CONSTITUTIVE EXPRESSION OF XENOBIOTIC-METABOLIZING ENZYMES IN RAT TESTIS: IMMUNOCHEMICAL CHARACTERIZATION AND REGULATION BY 17-BETA-ESTRADIOL BENZOATE AND BISPHENOL A

by

RAVINDRANATH REDDY GILIBILI

B.Pharm., Jawaharlal Nehru Technological University, 2004

M.Pharm., Manipal University, 2006

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Abstract

Relatively little is known about the expression, localization and regulation of rat testicular xenobiotic-metabolizing enzymes, including cytochrome P450s (CYP) and microsomal epoxide hydrolase (mEH), which are involved in the metabolism of xenobiotics including drugs and toxicants, and of endobiotics such as steroid hormones and prostaglandins. Suppression or induction of these enzymes in testis may alter the magnitude of tissue exposure to xenobiotics and endobiotics levels. In the present study, constitutive expression of various xenobiotic-metabolizing enzymes (Study 1) and their regulation by 17β-estradiol benzoate (EB) (Study 2) and an endocrine disrupting chemical, bisphenol A (BPA) (Study 3), were investigated in adult rat testis. As shown in Study 1, immunoblot analysis of testicular microsomes prepared from untreated rats revealed the presence of CYP1B1, CYP2A1, CYP17A1, NADPH-cytochrome P450 reductase (POR) and mEH, and absence of CYP1A1, CYP1A2, CYP2B1, CYP2E1, CYP2D1, CYP2D2, CYP2C6, CYP2C7, CYP2C11, CYP2C12, CYP2C13, CYP3A1, CYP3A2, CYP4A1, CYP4A2 and CYP4A3. Immunohistochemical analysis of tissue sections prepared from frozen testis indicated that CYP1B1, CYP2A1 and CYP17A1 were localized in interstitial cells, but not in seminiferous tubules, whereas mEH and POR were localized in both interstitial cells and in seminiferous tubules. In Study 2, subcutaneous (sc) treatment of EB at 0.004, 0.04, 0.4 or 4 μmol/kg once daily for 14 days suppressed testicular expression of CYP1B1, CYP2A1 and CYP17A1 at each of the dosages tested. EB also suppressed mEH and POR protein expression at dosages $\geq$ 0.04 μmol/kg. In Study 3, administration of BPA at 400, 800 or 1600 μmol/kg sc once daily for 14 days decreased testicular CYP1B1, CYP2A1, CYP17A1, mEH and POR protein expression at each of the dosages tested. EB and BPA did not produce a general down-regulation of testicular protein expression because neither of these chemicals decreased

calnexin protein (endoplasmic reticulum marker) levels. In summary, CYP1B1, CYP2A1, CYP17A1, mEH and POR were detected in rat testis and their expression was confined to interstitial cells (CYP1B1, CYP2A1, CYP17A1, mEH and POR) and seminiferous tubules (mEH and POR). Constitutive expression of these rat testicular enzymes was suppressed by exogenous administration of 17β-estradiol and BPA.

Preface

The Animal Care Committee at the University of British Columbia examined and approved the use of animals for the experimental project described in this thesis. The project was completed under the UBC Animal Care Protocol titled, "Constitutive Expression of Xenobiotic-Metabolizing Enzymes in Rat Testis: Immunochemical Characterization and Regulation by 17β-Estradiol Benzoate and Bisphenol A", Animal Care Certificate A10-0225, issued on May 1, 2010.

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List of Abbreviations

BaP	benzo[*a*]pyrene
BPA	bisphenol A
CYP	cytochrome P450
DMBA	7,12-dimethylbenz[*a*]anthracene
EB	17β-estradiol benzoate
EDTA	ethylene diamine tetraacetic acid
EH	epoxidehydrolase
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
FSH	follicle stimulating hormone
GH	growth hormone
GnRH	gonadotropin-releasing hormone
HCl	hydrochloric acid
KCl	potassium chloride
HPLC	high-performance liquid chromatography
HSD	hydroxysteroid dehydrogenase
LH	luteinizing hormone
mg	milligram
µg	microgram
ml	milliliter
µl	microliter
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)

NBT	*p*-nitro-blue tetrazolium chloride
PAH	polycyclic aromatic hydrocarons
POR	NADPH-cytochrome P450 oxidoreductase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TEMED	*N*,*N*,*N´*,*N´*-tetramethylethylenediamine
Tris	tris-(hydroxymethyl)aminoethane
TSH	thyroid stimulating hormone
UV	ultraviolet

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This thesis is dedicated to my parents, brother and sisters

1. Introduction

Cytochrome P450 (CYP) enzymes are a large superfamily of enzymes involved in the oxidative biotransformation of a variety of xenobiotics (foreign compounds) such as drugs and environmental chemicals, and of endogenous compounds such as steroids and prostaglandins. CYP enzymes function to structurally modify lipophilic substrates into more water soluble forms and thereby enhancing their elimination from the body. In rats and other mammals, CYP enzymes are highly expressed in liver and relatively expressed at much lower levels in extrahepatic tissues including testis (Guengerich et al., 2006).

Testis is the male reproductive organ involved in the production of sex hormones and spermatogenic cells. The expression and regulation of CYP enzymes in the testis is important, because changes in CYP expression could indirectly disturb testicular functions. For example, suppression of CYP expression in testis could lead to accumulation of drugs and endogenous compounds within the testis and may cause testicular toxicity (Park et al., 1981). To date, only a few CYP enzymes (CYP1B1, CYP2A1 and CYP2E1) have been reported to be present in rat testis. As far as I am aware, no comprehensive study has been carried out on the expression of other CYP enzymes in testis including those that are known to be highly expressed in the rat liver (Seng et al., 1991; Jiang et al., 1998; Lee et al., 1980; Otto et al., 1992; Leung et al., 2009; Sonderfan et al., 1989). Thus, the first study in this thesis was aimed at characterizing CYP expression in rat testis using immunochemical methods.

The second study in this thesis is an extension of a previous study from our laboratory (Leung et al., 2009). In this earlier work, exogenous administration of 17β-estradiol benzoate at 1.5 mg/kg for 14 days decreased CYP1B1 expression in Sprague-Dawley rat testis. The experiments were designed to investigate the effect of treatment with varying dosages of 17β-

estradiol benzoate on CYP expression and to determine the minimum dose that is required to produce a suppressive effect on the expression of CYP1B1 and other drug metabolizing enzymes in Sprague-Dawley rat testis.

There is a growing body of evidence suggest that human exposure to endocrine disrupting chemicals and in particular to estrogenic compounds (xenoestrogens), may cause reproductive abnormalities such as infertility and reduced sperm production (Vandenberg et al., 2007). Bisphenol A (BPA) is a xenoestrogen that is widely used in the synthesis of plastics and human exposure is common. BPA has been detected in approximately 95% of the US population tested in their urine (Calafat et al., 2005). Due to the estrogenic nature of BPA, BPA may produce similar effects on expression of testicular CYP enzymes as was observed with 17β-estradiol benzoate. The third study in this thesis was designed to investigate the effect of BPA at varying dosages on testicular expression of CYP and other metabolizing enzymes in male Sprague-Dawley rats.

As an introduction to the work described in this thesis, I will review the organization of testis, the hormonal regulation of testicular function, and provide a current review on the presence of xenobiotic-metabolizing enzymes in rat testis and their regulation by hormones. I will also summarize the physiological role of estrogens in men and their role in regulation of drug metabolizing enzymes. Finally, I will present an overview of the endocrine-disrupting activity of BPA.

1.1 Organization of the Mammalian Testis

The testis plays an important role in the production of sperm and sex hormones including testosterone and 17β-estradiol. Each testis is covered by a fibrous capsule of connective tissue called tunica albugenia (Figure 1.1A). A cross-section of testis shows two major compartments, the seminiferous tubules and the interstitium (Russell et al., 1990) (Figure 1.1B). The space between the tubules is called the interstitium. The majority of cells present in the interstitial space are Leydig cells. In general, there are three different populations of Leydig cells (progenitor, immature and adult Leydig cells) present in adult rat testis. Among these, adult Leydig cells are the predominant population. They are morphologically identified as round in shape and contain a larger amount of smooth endoplasmic reticulum with few or no lipid droplets when compared to immature and progenitor Leydig cells (Figure 1.1C). Lipid droplets are characteristic of immature Leydig cells and are useful markers to identify Leydig cells from other interstitial cells in immunohistochemical experiments. Leydig cells are the predominate site for the *de novo* synthesis of steroid hormones, in particular, testosterone and estrogens, which are essential for the maintenance of reproductive functions and male sexual characteristics (Roberts and Zirkin, 1991).

Besides Leydig cells, the interstitium also contains blood capillaries, lymphatic vessels and various immune cells such as macrophages, lymphocytes and mast cells. For this reason, the interstitium acts as a first line immune-barrier that provides protection to the testis against the pathogens in the bloodstream (Li et al., 2012).

