altered estrogen homeostasis and increased estrogen action has an inhibitory effect on Leydig cell steroidogenesis. In addition, a previous study showed that E_2 administration to male rats increased body fat accumulation not due to food intake (Levine and Emery, 1987). Interestingly, feeding HFD to male rats decreased EST expression in both Leydig cells and gonadal adipose tissue, this effect is probably an adaptive response to decreased endogenous E_2 levels (Figure 20). However, decreased EST levels may potentiate toxicity of exogenous estrogens since EST is a critical detoxifying enzyme for xenoestrogens (Song, 2001). Decreased EST might also contribute to enhanced adipogenesis and thereby predispose individuals to obesity (Wada et al., 2011). Altogether, BPA and HFD have the capacity to alter estrogen metabolism arising from changes in EST activity, which may predispose to endocrine-related diseases.

The finding that perinatal BPA exposure induced oxidative stress in adult Leydig cells from sexually mature male rats is similar to a previous report in mice (Kabuto et al., 2004). However, the present study showed that higher levels of ROS were generated in Leydig cells from adult rats long after BPA exposure was terminated. Although oxidative stress status in the testis during the fetus or neonatal period was not determined, results from the present study are similar to observations from the intrauterine growth restriction (IUGR) rat model. For example, increased oxidative stress induced by IUGR in pancreatic islets in the fetal period due to defective mitochondrial programming was persistent into adulthood leading to β-cell failure and development of diabetes in adults (Simmons et al., 2005). Furthermore, it was observed that offspring from BPA-exposed dams and IUGR models compensated for increased ROS by up-regulating antioxidant enzyme capacity (Kabuto et al., 2004; Simmons et al., 2005). These observations are similar to present results showing increased catalase and GPx enzyme activities in BPA-exposed Leydig cells.

There is evidence that accumulative free radical damage is a contributing factor to Leydig cell dysfunction in aged individuals (Chen et al., 2005). Therefore, increased ROS by BPA exposure may accelerate Leydig cell aging and low serum T levels, which contribute to early reproductive senescence (Luo et al., 2006). This is relevant to public health because low serum T levels in men are a risk factor for metabolic syndromes (Muller et al., 2005), insulin resistance and diabetes (Pitteloud et al., 2005). However, low but chronic increases in ROS production may protect against Leydig cell dysfunction through hormesis (Calabrese et al., 2011; Chen et al.,

2005; Gems and Partridge, 2008). Thus, additional studies are required to demonstrate the long term effects of increased ROS in Leydig cells following chemical exposures.

Leydig cells from HFD-fed animals showed increased ROS levels compared to NFD-fed animals. This is probably due to greater steroidogenic enzyme activity (CYP11A1 and CYP17A1) and mitochondrial ROS generation by LH/cAMP signaling (Aggarwal et al., 2009; Sebokova et al., 1988; Tai and Ascoli, 2011). Furthermore, BPA at the 25 μg/kg bw dose acting alone or with HFD feeding increased ROS levels in Leydig cells at ~25% and ~50% greater than NFD controls, respectively, suggesting additive effects of BPA and HFD. Furthermore, increased ROS in Leydig cells from BPA-exposed and HFD-fed animals might arise in part due to lack of adiponectin signaling in Leydig cells (Chapter 3) and the consequent decrease in antioxidant effects due to adiponectin.

Previously, BPA at 0.01 nM (18h), but not 10 nM, caused a decrease in T production by adult Leydig cells (Akingbemi et al., 2004). In the present study, results of *in vitro* experiments demonstrated that 0.01 nM BPA, but not 10 nM, decreased SOD and GPx activities and depleted GSH content without increasing ROS production in Leydig cells. In contrast, the 10 nM BPA concentration increased ROS production in Leydig cells without affecting antioxidant enzyme capacity. The reasons for these discrepancies are not known; however, it may be related to the non-monotonic effects ascribed to estrogenic compounds and endocrine disruptors (Welshons et al., 2003). Nonetheless, these results suggest that BPA at low

concentrations, relevant to the levels found in humans (1-20 nM), may act directly in Leydig cells to induce oxidative stress and or decrease antioxidant enzyme capacity, which potentially predispose Leydig cells to oxidative damage. Surprisingly, a previous report has shown that H_2O_2, one of the prototype ROS, at physiological concentrations (20, 30 and 50 μM, 6h) inhibits Leydig cell androgen secretion *in vitro* (Gautam et al., 2006). Therefore, increased ROS production in Leydig cells after perinatal BPA exposure may contribute, at least in part, to the decrease in Leydig cell T production observed previously (Chapter 3).

Feeding of HFD to BPA-exposed animals increased serum levels of TBARS—a biomarker of lipid peroxidation and peroxidative tissue injury (Janero, 1990). Serum GSH levels were also up-regulated in BPA-exposed animals maintained on HFD. Thus, increased TBARS levels are likely the result of oxidative injury due to BPA exposure and HFD consumption. This is interesting because oxidative stress has been associated with the pathogenesis of many diseases including cancers and neurodegenerative diseases (Spector, 2000). Furthermore, the free radical theory of aging postulates that accumulated damage from oxidative stress advances the aging process probably due to accelerated rates of telomere shortening (von Zglinicki, 2002).

In conclusion, perinatal exposure to environmentally relevant BPA levels induced oxidative stress in Leydig cells in adulthood, which possibly contributes to decreased androgen secretion. In addition, the present data demonstrated that BPA can act directly in Leydig cells to affect antioxidant enzyme capacity and ROS

production. Although oxidative stress predisposes to accelerated aging of several tissues, additional studies are required to elucidate the long-term effects of BPA regulation of antioxidant capacity in Leydig cells and testicular function.

CHAPTER 5

SUMMARY AND CONCLUSION

In the rat, Leydig cell development progresses through three stages and is defined by two features: proliferation and steroid hormone secretion. Early in the neonatal period, mesenchymal Leydig cell precursors differentiate into highly proliferative progenitor Leydig cells (PLCs) from PND 14 to 21 and then into immature Leydig cells (ILCs) between PND 21 and 35. Although ILCs are less able to divide than PLCs, they gradually differentiate into fully steroidogenic adult Leydig cells (ALCs) by PND 56. ALCs are devoid of any mitotic capacity but have a 150- and 5-fold greater capacity for T production than PLCs and ILCs, respectively. However, exposure to BPA in the perinatal period, as in this study, affected the two features of Leydig cell development. Importantly, results indicated that developmental effects of BPA persisted into adulthood. For example, the greater number of Leydig cells in the testis of BPA-exposed adult rats at 90 days of age is the result of increased Leydig cell proliferative activity early in development. In contrast to stimulatory effects on cell division, perinatal exposure of male rats to BPA suppressed development of steroidogenic capacity in Leydig cells, which was evident at the end of the exposure period (day 21 postpartum) and into adulthood. Overall, these findings showed that developmental exposures to BPA impact testis development in the male rat.

Moreover, the present study demonstrated that BPA has the capacity to alter Leydig cell function by affecting other endocrine factors which regulate testis function. For example, there is a direct relationship between energy homeostasis and reproduction. The present study demonstrated that developmental BPA exposure or HFD feeding alone decreased serum adiponectin levels. In addition, HFD feeding had the effect of modifying BPA-induced changes. Moreover, BPA and HFD feeding together decreased adiponectin and AdipoR2 protein expression in Leydig cells. Thus, BPA exposure may affect the endocrine and/or autocrine loop of adiponectin regulation of Leydig cells. Surprisingly, feeding of HFD increased Leydig cell T secretion by up-regulating HSD17B3 enzyme. This enzyme catalyzes formation of T from androstenedione, the final enzymatic step in androgen biosynthesis. However, BPA acting alone had the opposite effect and decreased T secretion by Leydig cells.

Finally, feeding of HFD increased serum lipid peroxidation in BPA-exposed male rats and decreased serum E_2 levels in all groups independent of BPA exposure. Furthermore, developmental exposure to BPA induced oxidative stress in Leydig cells and affected antioxidant capacity. For example, perinatal BPA exposure induced ROS generation in Leydig cells, which was further enhanced by HFD feeding.

In conclusion, the present results demonstrated that perinatal exposure of male rats to BPA impaired postnatal Leydig cell differentiation. Furthermore, results suggest that early-life exposures to BPA alone or with high fat diet feeding

affected adipose tissue-derived molecules (i.e., adiponectin), which integrate energy homeostasis and reproduction and may induce a metabolic syndrome with implication for androgen secretion by the testis. Interestingly, the exact mechanisms that link hypogonadism and metabolic syndrome and type 2 diabetes are not known. However, altered secretion of adipokines either by developmental exposure to estrogens or as a result of diet-induced obesity and metabolic syndrome may have a role in the development of hypogonadism. Moreover, BPA exposure along with HFD feeding induced oxidative stress in Leydig cells. Therefore, the long term effects of perinatal BPA exposure on Leydig cell function warrant further investigation because BPA-induced changes in Leydig cells may accelerate Leydig cell aging. Taken together, data from the present study reinforce the view that early-life BPA exposure causes biological effects in Leydig cells at environmentally relevant exposure levels and its presence in consumer products potentially has implication for public health.

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Appendix I: Statistical Analysis

Chapter 2: One-way ANOVA table for serum T levels from 90 day old male rats (figure 13C)

Table Analyzed				
Data 1				
One-way analysis of variance				
P value	0.1645			
P value summary	ns			
Are means signif. different? (P < 0.05)	No			
Number of groups	3			
F	1.961			
R squared	0.1513			
Bartlett's test for equal variances				
Bartlett's statistic (corrected)	2.39			
P value	0.3027			
P value summary	ns			
Do the variances differ signif. (P < 0.05)	No			
ANOVA Table	SS	df	MS	
Treatment (between columns)	13.45	2	6.726	
Residual (within columns)	75.46	22	3.43	
Total	88.91	24		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
Column A vs Column B	0.9986	1.137	P > 0.05	-1.079 to 3.076
Column A vs Column C	1.893	1.975	P > 0.05	-0.3742 to 4.159

Number of values	8	10	7
Minimum	0.7407	1.557	1.374
25% Percentile	2.341	1.984	1.493
Median	4.865	2.883	2.319
75% Percentile	6.138	5.111	2.497
Maximum	7.607	6.529	5.25
Mean	4.38	3.381	2.487
Std. Deviation	2.403	1.664	1.298
Std. Error	0.8495	0.5263	0.4908
Lower 95% CI	2.371	2.19	1.286
Upper 95% CI	6.388	4.571	3.688

Chapter 3: Two-way ANOVA table for Leydig cell T production (figure 15B); tabular results showing comparison among BPA-treated animals in NFD or HFD groups.

Table Analyzed	Data 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	2.4	0.0147		
BPA	15.28	P<0.0001		
Diet	58.46	P<0.0001		
Source of Variation	P value summary	Significant?		
Interaction	*	Yes		
BPA	***	Yes		
Diet	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	2	383.2	191.6	4.432
BPA	2	2444	1222	28.27
Diet	1	9349	9349	216.3
Residual	87	3761	43.23	
Number of missing values	3			
Bonferroni posttests				
Column A vs. Column B				
Diet	Column A	Column B	Difference	95% CI of diff.
Normal Fat Diet	38.49	30.78	-7.71	-14.19 to -1.227
High Fat Diet	62.19	52.87	-9.316	-15.59 to -3.040
Diet	Difference	t	P value	Summary
Normal Fat Diet	-7.71	3.211	P<0.01	**
High Fat Diet	-9.316	4.008	P<0.001	***
Column A vs. Column C				
Diet	Column A	Column C	Difference	95% CI of diff.
Normal Fat Diet	38.49	30.88	-7.606	-14.09 to -1.124
High Fat Diet	62.19	45.27	-16.91	-23.19 to -10.64
Diet	Difference	t	P value	Summary
Normal Fat Diet	-7.606	3.168	P<0.01	**
High Fat Diet	-16.91	7.276	P<0.001	***
Column B vs. Column C				
Diet	Column B	Column C	Difference	95% CI of diff.
Normal Fat Diet	30.78	30.88	0.1034	-6.379 to 6.586
High Fat Diet	52.87	45.27	-7.597	-13.87 to -1.321
Diet	Difference	t	P value	Summary
Normal Fat Diet	0.1034	0.04309	P > 0.05	ns
High Fat Diet	-7.597	3.268	P<0.01	**