The seminiferous tubule is another important compartment in the testis (Figure 1.1B). The seminiferous tubules are the primary sites where production of germ cells takes place by the process of spermatogenesis. Spermatogenesis is a complex and organized process. It involves 14

different steps, starting with spermatogonia at the basal germinal epithelium to formation of mature sperm and released into the lumen. The whole process takes 40 to 60 days in rat (Russell et al., 1990). Seminiferous tubules are highly convoluted and each tubule originates and ends at the rete testis, which forms a duct system that connects to the epididymis (Figure 1.1). The outer layer of the tubule is bound by lymphatic epithelium and peritubular myloid cells (Skinner et al., 1991). This outer layer forms a basement membrane for the columnar Sertoli cells and the basal germinal epithelium (Russell et al., 1990).

Sertoli cells have several functions in the testis. First, Sertoli cells are secretory cells and responsible for the fluid filled tubular lumen (Skinner et al., 1991). Second, Sertoli cells act as nursing cells and provide physical and nutritional support for spermatogenic cell development (Vogl, 1988). Third, Sertoli cells form tight junctions with adjacent Sertoli cells. These tight junctions are called the "blood-testis barrier" and form a physical barrier that protects spermatogenic cells from exposure to xenobiotics and pathogens (Vogl et al., 1991; Skinner et al., 1991; Russell et al., 1990). Finally, Sertoli cells are directly associated with the initial four cycles of the spermatogenesis process (Russell et al., 1990).

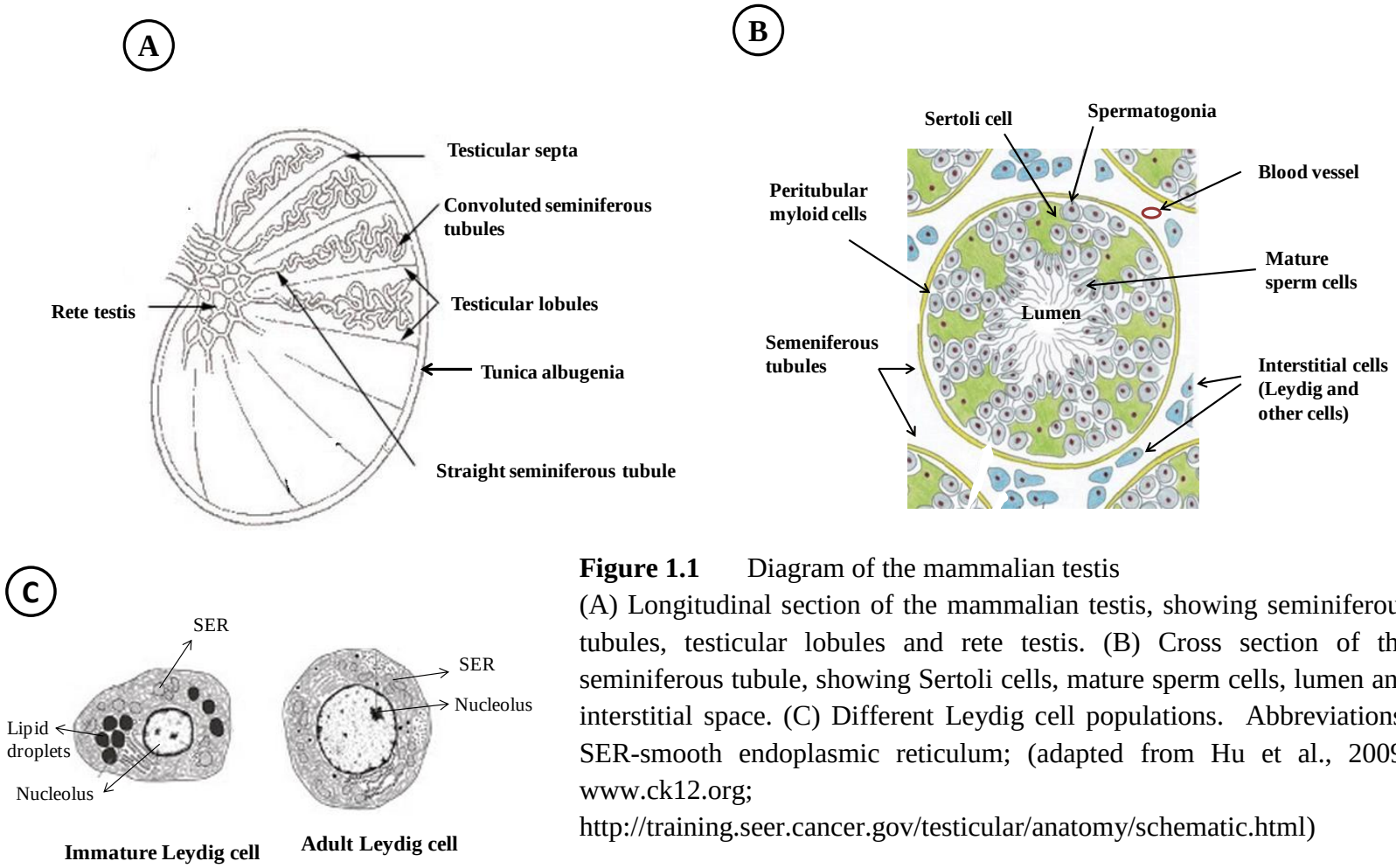

Figure 1.1 Diagram of the mammalian testis

(A) Longitudinal section of the mammalian testis, showing seminiferous tubules, testicular lobules and rete testis. (B) Cross section of the seminiferous tubule, showing Sertoli cells, mature sperm cells, lumen and interstitial space. (C) Different Leydig cell populations. Abbreviations: SER-smooth endoplasmic reticulum; (adapted from Hu et al., 2009; www.ck12.org; http://training.seer.cancer.gov/testicular/anatomy/schematic.html)

1.2 Hormonal Regulation of Testicular Functions

The functions of the testis, namely steroidogenesis and spermatogenesis, are regulated by various hormones such as pituitary, hypothalamus and sex hormones. Together, these hormones form a complex interdependent loop known as the hypothalamo-pituitary-testicular (HPT) axis (Weinbauer and Nieschlag, 1995, de Krester, 1987). It starts with the production of hypothalamic GnRH that regulates the secretion of gonadotropins, including LH and FSH, from the anterior pituitary gland. In turn, LH acts on testis, in particular, on Leydig cells and stimulates the synthesis and secretion of steroid hormones, testosterone and 17β-estradiol. Both of these two hormones in turn regulate the release of GnRH from hypothalamus, and LH and FSH from the pituitary through feedback inhibition mechanisms (Figure 1.2) (Weinbauer and Nieschlag, 1995; de Krester, 1987).

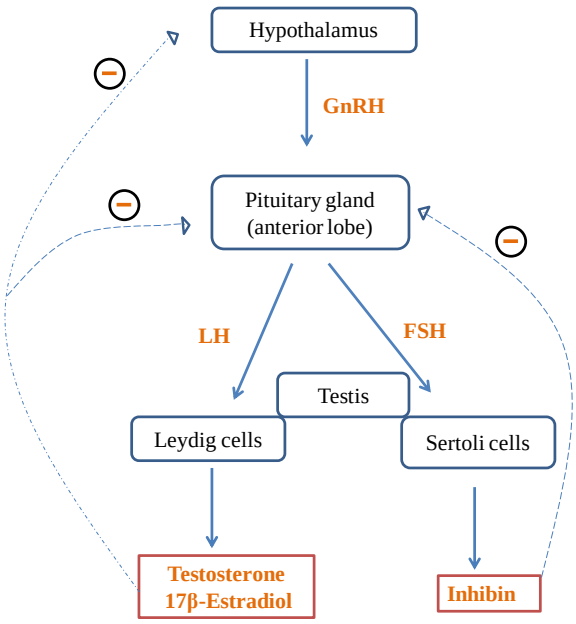

Figure 1.2 The hypothalamo-pituitary-testicular axis. Release of GnRH from the hypothalamus activates the pituitary secretion of gonadotropins, LH and FSH, which act on the testis and activate the production of testicular hormones (testosterone, estradiol and inhibin), which in turn produce feedback inhibition at the pituitary and hypothalamus (adapted from Weinbauer and Nieschlag, 1995).

The process of steroidogenesis is directly regulated by LH and 17β-estradiol. LH is a positive regulatory hormone. It increases the synthesis of androgens, such as testosterone and androstenedione, in Leydig cells by acting through LH receptors (Leung and Steele, 1992; Roberts and Zirkin, 1991). In contrast, 17β-estradiol is a negative regulatory hormone. It reduces androgen production in the Leydig cells by acting through estrogen receptors. Exogenous administration of 17β-estradiol decreased serum testosterone levels in male hypophysectomized (pituitary gland is removed) rats (Brinkmann et al., 1980). Also, studies with estrogen receptor-knockout male mice confirmed that 17β-estradiol has direct effects on the testis and these effects are mediated via estrogen receptors in the testis (Walker and Korach, 2004).