Chapter 3: Two-way ANOVA table for Leydig cell T production (figure 15B); narrative results showing comparison among BPA-treated animals in NFD or HFD groups.

Data analyzed: Data 1				
Source of Variation	Degrees of Freedom	Sum of Squares	Mean square	
BPA	2	2444	1222	
Diet	1	9349	9349	
Interaction	2	383.2	191.6	
Residual (error)	87	3761	43.23	
Total	92			
Does BPA have the same effect at all values of Diet?				
Interaction accounts for approximately 2.40% of the total variance.				
F = 4.43. DFn=2 DFd=87				
The P value = 0.0147				
If there is no interaction overall, there is a 1.5% chance of randomly observing so much				
interaction in an experiment of this size. The interaction is considered significant.				
Since the interaction is statistically significant, the P values that				
follow for the row and column effects are difficult to interpret.				
Does BPA affect the result?				
BPA accounts for approximately 15.28% of the total variance.				
F = 28.27. DFn=2 DFd=87				
The P value is < 0.0001				
If BPA has no effect overall, there is a less than 0.01% chance of randomly observing an				
effect this big (or bigger) in an experiment of this size. The effect is considered				
extremely significant.				
Does Diet affect the result?				
Diet accounts for approximately 58.46% of the total variance.				
F = 216.26. DFn=1 DFd=87				
The P value is < 0.0001				
If Diet has no effect overall, there is a less than 0.01% chance of randomly observing an effect				
this big (or bigger) in an experiment of this size. The effect is considered extremely significant.				

Chapter 3: Two-way ANOVA table for Leydig cell T production (figure 15B); tabular results showing comparison age-mates of NFD and HFD groups.

Table Analyzed	Data 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	2.4	0.0147		
Diet	58.46	P<0.0001		
BPA	15.28	P<0.0001		
Source of Variation	P value summary	Significant?		
Interaction	*	Yes		
Diet	***	Yes		
BPA	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	2	383.2	191.6	4.432
Diet	1	9349	9349	216.3
BPA	2	2444	1222	28.27
Residual	87	3761	43.23	
Number of missing values	3			
Bonferroni posttests				
Normal Fat Diet vs. High Fat Diet				
BPA	Normal Fat Diet	High Fat Diet	Difference	95% CI of diff.
0	38.49	62.19	23.7	17.93 to 29.47
2.5	30.78	52.87	22.09	16.33 to 27.86
25	30.88	45.27	14.39	8.626 to 20.16
BPA	Difference	t	P value	Summary
0	23.7	10.03	P<0.001	***
2.5	22.09	9.35	P<0.001	***
25	14.39	6.091	P<0.001	***

Chapter 3 Two-way ANOVA table for Leydig cell T production (figure 15B); narrative results showing comparison age-mates of NFD and HFD groups.

Data analyzed: Data 1				
Source of Variation	Degrees of Freedom	Sum of Squares	Mean square	
Diet	1	9349	9349	
BPA	2	2444	1222	
Interaction	2	383.2	191.6	
Residual (error)	87	3761	43.23	
Total	92			
Does Diet have the same effect at all values of BPA?				
Interaction accounts for approximately 2.40% of the total variance.				
F = 4.43. DFn=2 DFd=87				
The P value = 0.0147				
If there is no interaction overall, there is a 1.5% chance of randomly observing so much				
interaction in an experiment of this size. The interaction is considered significant.				
Since the interaction is statistically significant, the P values that				
follow for the row and column effects are difficult to interpret.				
Does Diet affect the result?				
Diet accounts for approximately 58.46% of the total variance.				
F = 216.26. DFn=1 DFd=87				
The P value is < 0.0001				
If Diet has no effect overall, there is a less than 0.01% chance of randomly observing an				
effect this big (or bigger) in an experiment of this size. The effect is considered				
extremely significant.				
Does BPA affect the result?				
BPA accounts for approximately 15.28% of the total variance.				
F = 28.27. DFn=2 DFd=87				
The P value is < 0.0001				
If BPA has no effect overall, there is a less than 0.01% chance of randomly observing an effect				
this big (or bigger) in an experiment of this size. The effect is considered extremely significant.				

Chapter 3: Two-way ANOVA table for serum adiponectin levels (figure 16A); tabular results showing comparison among BPA-treated animals in NFD or HFD groups.