The process of spermatogenesis is regulated by testosterone, 17β-estradiol and FSH. 17β-Estradiol exerts its effects on spermatogenic cells by acting through estrogen receptors, whereas testosterone and FSH exert their effects by acting on Sertoli cell through androgen and FSH receptors, respectively (de Krester, 1987). Activation of Sertoli cells by FSH causes the cells to secrete a polypeptide hormone, inhibin, which in turn regulates the release of FSH from the pituitary by feedback mechanism (Weinbauer and Nieschlag, 1995).

Taken together, 17β-estradiol could regulate the physiological functions of the testis by acting directly on the testis through estrogen receptors or indirectly by affecting the hypothalamo-pituitary-testicular axis. This suggests that exogenous administration of 17β-estradiol or endocrine disrupting chemical such as BPA, could perturb the hypothalamo-pituitary-testicular axis or exert directly on the testis through estrogen receptors, and thereby cause reproductive abnormalities.

1.3 Xenobiotic-Metabolizing Enzymes

The xenobiotic-metabolizing enzymes are a diverse group of proteins that are involved in the metabolism of a wide variety of xenobiotic (foreign) compounds including drugs, environmental chemicals, and endogenous compounds such as cholesterol, steroid hormones and prostaglandins. Based on the type of metabolism, these enzymes are divided into two groups, Phase I and Phase II. Phase I enzymes typically catalyze oxidation, reduction and hydrolysis reactions. The end products of these reactions have a modified chemical structure, when compared with the parent compound, with an addition or removal of small functional groups such as hydroxyl (-OH), methyl ($–CH_3$) and carboxyl (-COOH) groups. Phase I category enzymes include CYP, epoxide hydrolase (EH), flavin monooxygenase and other oxidative enzymes.

Phase II metabolism includes several types of conjugation reactions such as glucuronidation, sulfation, and glutathione and amino acid conjugation, in which substrates (including end products of Phase I metabolism) undergo addition of endogenous small molecules as a conjugate and become more water soluble. The conjugative enzyme families include the uridine 5´-diphospho-glycosyltransferases, glutathione S-transferases, sulfotransferases and *N*-acetyltransferases (Guengirich 2009).

Among the Phase I and Phase II enzymes, cytochrome P450 are the most important xenobiotic-metabolizing enzymes, due to their versatile nature of their catalytic activity and their broad range of substrate specificity (Guengirich 2009). To date, nearly 75% of the marketed drugs are metabolized by CYP enzymes.

1.3.1 Cytochrome P450 Enzymes

All the CYP enzymes contain a heme prosthetic group (protoporphyrin IX) that is attached to a single polypeptide chain (apoprotein) consisting of approximately 500 amino acids. The iron atom in the heme prostetic group forms as a part of catalytic site of the CYP enzymes. In the reduced state, the iron atom of the heme prosthetic group produces a unique spectrum with maximum absorption at 450 nm when co-incubated with carbon monoxide. For this reason, the enzymes were named as cytochrome P450 ("P" for pigment and 450 nm is wavelength) (Omura and Sato, 1964).

CYP enzymes have been characterized in bacteria, fungi, plants, insects, birds and mammals. In mammals, CYP enzymes are highly expressed in liver but are also found in extrahepatic tissues including kidney, intestine, brain, lung, skin, adrenal, heart and gonads (Pavek and Dvorak, 2008). At the cellular level, these enzymes are localized predominantly on the smooth endoplasmic reticulum and to a lesser extent in mitochondria of mammalian cells (Omura, 2006).

To date, 57 CYP enzymes have been identified in humans and 83 CYP enzymes have been identified in rats. Based on amino acid sequence homology, the CYP enzymes have been classified into 18 families and 43 subfamilies. CYP enzymes that share more than 40% amino acid sequence homology are grouped into the same family, designated by an Arabic numeral (e.g., CYP1, CYP2 and CYP3). Within a family, CYP enzymes that share more than 55% amino acid sequence homology are grouped into the same subfamily, designated by a capital letter (e.g., CYP1A, CYP2B and CYP3A). The last number represents an individual enzyme within the subfamily (e.g., CYP1A1) (Hasler, 1999; Hoffman et al., 2001; Nelson et al., 2004).

Hepatic CYP enzymes belonging to families 1, 2, and 3 are involved mainly in the metabolism of xenobiotics including drugs, carcinogens and procarcinogens, and endobiotics like steroid hormones and fatty acids (Table 1.1). Within these families, CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP3A, subfamilies have been identified as major CYP enzymes responsible for xenobiotic metabolism in the liver (Nelson et al., 1993; Sutter et al., 1994).

Table 1.1 List of CYP enzymes that belongs to CYP1, CYP2 and CYP3 family in humans and rats (modified from Martignoni et al., 2006).

Family	Subfamily	Human	Rat
CYP1	A	1A1, 1A2	1A1, 1A2
	B	1B1	1B1
CYP2	A	2A6, 2A7, 2A13	2A1, 2A2, 2A3
	B	2B6	2B1, 2B2, 2B3
	C	2C8, 2C9, 2C19	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23
	D	2D6	2D1, 2D2, 2D3, 2D4, 2D5, 2D18
	E	2E1	2E1
CYP3	A	3A4, 3A5, 3A7, 3A43	3A1, 3A2, 3A9, 3A18, 3A62

1.3.1.1 Testicular Steroidogenic CYP Enzymes

The testis contains two types of CYP enzymes, steroidogenic CYP enzymes and the xenobiotic-metabolizing CYP enzymes (Leung et al., 2009). The steroidogenic CYP enzymes are responsible for the biosynthesis of testosterone and 17β-estradiol in the testis (Shan et al., 1993) using cholesterol as a starting material. These steroidogenic enzymes include CYP11A1 (cholesterol-side chain cleavage), CYP17A1 (17, 20 lyase) and CYP19A1 (reviewed in Sanderson, 2006; Payne and Hales, 2004). Hydroxysteroid dehydrogenase enzymes (HSD) are not CYP enzymes, but are actively involved in the steroidogenesis. Leydig cells are the primary

site for the *de novo* synthesis of steroids and also for the expression of steroidogenic CYP and HSD enzymes. The steps involved in steroidogenic pathway have been clearly illustrated in Figure 1.3.

CYP11A1 is primarily localized in the mitochondria, but not in the smooth endoplasmic reticulum, where most of CYP enzymes are expressed. CYP11A1 expression in the testis is limited to Leydig cells only. CYP11A1 is involved in the conversion of cholesterol into pregnenolone, which is a rate limiting and first step in the biosynthesis of steroids (Miller, 1988; Sanderson, 2006; Payne and Hales, 2004; Guo et al., 2007) (Figure 1.3).

The HSD enzymes including 3β- and 17β-HSD are the most important enzymes in the steroidogenesis process. 3β-HSD is involved in the conversion of pregnenolone to progesterone (Figure 1.3). In rats, 4 different isoforms of 3β-HSD (3β-HSD1-4) are detected at mRNA level and their expression pattern is tissue specific (Simard et al., 1993). In the adrenal and ovary, all forms of 3β-HSD are found except 3β-HSD-3. In the testis, 3β-HSD-1&2 are detected (3β-HSD-1 is predominant), but not 3β-HSD-3&4. In the testis, 3β-HSD enzymes are primarily localized in the Leydig cells (Simard et al., 1993; Payne and Hales, 2004). For this reason, 3β-HSD protein is used as a Leydig cell marker in the various histochemical experiments.

CYP17A1 is one of the critical enzymes in the steroidogenesis process. Functionally, CYP17A1 involved in the catalysis of two reactions, 17α-hydroxylation of the C21 steroids, pregnenolone or progesterone, followed by the cleavage of the C17–20 bond to produce the C19 steroids, dehydroepiandrosterone or androstenedione, respectively (Figure 1.3) (Fevold et al., 1989; Nakajin et al., 1981; Payne and Hales, 2004). The weak androgen androstenedione is further converted to testosterone by 17β-HSD. Further, testosterone and androstenediones are converted into 17β-estradiol and estrone, respectively, in the presence of CYP19A1 (aromatase).

In humans and rodents, CYP19A1 is predominantly expressed in adipose and brain and to a lesser extent in skin, liver, testis and other organs (Yanase et al., 2001; Zhao et al., 2005). In rat testis, it is expressed in all cell types including spermatogenic, Sertoli and Leydig cells (Levallet et al., 1998).

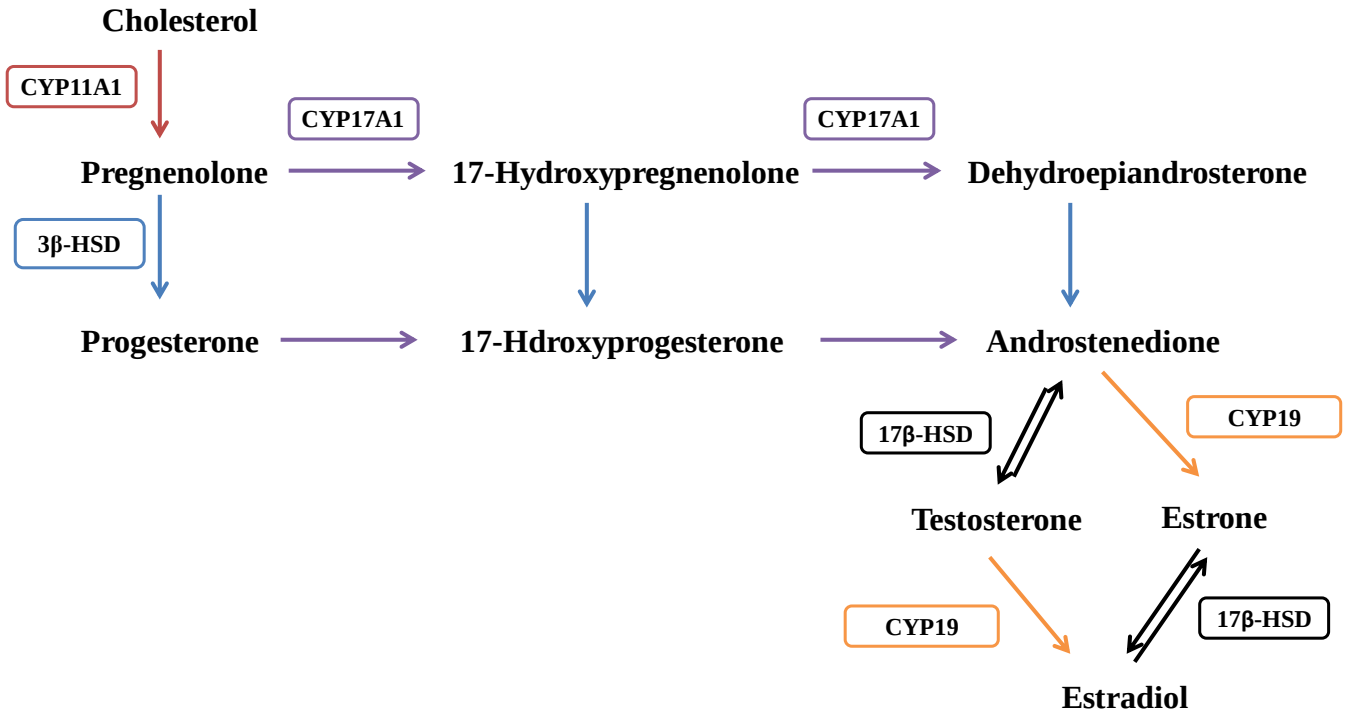

Figure 1.3 Schematic representation of various steps catalyzed by steroidogenic cytochrome P450 and hydroxysteroid dehydrogenase enzymes in the biosynthesis of steroid hormones in the testis (modified from Payne and Hales, 2004). Steroidogenic enzymes are highlighted in boxes and their respective reactions are denoted with similar color arrows.

1.3.1.2 CYP Enzymes Involved in the Xenobiotic Metabolism

Little is known about the expression and physiological functions of xenobiotic-metabolizing CYP enzymes that are present in the testis. Gene expression profiling of mouse and human testicular tissue has shown the presence of several CYP mRNA transcripts such as CYP1A1, CYP1B1, CYP2E1, CYP2R1, CYP2S1 and CYP2W1 (Choudhary et al., 2003; Bieche et al., 2007), but the translation of these mRNA transcripts into functional proteins has not been described. Other studies reported the presence of CYP1B1, CYP2A1 and CYP2E1 mRNA and protein in rat Leydig cells (Seng et al., 1991; Jiang et al., 1998; Lee et al., 1980; Otto et al., 2001; Leung et al., 2009; Sonderfan et al., 1989). Further details of these enzymes are given below.

1.3.1.2.1 CYP1B Subfamily

In rats and humans, CYP1B1 is the only reported member of the CYP1B subfamily. CYP1B1 is an extrahepatic CYP enzyme, highly expressed in steroidogenic tissues such as adrenal, testes and ovary (Walker et al., 1995, Otto et al., 1992), and is expressed to a lesser extent in steroid-responsive tissues, such as the breast, uterus and prostate (Bhattacharyya et al., 1995; Brake et al., 1999; Sutter et al., 1994). In human liver, only CYP1B1 mRNA was observed with no detectable levels of protein (Chang et al., 2003). Recent research reports suggest that CYP1B1 is up regulated in a variety of human cancers including breast, colon, lung, esophagus, skin and brain (Gajjar et al., 1994).

To date, there is no information on the physiological role of CYP1B1 in testis. Recombinant human CYP1B1 is reported to catalyze the oxidation of various xenobiotics, such as ethoxyresorufin, theophylline, caffeine and bufuoralol to their respective oxidative metabolites, *in vitro* (Shimada et al., 1997). CYP1B1 can participate in the bioactivation of

environmental procarcinogens such as benzo[a]pyrene to genotoxic metabolites and can play a role in the testicular toxicity (Shimada et al., 1996, Archibong, 2008).

The expression of CYP1B1 in reproductive tissues is under complex endocrine regulation. A study by Leung et al. (2009) revealed that testicular CYP1B1 protein expression is developmentally and hormonally regulated in male rats. The highest expression was observed in adult rats compared to pubertal and prepubertal rats. Testicular CYP1B1 expression was positively regulated by pituitary hormones and negatively regulated by 17β-estradiol. Hypophysectomy of male rats decreased testicular CYP1B1 expression by 90%, when compared with intact and untreated male rats. Further, CYP1B1 levels were restored in hypophysectomized rats following treatment with pituitary hormones, LH, FSH and prolactin. Maximum restoration was observed with mixture of LH, FSH and prolactin than LH alone (Leung et al., 2009). More recently, mechanism-based studies with MA-10 mouse Leydig cells and R2C rat Leydig cells revealed that CYP1B1 induction by LH is concentration- and time-dependent, and mediated by protein kinase A, but not by protein kinase C and G (Deb and Bandiera, 2011).

1.3.1.2.2 CYP2A Subfamily

The CYP2A subfamily includes three enzymes CYP2A6, CYP2A7 and CYP2A13 in humans. Among these, CYP2A6 is catalytically active in the metabolism of xenobiotics such as coumarin, nicotine, cyclophosphamide and aflatoxin B1 (Guengerich, 1997). CYP2A6 is highly expressed in liver and its protein level accounts for up to ~4% of total hepatic CYP content (Honkakoski and Negishi, 1997). In contrast, CYP2A7 and CYP2A13 expression levels are low in liver. CYP2A13 is predominantly expressed in the respiratory tract and plays a role in the

bioactivation of aflatoxin B1 to carcinogenic metabolites (He et al., 2006; Martignoni et al., 2006).

The CYP2A subfamily consists of three members CYP2A1, CYP2A2 and CYP2A3 in rats. CYP2A1 and CYP2A2 are predominantly expressed in the liver, but their expression is gender dependent, CYP2A1 and CYP2A2 are female and male dominant isoforms, respectively. CYP2A1 catalyzes the metabolism of testosterone to a 7α-hydroxylated metabolite. This reaction is characteristic and is used as a catalytic marker for CYP2A1 in rat hepatic microsomes (Ryan and Levin, 1993). CYP2A2 is involved in the biotransformation of testosterone to 7α-, 16α-, 6β- and predominantly 15α-hydroxylated metabolites.

CYP2A1 expression in rat liver is hormonally and developmentally regulated. Its highest expression was observed at the prepubertal stage in both male and female rats, but CYP2A1 protein levels gradually declined by approximately 50% in adult male rats, but not in adult female rats (Waxman et al., 1985). Hormonal regulation of CYP2A1 in rat liver is quite complex. Studies with hypophysectomized animals revealed that hepatic expression of CYP2A1 is under the suppressive control of pituitary hormones in the adult male rats (Waxman et al., 1989a). Hypophysectomy of adult rats resulted in elevated hepatic levels of CYP2A1 in males, but no effect was observed in females. Expression levels were increased in hypophysectomized rats after administration of GH in a female specific secretion pattern, but expression levels were reduced with administration of GH in a male specific secretion pattern (Waxman et al., 1989a; Waxman and Chang, 1995). This could be a plausible reason for the gender-dependent expression of CYP2A enzymes in rat liver. Neonatally castrated rats had increased hepatic-expression of CYP2A1 level, but expression was reduced to normal levels after treatment with testosterone. Conversely, estradiol treatment restored expression levels of CYP2A1 in

ovariectomized adult female rats, in which hepatic expression was decreased (Waxman et al., 1989a). This suggests that hepatic expression of CYP2A1 is negatively regulated by testosterone in male rats, but positively regulated by estrogens in female rats.