Table Analyzed	Data 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	6.36	0.0629		
BPA	22.33	0.0002		
Diet	16.47	0.0003		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
BPA	***	Yes		
Diet	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	2	34.8	17.4	2.925
BPA	2	122.2	61.09	10.27
Diet	1	90.14	90.14	15.16
Residual	50	297.4	5.948	
Number of missing values	4			
Bonferroni posttests				
Column A vs. Column B				
Diet	Column A	Column B	Difference	95% CI of diff.
Normal Fat Diet	12.41	13.62	1.205	-1.874 to 4.283
High Fat Diet	9.299	9.434	0.1355	-2.861 to 3.132
Diet	Difference	t	P value	Summary
Normal Fat Diet	1.205	1.075	P > 0.05	ns
High Fat Diet	0.1355	0.1242	P > 0.05	ns
Column A vs. Column C				
Diet	Column A	Column C	Difference	95% CI of diff.
Normal Fat Diet	12.41	8.193	-4.22	-7.398 to -1.042
High Fat Diet	9.299	7.852	-1.446	-4.525 to 1.632
Diet	Difference	t	P value	Summary
Normal Fat Diet	-4.22	3.648	P<0.01	**
High Fat Diet	-1.446	1.291	P > 0.05	ns
Column B vs. Column C				
Diet	Column B	Column C	Difference	95% CI of diff.
Normal Fat Diet	13.62	8.193	-5.424	-8.680 to -2.169
High Fat Diet	9.434	7.852	-1.582	-4.660 to 1.497
Diet	Difference	t	P value	Summary
Normal Fat Diet	-5.424	4.577	P<0.001	***
High Fat Diet	-1.582	1.412	P > 0.05	ns

Chapter 3: Two-way ANOVA table for serum adiponectin levels (figure 16A); narrative results showing comparison among BPA-treated animals in NFD or HFD groups.

Data analyzed: Data 1			
Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
BPA	2	122.2	61.09
Diet	1	90.14	90.14
Interaction	2	34.8	17.4
Residual (error)	50	297.4	5.948
Total	55		
Does BPA have the same effect at all values of Diet?			
Interaction accounts for approximately 6.36% of the total variance.			
F = 2.93. DFn=2 DFd=50			
The P value = 0.0629			
If there is no interaction overall, there is a 6.3% chance of randomly observing so much			
interaction in an experiment of this size. The interaction is considered not quite significant.			
Does BPA affect the result?			
BPA accounts for approximately 22.33% of the total variance.			
F = 10.27. DFn=2 DFd=50			
The P value = 0.0002			
If BPA has no effect overall, there is a 0.018% chance of randomly observing an			
effect this big (or bigger) in an experiment of this size. The effect is considered			
extremely significant.			
Does Diet affect the result?			
Diet accounts for approximately 16.47% of the total variance.			
F = 15.16. DFn=1 DFd=50			
The P value = 0.0003			
If Diet has no effect overall, there is a 0.029% chance of randomly observing an effect			
this big (or bigger) in an experiment of this size. The effect is considered extremely significant.			

Chapter 3: Two-way ANOVA table for serum adiponectin levels (figure 16A); tabular results showing comparison age-mates of NFD and HFD groups.

Table Analyzed	Data 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	6.36	0.0629		
Diet	16.47	0.0003		
BPA	22.33	0.0002		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
Diet	***	Yes		
BPA	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	2	34.8	17.4	2.925
Diet	1	90.14	90.14	15.16
BPA	2	122.2	61.09	10.27
Residual	50	297.4	5.948	
Number of missing values	4			
Bonferroni posttests				
Normal fat diet vs. High fat diet				
BPA	Normal fat diet	High fat diet	Difference	95% CI of diff.
0	12.41	9.299	-3.114	-5.816 to -0.4125
2.5	13.62	9.434	-4.183	-6.959 to -1.407
25	8.193	7.852	-0.3407	-3.276 to 2.595
BPA	Difference	t	P value	Summary
0	-3.114	2.855	P < 0.05	*
2.5	-4.183	3.733	P<0.01	**
25	-0.3407	0.2875	P > 0.05	ns

Chapter 3: Two-way ANOVA table for serum adiponectin levels (figure 16A); narrative results showing comparison age-mates of NFD and HFD groups.

Data analyzed: Data 1				
Source of Variation	Degrees of Freedom	Sum of Squares	Mean square	
Diet	1	90.14	90.14	
BPA	2	122.2	61.09	
Interaction	2	34.8	17.4	
Residual (error)	50	297.4	5.948	
Total	55			
Does Diet have the same effect at all values of BPA?				
Interaction accounts for approximately 6.36% of the total variance.				
F = 2.93. DFn=2 DFd=50				
The P value = 0.0629				
If there is no interaction overall, there is a 6.3% chance of randomly observing so much				
interaction in an experiment of this size. The interaction is considered not quite significant.				
Does Diet affect the result? (Are the curves different?)				
Diet accounts for approximately 16.47% of the total variance.				
F = 15.16. DFn=1 DFd=50				
The P value = 0.0003				
If Diet has no effect overall, there is a 0.029% chance of randomly observing an				
effect this big (or bigger) in an experiment of this size. The effect is considered				
extremely significant.				
Does BPA affect the result? (Are the curves horizontal?)				
BPA accounts for approximately 22.33% of the total variance.				
F = 10.27. DFn=2 DFd=50				
The P value = 0.0002				
If BPA has no effect overall, there is a 0.018% chance of randomly observing an effect				
this big (or bigger) in an experiment of this size. The effect is considered extremely significant.				
Note that these calculations use only the Y values and totally ignore the numbers you entered into the				
X column.				

Chapter 3 Two-way ANOVA table for adipose tissue EST protein expression (figure 18D); tabular results showing comparison among BPA-treated animals in NFD or HFD groups.

Table Analyzed	Data 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	12.21	0.0051		
BPA	7.11	0.0415		
Diet	10.41	0.0026		
Source of Variation	P value summary	Significant?		
Interaction	**	Yes		
BPA	*	Yes		
Diet	**	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	2	0.3057	0.1528	5.736
BPA	2	0.1781	0.08903	3.341
Diet	1	0.2605	0.2605	9.773
Residual	66	1.759	0.02665	
Number of missing values	0			
Bonferroni posttests				
Column A vs. Column B				
Diet	Column A	Column B	Difference	95% CI of diff.
Normal Fat Diet	0.4626	0.4628	0.0002233	-0.1811 to 0.1815
High Fat Diet	0.4837	0.3741	-0.1096	-0.2909 to 0.07164
Diet	Difference	t	P value	Summary
Normal Fat Diet	0.0002233	0.00335	P > 0.05	ns
High Fat Diet	-0.1096	1.645	P > 0.05	ns
Column A vs. Column C				
Diet	Column A	Column C	Difference	95% CI of diff.
Normal Fat Diet	0.4626	0.6867	0.2241	0.04286 to 0.4054
High Fat Diet	0.4837	0.3934	-0.09035	-0.2716 to 0.09093
Diet	Difference	t	P value	Summary
Normal Fat Diet	0.2241	3.363	P<0.01	**
High Fat Diet	-0.09035	1.356	P > 0.05	ns
Column B vs. Column C				
Diet	Column B	Column C	Difference	95% CI of diff.
Normal Fat Diet	0.4628	0.6867	0.2239	0.04264 to 0.4052
High Fat Diet	0.3741	0.3934	0.0193	-0.1620 to 0.2006
Diet	Difference	t	P value	Summary
Normal Fat Diet	0.2239	3.36	P<0.01	**
High Fat Diet	0.0193	0.2895	P > 0.05	ns

Chapter 3 Two-way ANOVA table for adipose tissue EST protein expression (figure 18D); narrative results showing comparison among BPA-treated animals in NFD or HFD groups.

Data analyzed: Data 1				
Source of Variation	Degrees of Freedom	Sum of Squares	Mean square	
BPA	2	0.1781	0.08903	
Diet	1	0.2605	0.2605	
Interaction	2	0.3057	0.1528	
Residual (error)	66	1.759	0.02665	
Total	71	2.503		
Does BPA have the same effect at all values of Diet?				
Interaction accounts for 12.21% of the total variance.				
F = 5.74. DFn=2 DFd=66				
The P value = 0.0051				
If there is no interaction overall, there is a 0.51% chance of randomly observing so much				
interaction in an experiment of this size. The interaction is considered very significant.				
Since the interaction is statistically significant, the P values that				
follow for the row and column effects are difficult to interpret.				
Does BPA affect the result?				
BPA accounts for 7.11% of the total variance.				
F = 3.34. DFn=2 DFd=66				
The P value = 0.0415				
If BPA has no effect overall, there is a 4.1% chance of randomly observing an				
effect this big (or bigger) in an experiment of this size. The effect is considered				
significant.				
Does Diet affect the result?				
Diet accounts for 10.41% of the total variance.				
F = 9.77. DFn=1 DFd=66				
The P value = 0.0026				
If Diet has no effect overall, there is a 0.26% chance of randomly observing an effect				
this big (or bigger) in an experiment of this size. The effect is considered very significant.				

Chapter 3 Two-way ANOVA table for adipose tissue EST protein expression (figure 18D); tabular results showing comparison among age-mates in NFD or HFD groups.

Table Analyzed	Data 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	12.21	0.0051		
Diet	10.41	0.0026		
BPA	7.11	0.0415		
Source of Variation	P value summary	Significant?		
Interaction	**	Yes		
Diet	**	Yes		
BPA	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	2	0.3057	0.1528	5.736
Diet	1	0.2605	0.2605	9.773
BPA	2	0.1781	0.08903	3.341
Residual	66	1.759	0.02665	
Number of missing values	0			
Bonferroni posttests				
Normal Fat Diet vs. High Fat Diet				
BPA	Normal Fat Diet	High Fat Diet	Difference	95% CI of diff.
0	0.4626	0.4837	0.02116	-0.1426 to 0.1849
2.5	0.4628	0.3741	-0.08871	-0.2524 to 0.07501
25	0.6867	0.3934	-0.2933	-0.4570 to -0.1296
BPA	Difference	t	P value	Summary
0	0.02116	0.3176	P > 0.05	ns
2.5	-0.08871	1.331	P > 0.05	ns
25	-0.2933	4.401	P<0.001	***

Chapter 3 Two-way ANOVA table for adipose tissue EST protein expression (figure 18D); narrative results showing comparison among age-mates in NFD or HFD groups.

Data analyzed: Data 1			
Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Diet	1	0.2605	0.2605
BPA	2	0.1781	0.08903
Interaction	2	0.3057	0.1528
Residual (error)	66	1.759	0.02665
Total	71	2.503	
Does Diet have the same effect at all values of BPA?			
Interaction accounts for 12.21% of the total variance.			
F = 5.74. DFn=2 DFd=66			
The P value = 0.0051			
If there is no interaction overall, there is a 0.51% chance of randomly observing so much			
interaction in an experiment of this size. The interaction is considered very significant.			
Since the interaction is statistically significant, the P values that			
follow for the row and column effects are difficult to interpret.			
Does Diet affect the result?			
Diet accounts for 10.41% of the total variance.			
F = 9.77. DFn=1 DFd=66			
The P value = 0.0026			
If Diet has no effect overall, there is a 0.26% chance of randomly observing an			
effect this big (or bigger) in an experiment of this size. The effect is considered			
very significant.			
Does BPA affect the result?			
BPA accounts for 7.11% of the total variance.			
F = 3.34. DFn=2 DFd=66			
The P value = 0.0415			
If BPA has no effect overall, there is a 4.1% chance of randomly observing an effect			
this big (or bigger) in an experiment of this size. The effect is considered significant.			