Little is known about CYP2A1 expression in rat testis. Sonderfan et al., (1989) first reported the presence of CYP2A1 in rat testis microsomes. Later in 1991, Seng and coworkers reported that CYP2A1 was localized in the Leydig cells, and played an important role in the metabolism of testosterone. It is not known, however, whether, if the CYP2A1 expression in rat testis is regulated by hormones or affected by exposure to endocrine disrupting chemicals.

1.3.1.2.3 CYP2E Subfamily

CYP2E1 is the only gene of the CYP2E subfamily in rats and humans. It is constitutively expressed in liver and other extrahepatic tissues such as lung, intestine and other organs (Martignoni et al., 2006). CYP2E1 was initially designated as the "membrane-bound ethanol-oxidizing enzyme" due to its role in the metabolism of ethanol (Lierber et al., 1970). CYP2E1 is thought to play an important role in the conversion of acetone to acetol. Acetol is further converted to glucose through the gluconeogenesis pathway (Koop and Casazza, 1985). However, studies on C*yp2e*1-null mice have shown that CYP2E1 is not required for mammalian development and physiological homeostasis (Lee at al., 1996).

CYP2E1 has been extensively studied because of its pivotal role in the metabolism of toxic xenobiotics such as acetaminophen, azoxymethane and low molecular weight organic compounds including, aniline, acrylamide, benzene, butanol and carbon tetrachloride (Guengerich et al., 1991). For example, hepatic CYP2E1 mediated metabolism of acetaminophen

and azoxymethane produce reactive intermediates that cause hepatotoxicity and colon cancer, respectively (Jollow et al., 1973, Shone et al., 1991).

Due to the role of CYP2E1 in the bioactivation of toxic chemicals, it is important to investigate the presence of CYP2E1 in reproductive tissues. Little is known about the CYP2E1 expression in gonadal tissues. To date, only one study (Jiang et al., 1998) reported the presence of CYP2E1 at the mRNA and protein level in rat testis.

1.3.1.2.4 NADPH-Cytochrome P450 Oxidoreductase

NADPH-cytochrome P450 oxidoreductase (POR) is a membrane bound protein expressed in the endoplasmic reticulum of all eukaryotic cells (Porter, 1991). In mammals, POR is expressed in all tissues that have been tested, with the highest expression in liver (Ram and Waxman 1992). POR is not involved in the metabolism of xenobiotics or endogenous hormones, but it is an obligatory partner for CYP enzymes, including both steroidogenic and xenobiotic-metabolizing CYP enzymes.

CYP enzymes convert lipophilic compounds to more water soluble metabolites by addition of one oxygen atom. In order to carry out this catalytic reaction, CYP enzymes require electrons from NADPH. However, microsomal CYP enzymes are not capable of receiving the electrons directly from NADPH. CYP enzymes receive electrons via POR. As shown Figure 1.4, POR contains two subunits, a flavin adenine dinucleotide (FAD) and a flavin mononucleotide (FMN) (Porter and Kasper, 1985; Wang et al., 1997; Tee et al., 2011). Initially, the FAD moiety receives electrons from the reduced form of NADPH and transfers the electrons to the FMN. FMN then transfers the electrons to CYP enzymes. In this manner, POR participates and acts as

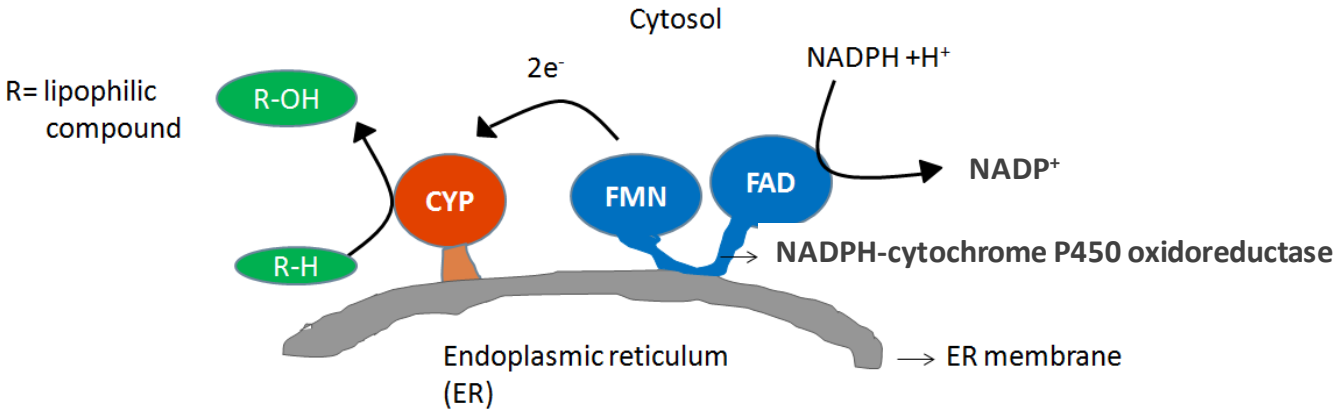

Figure 1.4 Diagrammatic representation of the function of NADPH-cytochrome P450 oxidoreductase (POR). Electrons flow from the reduced form of nicotinamide adenine dinucleotide phosphate ($NADPH+H^+$) to the flavin adenine dinucleotide (FAD) moiety of POR, which is bound to the endoplasmic reticulum membrane. The FAD moiety undergoes conformational changes and transfers the electrons to flavin mononucleotide (FMN). These electrons are essential for the catalytic activity of cytochrome P450 (CYP) enzymes, which convert lipophilic compounds (xenobiotics) to more water soluble metabolites by addition of an oxygen atom (modified from Teen et al ., 2011).

rate limiting factor in CYP-mediated catalytic activity. POR also serves as a cofactor for several non-P450 enzymes, including heme oxygenase (Wilks et al., 1995), and fatty acid elongase (IIan et al., 1981). In rats, POR is highly expressed in liver, and, to a lesser extent in extrahepatic tissues such as kidney, lung, adrenal and testis. POR expression in these tissues is regulated mainly by the pituitary hormones (Waxman et al., 1989b; Ram and Waxman 1992). Hypophysectomy of adult male rats resulted in decreased hepatic expression of POR by 85%, when compared with intact male rats. Expression levels of POR in hypophysectomised rats were restored to normal levels after treatment with thyroxin, and to a lesser extent with Adrenocorticotropic hormone (Waxman et al., 1989b). In the same study, administration of human chorionic gonadotropin to hypophysectomised rats had no effect on the reduced levels of POR. Hypophysectomy decreased expression and activities of POR in kidney, lung, adrenal and testis (Ram and Waxman 1992). This decrease in POR expression was restored to normal levels in kidney, lung, adrenal, but not in testis, after administration of thyroxin to hypophysectomized rats (Ram and Waxman 1992). No published reports are available on effect of steroid hormones or of endocrine disrupting chemicals on the expression of POR in adult male rat testis.

1.3.2 Epoxide Hydrolase

Epoxide hydrolase (EH) is an important xenobiotic-metabolizing enzyme that is involved in the hydrolysis of epoxides. Several forms of EH are present in mammals including cholesterol EH, hepoxilin A_3 EH, leukotriene A_4 EH, soluble EH (sEH) and microsomal EH (mEH). Among these, microsomal EH (mEH) has been studied extensively due to its potential role in the detoxification of drugs and environmental compounds (Arnand et al., 2005; Decker et al., 2009).

In contrast, sEH and cholesterol EH are involved in the hydrolysis of endogenous compounds such as cholesterol and unsaturated fatty acids (Decker et al., 2009).

In rats and humans, mEH is highly expressed in liver and is present at relatively low levels in other extrahepatic tissues including intestine, lung, kidney and testis (Oesh et al., 1977; de Waziers et al., 1990). mEH catalyzes the hydrolysis of epoxides to corresponding trans-dihydrodiols, using water as a cofactor (Armstrong 1987; Oesch et al., 2000; Arand et al., 2005). In general, epoxides that are formed during the metabolism of xenobiotics and endogenous compounds and are highly unstable and often lead to the formation of reactive intermediates. Due to their unstable nature, epoxides tend to react with nucleophilic cellular components such as DNA, and produce localized tissue toxicity and mutagenesis resulting in cancer (Oesch et al., 2000). For this reason, mEH is an important enzyme in the detoxification of reactive epoxide intermediates that are produced via CYP enzymes. For example, oxidative metabolism of styrene, an industrial chemical, by various hepatic CYP enzymes produce styrene-7,8-oxide, which is a genotoxic epoxide metabolite. mEH catalyzes the hydrolysis of genotoxic styrene-7,8-oxide to a less toxic metabolite, called phenyl glycol (Sumner and Fennell, 1994; Decker et al., 2009). In this way, mEH acts as a detoxifying enzyme. In another example (Figure 1.5), CYP mediated metabolism of benzo[*a*]pyrene, a polycyclic aryl hydrocarbon chemical, produces optically active toxic epoxides (benzo[*a*]pyrene-7,8-oxide), which undergo hydrolysis to less toxic dihydrodiols (benzo[*a*]pyrene-7,8-dihydrodiol) by mEH.