Appendix II: Protocol for Leydig cell isolation

Two days prior to the day of isolation autoclave the following (for two isolations)

1. Two 1L, four 500mL, and four 250 ml bottles
2. Two sets of funnel with nylon mesh
3. Special scissor and forceps (2 sets)
4. Two special centrifuge tubes (2 sets)

One day prior to the isolation

1. Preparation of media

a) Dissociation buffer (DB) b) Percoll buffer (PB) c) Leydig Cell Medium (LCM) d) Sedimentation buffer (SB) for adult Leydig cell isolation

Ingredients	Company	DB (1L)	SB (1L)	PB (1L)	LCM (1L)
M-199	#31100-035, Gibco	1 packet	1 packet	-	-
DMEM & F12	#D2906, Sigma	-	-	1 vial	-
HBSS	#14185, Gibco	-	-	-	100 ml
Millipore water		900 ml	900 ml	900 ml	900 ml
Sodium bicarbonate	S5761, Sigma	0.71g	0.71g	0.35g	1.2g
HEPES	#H4034, Sigma	2.1g	2.1g	2.1g	-
Albumin bovine fraction 5	#103703, MP Biomedicals	1g	10g	2.5g	-
Albumin from bovine serum	#A4919, Sigma	-		-	1g
SBTI	#T9003, Sigma	25mg		25mg	-
Gentamicin	#G1397, Sigma	-		-	12mg (240µl)
Set the pH between 7.1-7.2 using NaOH					
Sterilize by vacuum filter (using extra filter)					

On the day of Leydig cell isolation

1. Clean and turn on the UV light of bio-safety cabin
2. Prepare 75 % ethanol if required
3. Take out DB for thawing
4. Switch on the water bath set to 34°C
5. Switch on centrifuge (4°C)
6. Chilled D-PBS on ice
7. Two pairs of scissors and curved forceps

Animals required for single isolation; thirty 21 day old for progenitor Leydig cells (PLCs), eighteen 35 day old for immature Leydig cells (ILCs), eight 90 day old for adult Leydig cells (ALCs).

1. Remove testes, put into ice-chilled Dulbecco's PBS (D-PBS)

   Perfuse 0.5 ml of DB (1mg per ml collagenase + one 1 ml aliquot of 2.4 U/ml dispase; final concentration = 0.185 U/ml in 13 ml) in to testicular artery of each testis. Preparation of internal collagenase: 12 mg of collagenase + one 1 ml aliquot dispase +12 ml of DB. Filter sterilize the internal collagenase.
2. Put each perfused testis into beaker containing fresh chilled D-PBS.

   Note: Skip step 2 and 3 if your are isolating PLCs and ILCs
3. Decapsulate 2 testes into a 50 ml centrifuge tube containing 5 ml of DB. Repeat for remaining 5 pairs of testes for ALCs. For isolation of PLCs or ILCs, decapsulate 10 or 6 testes per 50 ml tube containing 5 ml of DB (i.e. total of six 50 ml tubes).
4. Add 5 ml of external collagenase to 50 ml centrifuge tubes and seal the cap and cover with parafilm. Shake @ 90 cycles per minute @ 34°C for 10 to 20 minutes

(monitor) in waterbath. At end of 20 minutes gently shake the tubes with hands to facilitate dissolution of testicular paranchyma. Repeat this for three times up to 1 hour.

Preparation of external collagenase: 16 mg of collagenase + 32 ml of DB + one 0.42 mg aliquot DNase + one 1 ml aliquot of dispase. Filter sterilize the external collagenase.

5. **For PLC and ILC isolations**: Bring volume of each tube up to 50 ml with DB, then pour the contents of each tube into three new 220 ml centrifuge tubes (2 per one 220 ml tubes), through two layers of 100 micron nylon mesh. Repeat for the five remaining pairs. Bring each collection tube up to 220 ml with DB, then spin down at 250g for 10 minutes.

   For Adult Isolations (using SB). Bring volume of each tube up to 50 ml with SB. Recap tube and invert the tube several times. Let the seminiferous tubules settle for several minutes. With a 10 or 25 ml pipette, draw up the supernate, without disturbing the pellet. Save the supernate. Once the supernate has been collected, refill the tube containing the tubules with SB again invert the tube, then allow the tubules to settle, and collect the supernate. Repeat 1 to 2 more times. Spin down the tubes at 250g for 15 to 20 minutes.

6. Percoll density gradient centrifugation. To form in situ gradient: 4 ml 10X HBSS + 44 ml Percoll (= stock isotonic Percoll, SIP). 19 ml SIP + 16 ml of PB = 35 ml of 55% pH 7.4 SIP. To the tube that will receive the cells add one aliquot of DNase. Add 35 ml 55% SIP to dummy. To the dummy tube pipette 20 µl each density marker beads (DMB) 3, 4 and 5.

7. From step 5, pour off the supernatant, then using PB (14ml), resuspend the three cell pellets from each tubes. The final solution volume should be 16ml due to the pellet. Load the solution on 19 ml percoll solution (total of 35 ml and 55% SIP).
8. Balance the dummy percoll tube with percoll tube containing cells pellets and using a Beckman JA-20 rotor, spin @ 14,500 RPM (approx. 20,000 X g_{ave}) for 1 hour at 4°C.
9. Pipette of the upper layer of the gradient carefully, from the meniscus to DMB 4. From DMB 4 to the midpoint between DMB's 4 & 5 is P_0. From the midpoint to the bottom of the gradient is GRK's P_2 fraction. Fill both tubes with PB to dilute out the Percoll and centrifuge @ 250g for 10 minutes.
10. Respuspend the final pellet in 4-8 ml of LCM. Use 25 µl droplets for 3β-HSD staining to test purity of the preparation, and hemocytometer count (the total number of cells falling within the boundaries of 5 squares on Neubauer hemocytometer X 0.05 = the number of cells in millions per ml. Culture the isolated cells with freshly prepared medium.

    Cell culture media for 50 ml; LCM- 48.875 ml + Lipoprotein- 1.25 ml (#L4646, Sigma) + LH @ 10 ng/ml, (Stock of LH is 5 ng/µL).