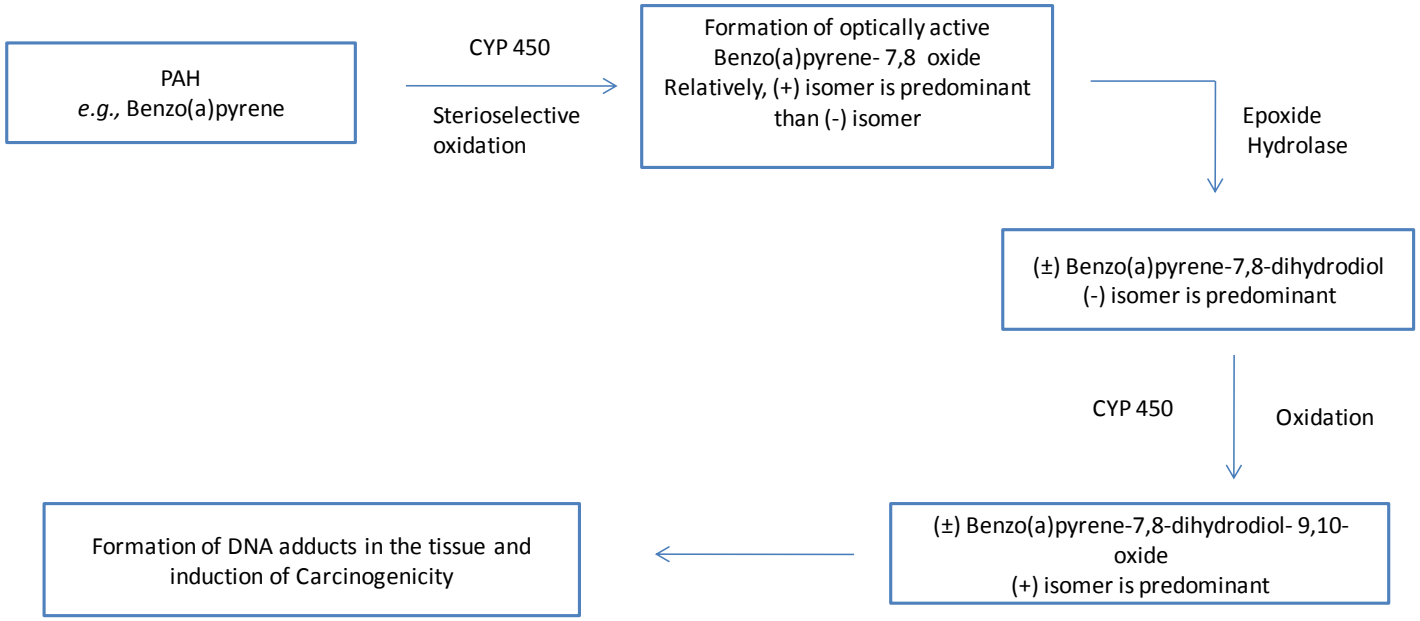

Figure 1.5 Schematic representation of the interplay of testicular CYP enzymes and EH in the bioactivation of benzo[*a*]pyrene (Levin et al., 1982).

mEH is expressed in interstitial cells and spermatogenic cells in rat testis (Ishii-Ohba et al., 1984; Mukhtar et al 1978). Based on the reported catalytic activity studies using microsomes, it is presumed that predominant expression could be in spermatogenic cells (Lee at al., 1980).

mEH expression levels are developmentally regulated in rat testis as mEH catalytic activity levels increased from the prepubertal stage to the onset of puberty (45 days of age) (Mukhtar et al., 1978). It has been reported that testicular mEH is regulated by pituitary and gonadal hormones (Lee et al., 1980). Hypophysectomy of adult male rats decreased mEH activity in testicular micrososmes, but activity levels were restored to normal in microsomes prepared from LH-, FSH- and testosterone-treated hypophysectomized rats (Lee et al., 1980). The effect of 17β-estradiol and estrogen-mimicking compounds such as Bisphenol A on testicular mEH expression in intact male rats has not been explored.

1.4 Overview of Estrogens

Estrogens are well recognized as female sex hormones. They are also found in men at circulating levels that are higher than those in postmenopausal women (Janssen et al., 1998). There are three forms of circulating estrogens, 17β-estradiol, estrone and estriol. Although all three forms are biologically active, 17β-estradiol has highest affinity for estrogen receptors (Weichman and Notides, 1980). Circulating levels of 17β-estradiol in men mostly (75-90%) arise from brain and adipose tissue. A smaller extent (10-25%) is produced in the testes (Levine et al., 1997). Estrogens are synthesized from androstenedione and testosterone, in the presence of CYP19A1 (aromatase). In humans and rodents, CYP19A1 is predominantly expressed in adipose tissues and brain, and to a lesser extent in the skin, liver, bone, ovary, placenta, testis and other organs (Yanase et al., 2001; Zhao et al., 2005). The biosynthesis of estrogens is shown in Figure 1.6.

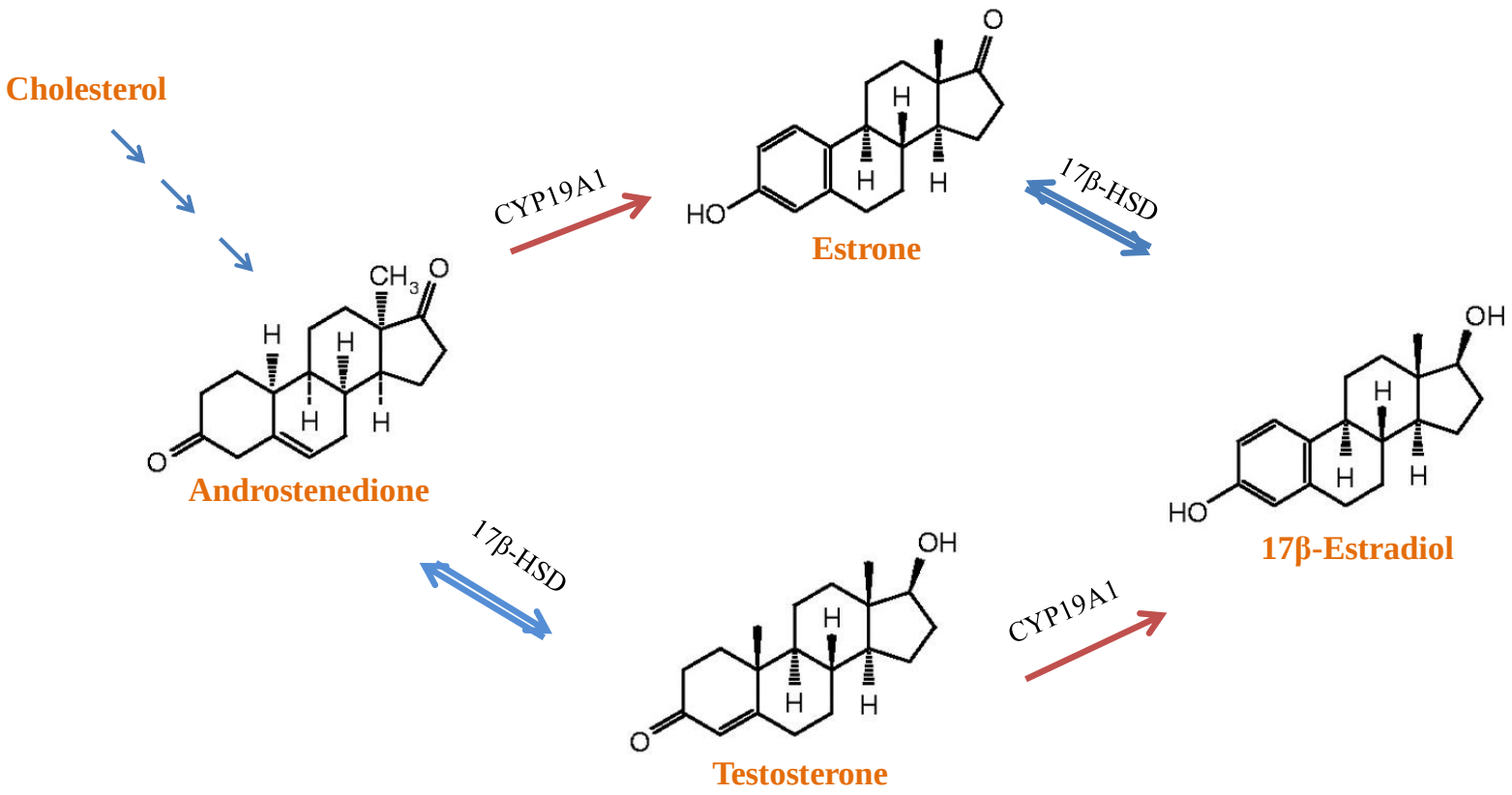

Figure 1.6 Synthesis of estrogens. The circulating androgens, androstenedione and testosterone, are converted to estrone and 17β-estradiol, respectively, in the presence of CYP19A1 (modified from Payne and Hales, 2004). Abbreviations: 17β-HSD, 17β-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxystroid dehydrogenase; CYP19A1, cytochrome P450 19A1.

1.4.1 Physiological Functions of Estrogens

Estrogens have pronounced role in various physiological functions in men such as maintenance of bone health, cardiovascular function and neuroendocrinal functions (Rochera et al., 2007; Sader and Gillies and McArthur, 2010). Estrogens are essential for normal bone growth. Men with congenital aromatase deficiency or low levels of 17β-estradiol shown to exhibit delayed bone maturation, continued and linear growth of bone, reduced mineralization of bone and osteoporosis (Rochera et al., 2007). It has been reported that these men suffer from metabolic syndrome disorder, which includes deposition of abdominal fat and insulin resistance (Maffei et al., 2004; Rochira et al., 2007). Treatment with exogenous 17β-estradiol improves bone health and glycemic control in these patients. Abnormalities those observed in men with congenital aromatase deficiency were observed in aromatase gene knock-out mice (Fischer et al., 1998; Murata et al., 2002).

Estrogens also play a role in spermatogenesis process. Men with congenital aromatase deficiency and estrogen receptor gene knock-out mice have small testis and a great reduction in sperm count and quality (reviewed in Akingbemi, 2005). This indicates that 17β-estradiol is essential for the normal development and functional maintenance of male reproductive organs.

Most of the effects of 17β-estradiol are mediated through estrogen receptors (ERs). There are two forms of ERs, ERα and ERβ. These two forms belong to the steroidal nuclear receptor family and are localized in cytosol. In rats, ERs are expressed in liver, brain, testis and other tissues (Fischer et al., 1984; Azcoitia et al., 1999, Pelletier et al., 2000; Perez et al., 2003). In the testis, ERα is mainly localized to Leydig cells and ERβ is localized to Sertoli and spermatogenic cells (Fisher et al., 1997; Saunders et al., 1998; Pelletier et al., 2000; Bois et al., 2010). Some

studies also reported the presence of ERα in Sertoli and spermatogenic cells and ERβ in Leydig cells (Nakamura et al., 2010; Lucas et al., 2008).

1.4.2 Biotransformation of Estrogens

17β-Estradiol is mainly metabolized in the liver and to smaller extent in extrahepatic tissues to water-soluble metabolites. 17β-Estradiol is metabolized by two pathways; oxidation and conjugation. In the conjugation pathway, 17β-estradiol or its metabolites undergoes conjugation with sulfate and glucuronic acid to produce hydrophilic conjugates. Circulating 17β-estradiol is always in dynamic equilibrium with estrone, thus estrone sulfate is the major metabolite of 17β-estradiol that excreted in the urine (Tsuchiya et al., 2005).

Another major pathway of 17β-estradiol metabolism is oxidation (Figure 1.6). Studies by Lee et al. (2001 and 2002) revealed that 2- and 4-hydroxyestradiols are the major oxidative metabolites of 17β-estradiol after incubation with human hepatic microsomes. Similar results were also observed with extrahepatic tissue homogenates, such as brain, pituitary and human fetus (Ball and Knuppen, 1978).

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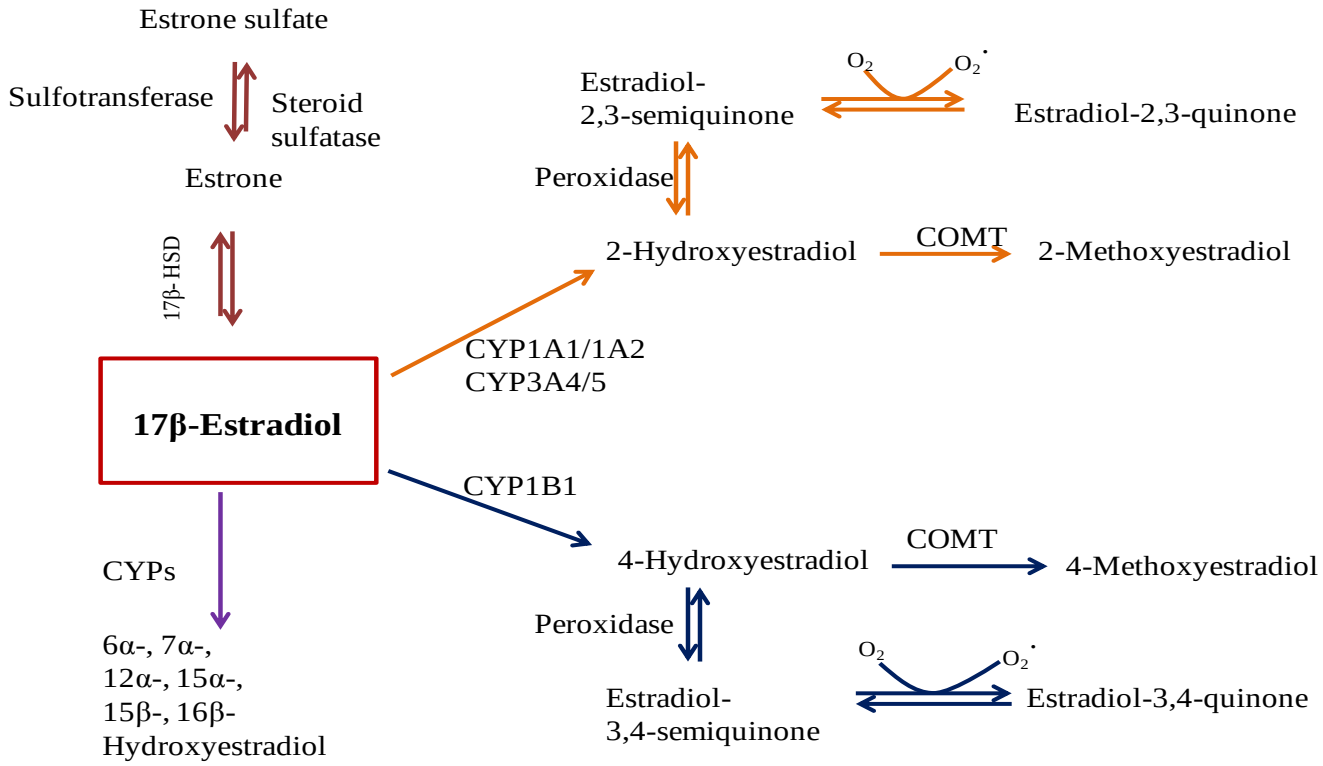


Figure 1.7 Schematic representation of various metabolic pathways of 17β-estradiol. 17β-Estradiol can be metabolized to 2- and 4-hydroxyestradiol, which is further metabolized to 2- and 4-methoxyestradiol, respectively, by catechol-O-methyl transferases (COMT). To a minor extent, 2- and 4-hydroxy metabolites can undergo redox cycling and produces reactive semiquinones and oxygen free radicals, which cause DNA damage and localized tissue toxicity (adapted from Tsuchiya et al., 2005).

A study by Lee et al. (2003) characterized the CYP enzymes responsible for the oxidative metabolism of estradiol in the human liver microsomes and suggested that CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4 are mainly responsible for the formation of the 2-hydroxy metabolite, whereas CYP1B1 is primarily responsible for the formation of the 4-hydroxy metabolite (Lee et al 2003; Hayes et al., 1996).

These two metabolites are further converted to 2- and 4-methoxyestradiol, respectively, by catechol-O-methyltransferase (COMT). To a lesser extent, 2- and 4-methoxyestradiol can undergo redox-cycling to produce semiquinones and oxygen free radicals. Due to the slow turnover of 4-hydroxyestradiol by COMT, more of the 4-hydroxyestradiol is converted to semiquinones and oxygen free radicals, which are highly unstable and tend to react with nucleophilic components of cellular components such as DNA and cause localized tissue toxicity or carcinogenicity, *in vivo* and *in vitro* (Newbold and Liehr, 2000; Nutter et al., 1991; Nutter et al., 1991).