Appendix III: Protocol for Radioimmunoassay (RIA)

Day 1

1. Label the 72 glass tubes on a test tube holder [e.g., 22 for standards + 50 (for **25** unknown samples in duplicate) + 2 internal control (IC) is same 100 pg testosterone standard]

Tube number	**TBSG (µl)**	**Standards (µl) concentration (pg)**	**Unknown sample (µl)**	**Antibody (µl)**	**Tracer (µl)**	**Charcoal (µl)**
1	500			200	300	-
2	500			200	300	-
3	500			-	300	200
4	500			-	300	200
5	300			200	300	200
6	300	(0 pg)		200	300	200
7	-	300 (10 pg)		200	300	200
8	-	300 (10 pg)		200	300	200
9	-	300 (25 pg)		200	300	200
10	-	300 (25 pg)		200	300	200
11	-	300 (50 pg)		200	300	200
12	-	300 (30 pg)		200	300	200
13	-	**300 (100 pg)**		200	300	200
14	-	**300 (100 pg)**		200	300	200
15	-	300 (250 pg)		200	300	200
16	-	300 (250 pg)		200	300	200

17	-	300 (500 pg)		200	300	200
18	-	300 (500 pg)		200	300	200
19	-	300 (1000 pg)		200	300	200
20	-	300 (1000 pg)		200	300	200
21	-	300 (2000 pg)		200	300	200
22	-	300 (2000 pg)		200	300	200
23	290	-	10	200	300	200
24	290	-	10	200	300	200
25	290	-	10	200	300	200
26	290	-	10	200	300	200
27	290	-	10	200	300	200
28	290	-	10	200	300	200
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.	.	-	.	.	.	.
69	290	-	10	200	300	200
70	290	-	10	200	300	200
71 (IC)	-	**300 (100 pg)**	-	200	300	200
72 (IC)	-	**300 (100 pg)**	-	200	300	200

2. Add 500 µl of TBSG to tube numbers 1 to 4 and 300µl to tubes # 5 and 6
3. Add different standards (300 µl) from tube # 7 to 22
4. Add the unknown samples in duplicates to rest of tubes (dispense the sample deep in the bottom of the tubes and use single tip for each tube or sample)
5. Add TBSG (290 µl) tubes for unknown samples. The amount of TBSG volume can vary depending on the sample size (e.g. if sample size is 25µl the TBSG added will be 275). It is calculated by formula, TBSG = 300 – sample size
6. Add testosterone antibody (200 µl) using eppendorf pipette (5 ml capacity)
7. Add tracer for testosterone (300 µl) using eppendorf pipette (5 ml capacity)
8. Mix well on vertex after covering with aluminum foil
9. Keep it in the refrigerator at 4°C

Day 2

10. Label the scintillation tubes and dispense 5 ml of scintillation liquid by automatic pipette
11. Bring out the charcoal dextran solution from the refrigerator and keep it on a magnetic stirrer.
12. Bring out the samples from the refrigerator and add 200 µl of charcoal dextran to all tubes except tube # 1 and 2. Use using eppendorf pipette (5 ml capacity) for dispensing of charcoal.
13. Mix well after covering the aluminum foil on vertex
14. Keep the tubes on ice for 20 minutes
15. Meanwhile turn on the refrigerated centrifuge to bring down the temperature to 4° Celsius and ready to use after 20 minutes
16. After 20 minutes spin the tubes at 3000 RPM for 10 minutes at 4° Celsius
17. Take the tubes out of the centrifuge and keep them on ice with help of a test tube holder.
18. Without disturbing, decant the supernatant from the tube in to scintillation vials containing the scintillation cocktail and cap the glass vial. Decanting of

all the tubes to the scintillation vials should be done as soon as possible to avoid seepage of charcoal from pellet to the supernatant.

19. After transferring to the supernatant to scintillation vials from all the tubes, mix the content thoroughly on a vertex and read the activity of the tritium on a beta counter using protocol 11.
20. After the counting, pick up the vials and results in CPM (count per minutes).
21. The concentration of the unknown samples is calculated from the standard curve using the graphpad prism software and expressed in pg/ml or pg/number of Leydig cells for 3 hours.

    Note: The concentration of testosterone in the sample should lie with the range of the assay (i.e. between 10 to 2000 pg). A pilot assay should be done to determine the sample size.

TBSG Recipe (1L)

Gelatin (#D4751, sigma)	1 g
Trizma HCl (#T3253, Sigma)	4.44 g
Trizma Base (#T1503, Sigma)	5.65 g
NaCl (Sigma)	5.84 g
NaN3(sodium azide, #S2002, Sigma)	7 g
dd water	1L

Dissolve gelatin at the beginning in a beaker containing 100 ml of distilled water at 60°C with stirring.

Charcoal Solution

Dissolve 0.3 g of Dextran T-70 (#D4751, Sigma) in 100 ml of distilled water, and then add 3 g of activated charcoal (#C4386, Sigma).

Appendix IV: Western Blotting procedures

Day one

1. Assemble gel-casting module, including the spacer glass plates and short glass plates, on the white plastic tray near the wooden shelf on the bench. Gather a 100mL beaker and a 50mL beaker.
2. Prepare 10% ammonium persulfate (APS). To make APS, in a 1.7mL centrifuge tube, add 100µL distilled water to every 0.010g APS (600 µl). NOTE: The amount of APS will vary depending on how many gels you make, but you will always add 100µL distilled water to every 0.010g APS.
3. Prepare 10% acrylamide gel in the 100mL glass beaker by following recipe below (makes four gels total):

Distilled water	14.85mL
30% acrylamide	12.45mL
1.5M Tris	9.45mL
10% Sodium Dodecyl Sulfate (SDS)	375µL
10% APS	375µL (Save the remaining)
Tetramethylethylenediamine (TEMED)	15µL

NOTE: Recipes for a lower quantity gel (e.g., 2 gels instead of 4) or for different concentration gel (e.g., 6%, 15%) are located on the large, lined, yellow sticky notes taped to wooden shelf on the bench.

4. Add 2mL of 10% gel mixture to each of the glass plate cells. Wait 2 minutes to see if there is a leak from any of the cells. If no leakage, SLOWLY add 6mL of 10% gel mixture to each glass plate cell (each cell should have a total of 8mL 10% gel mixture).
5. Add a thin layer of isobutanol or ethanol on top of the 10% gel mixture in each glass plate cell.
6. Wait 15-20 minutes for gel to solidify.
7. Prepare the stack by following the recipe for 4-gels:

Distilled water	6.15mL
30% acrylamide	1.50mL
1.0M Tris	1.125mL
10% SDS	90µL
10% APS	90µL
TEMED	9µL

8. Mix, then add STACK on top of the isobutanol/10% gel mixture in each glass plate cell until the STACK liquid overflows from each cell.
9. IMMEDIATELY place combs in the STACK and allow 15-20minutes for the entire gel in each glass plate cell to solidify.
10. Remove combs from each glass plate cell and BEFORE LOADING SAMPLES, flush all lanes in each gel with distilled water, and place 2 glass plate cells into each of the 2 companion running modules (Fig. a) needed for running gels.

Fig. a

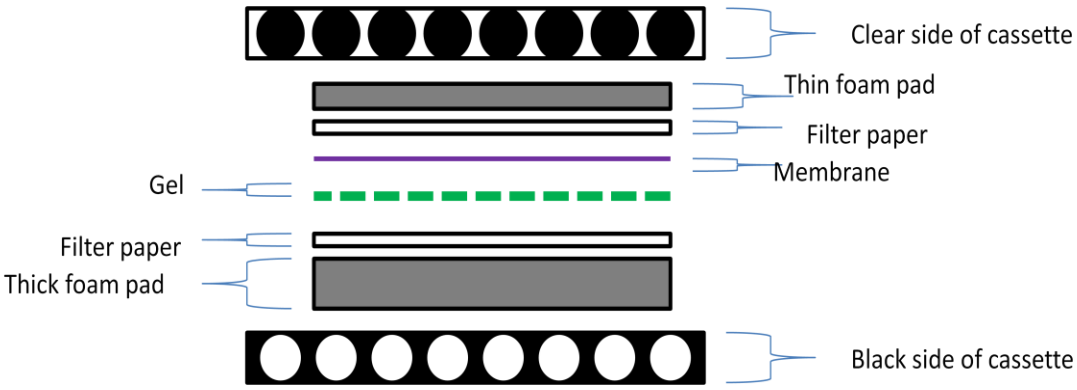
11. Add more distilled water into the lanes of each if water level has decreased. Load 4 µL protein ladder to first lane, followed by loading of protein samples.
12. Gently place the 2 companion running modules into a tank (Fig. b), then gently place the tank into a large plastic container. Place 2 ice packs in the large plastic container, the ice packs should be on opposite sides of the tank.

Fig. b

13. Pour 1X running buffer into each of the companion running modules until running buffer SLIGHTLY overflows. Then, pour additional 1X running buffer into the tank until the tank is approximately 1/3 full.
14. Place lid on the tank. Run gel at 200 V for 30 minutes.
15. While the gels are running, soak the following materials in used transfer buffer (used transfer buffer is transfer buffer that has been recycled from a previous Western blot):
    a. In one container: 2 thick foam pads, 2 thin foam pads, and 4 sheets of thick filter paper
    b. In another container: 4 nitrocellulose membranes
16. Remove gels from glass plate cells, cut gels to appropriate size, and place gels into used transfer buffer.

Fig. c

17. Assemble 2 cassettes (Fig. c), with each cassette containing the previously-listed materials in the following order (Fig. d):



a. MAKE SURE THAT THESE MATERIALS ARE AS MOIST AS POSSIBLE WHILE ASSEMBLING THE CASSSETTES.

18. Place the 2 cassettes into the central core (Fig. e). Put central core in the tank. Place Stirrer bar the bottom center of tank. Put a bio-ice cooling unit (Fig. f) into the tank next to the central core.

Fig. e

Fig. f

19. Place tank into a large plastic container. Then, put 2 ice packs in the large plastic container, with each ice pack on an opposite side of the tank. Place large plastic container (with the tank in it) in a stirrer plate.
20. Pour enough transfer buffer into tank until the bio-ice cooling unit begins to float. Turn on stirrer plate.
21. Put lid on tank. Run transfer at 100 V for 70-90 minutes.

22. Remove membranes from cassettes and place membranes in 5% blotto (5.00g non fat, dried milk in 100mL of 0.1% PBS Tween 20) for 60-minute blocking on a rocker at room temperature.
23. Remove blotto; incubate membranes in primary antibody solution (primary antibody diluted with 8mL of 5% blotto) on a rocker at 4°C overnight.

Day Two

(The following procedures are performed at room temperature):

1. Remove primary antibody from membranes.
2. Wash membranes in cold, distilled water for approximately 30 seconds.
3. Wash membranes in 0.1% PBS Tween 20 three times (5minutes in 0.1% PBS Tween 20 per wash) on a rocker.
4. Place membranes in secondary antibody solution for 90 minutes on a rocker.
5. Remove secondary antibody from membranes.
6. Wash membranes in cold, distilled water for approximately 30 seconds.
7. Wash membranes in 0.1% PBS Tween 20 **four** times (5 minutes in 0.1% PBS Tween 20 per wash) on a rocker.
8. If you are **NOT** ready to **IMMEDIATELY** expose membranes to film, place membranes in PBS tablet. When ready to expose membranes to film, move to step 9.

Fig. g

9. Place membranes in chemiluminescent antibody detection reagent for two minutes on a rocker (we use the Hyglo antibody detection product from Denville Scientific in Fig. g).

10. Lightly dab membranes on a Kim wipe to remove excess antibody detection reagent. Place membranes in a sheet protector.
11. Expose membranes to X-ray film(s) for desired time.
12. Place film(s) in developer for two minutes, then place film(s) in fixer for two minutes, then rinse film(s) in water.

Fig. a: gel-casting module	Fig. b: spacer glass plates	Fig. c: short glass plates