1.4.3 Effect of 17β-Estradiol on CYP Expression

Studies with experimental animals suggest that 17β-estradiol plays an important role in the regulation of CYP expression in liver and other extrahepatic tissues such as ovary and testis. In rats, hepatic expression of sex-specific enzymes such as CYP2C7 and CYP2C11 is regulated by 17β-estradiol (Waxman et al., 1985). CYP2C7 is female specific enzyme and its expression in female rats is 2-3 folds higher than that of male rats. Hepatic CYP2C7 expression in female rats is positively regulated by 17β-estradiol (Waxman et al., 1985). Administration of 17β-estradiol to ovariectomized rats restored the reduced level of CYP2C7 (Waxman et al., 1985). Similarly, induction of CYP2C7 was observed in intact male rats after treatment with 17β-estradiol

(Bandiera and Dworschak, 1992). CYP2C11 is a male specific enzyme and its expression in male rats is negatively regulated by 17β-estradiol. Neonatal administration of 17β-estradiol reduced the expression of CYP2C11 in male rats (Bandiera and Dworschak, 1992). In another study, administration of 17β-estradiol to adult female rats decreased CYP2A1 expression and increased CYP3A1 expression in liver (Ickenstein and Bandiera, 2002).

17β-Estradiol has a suppressive effect on the expression of xenobiotic-metabolizing CYP enzymes in rat testis. A study by Leung et al. (2009) demonstrated that testicular expression of CYP1B1 is suppressed by 90% in adult rats that were treated with 17β-estradiol benzoate at a dosage of 1.5mg/kg, once daily, for 14 days. Another study by Deb et al. 2011 made similar observations when neonatal and pubertal rats were administred with 17β-estradiol for 3 and 14 days, respectively. It has also been reported that 17β-estradiol elicits dose- and time-dependent suppression of CYP1B1 expression at the mRNA level in MA-10 mouse Leydig cells *in vitro* (Deb et al., 2011).

1.5 Endocrine-Disrupting Chemicals

The endocrine system, a complex network of various glands including pituitary, thyroid, adrenal, testis and ovary and the hormones these glands secrete, plays critical roles in the maintenance of physiological functions in the body. Endocrine-disrupting chemicals are either man-made or of natural origin and can mimic the endogenous hormones, interfere with their actions or disturb the entire endocrine network. According to the U.S. Environmental Protection Agency (EPA), an endocrine-disrupting chemical was defined as “an exogenous agent that interferes with 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" (Kavlock et al., 1996).

A wide variety of molecules that are used for agricultural and industrial purposes have been found to be endocrine disruptors. Example include the following synthetic chemicals used as industrial solvents/lubricants and their byproducts (polychlorinated biphenyls, polybrominated biphenyls, dioxins); chemicals used in plastics including bisphenol A and phthalates; chemicals used as pesticides including methoxychlor, chlorpyrifos; pharmaceutical agents (diethylstilbestrol); natural chemicals that are present in the human food and animal food (e.g., phytoestrogens such as genistein and coumesterol) (reviewed in Casals-casas and Desvergne, 2010).

In my research, I have chosen bisphenol A as a representative endocrine-disrupting chemical to investigate its effects on testicular expression of xenobiotic-metabolizing enzymes.

1.5.1 Bisphenol A

Bisphenol A [2,2-(4,4-dihydroxydiphenol) propane] is a high volume industrial chemical. The estimated production of bisphenol A (BPA) is nearly 8 billion pounds per day. The expected growth in demand for BPA is 6-10% and approximately 100 tons may be released into the atmosphere per year (Kavlock et al., 1996). BPA was first synthesized by A.P. Dianin in 1891. In 1936, BPA was rediscovered in a search for synthetic estrogens, but due to its weak estrogenic activity and the discovery of diethylstibesterol (DES), which is a more potent estrogen, BPA was abandoned from using as a synthetic estrogen (Dodds and Lawson, 1936). Later in 1957, BPA was rediscovered as a starting material for plastics and commercial production was started in U.S.A.

BPA belongs to a class of endocrine-disrupting chemicals that bind to ER and mimic the effects of endogenous 17β-estradiol. BPA produces adverse effects on estrogen-dependant tissues by perturbing the homeostasis of endogenous hormones. For many years, it was thought that BPA had no harmful effects on human health. However, in the past two decades, research interest in BPA and its effects on human health have increased tremendously because of its reported estrogenic activity *in vitro* and *in vivo*, and its ubiquitous presence in the environment (reviewed in Vandenberg et al., 2007).

As an industrial chemical, BPA is a monomer and is used as a building block for the synthesis of polycarbonate, epoxy, unsaturated polyester and polysulfone resins. BPA containing plastic materials have a wide variety of applications, for example as a food contact layer of food cans, as a packaging material to store food, beverages and other chemicals, and also as industrial floorings, protective coatings and adhesives. BPA containing polycarbonates are widely used in baby bottles, kitchen dishes and many other household appliances (reviewed in Vandenberg et al., 2007; Vandenberg et al., 2009; Rubin, 2011).

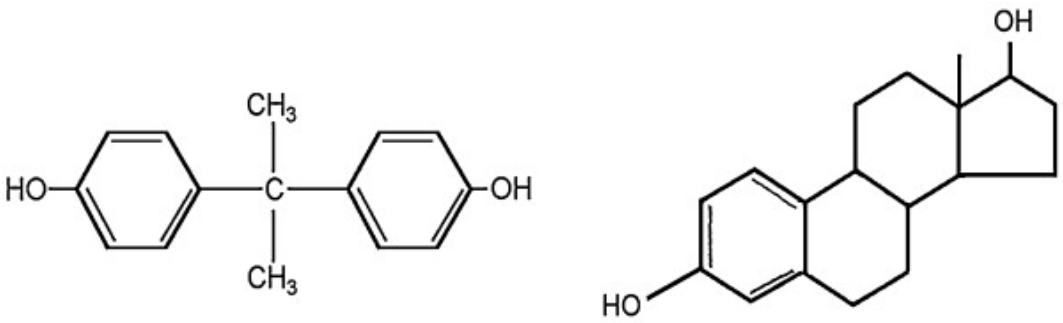
Polymerization (a chemical reaction to form polymer) of BPA monomers with ester bonds of diphenyl carbamate is a key step in the synthesis of polycarbonate plastics and epoxy resins that are used in many products. These ester bonds are easily hydrolyzed in the presence of acids, bases and also at higher temperatures (Welshons et al., 2006). Hydrolysis of ester bonds in finished products such as baby bottles and containers leads to the leaching of BPA monomers out of plastic and into the material contained in the bottle such as drinking water or food (Vandenberg et al., 2007).

BPA has been detected everywhere in the environment, including drinking water, food and vegetables, and also in human body fluids such as saliva and blood. Further, BPA levels

were quantified using ELISA and HPLC in serum from healthy men (1.49 ± 0.11 ng/ml) and urine from healthy men (5.18 μg/l), serum from non-pregnant women (2 ± 0.8 ng/ml), serum from pregnant women (1.5 ± 0.8 ng/ml), amniotic fluid from 15-18 weeks of gestation period women (8.3 ± 8.9 ng/ml) and serum from fetus (2.2 ± 1.8 ng/ml), in saliva after application of dental sealant (50 mg dental sealant implanted in humans and immediately collected saliva for 1hr, 90-931 μg of BPA detected) and vegetable food cans (4 - 23 μg of BPA recovered/can) (Ikezuki et al., 2002; Olea et al., 1996; Brotons et al 1995; Calafat et al., 2005). The concentrations of BPA that were detected in saliva (after application of dental sealants) and in food cans were sufficiently high to exert estrogenic activity *in vitro* (Olea et al., 1996; Brotons et al., 1995).

1.5.1.1 Chemical Structure and Receptor Binding Studies

Although BPA is structurally dissimilar to 17β-estradiol, the presence of two phenol functional groups in its chemical structure allows BPA to bind to ER and to act as an agonist (Figure 1.7). The two phenol groups are essential to bind and activate ERs. Based on results of the saturation ligand-binding and the luciferase transactivation studies, BPA has 10 times higher affinity for ERβ than ERα (Kuiper et al., 1997; Kuiper et al., 1998). However, the relative binding affinity and transactivation activity of BPA for ERα and ERβ is 10,000 and 2 times lower, respectively, when compared to 17β-estradiol. It was reported that BPA differentially influences the co-activator recruitment of ERs. For example, recruitment of co-activator TIF2 by the BPA/ERβ complex is 500 times greater potent than BPA/ERα complex (Routledge et al., 2000; and reviewed in Wetherill et al., 2007). Differences in ER binding affinity and co-activator recruitment with BPA could lead to complex tissue-specific and cell-type specific response.



Bisphenol A **17β-Estradiol**

Figure 1.8 Chemical structures of bisphenol A and 17β-estradiol (taken from Rubin, 2011).

1.5.1.2 Estrogenic Activities

The estrogenic activity of BPA was first reported by Dodds and Lawson in 1936, where induction of vaginal cornification was observed in ovariectomized rats following exposure to BPA at 85 mg/kg, three times daily, for 3 days. In 1993, Feldman and coworkers (Krishnan et al., 1993) rediscovered the estrogenic property of BPA during an investigation to find the estrogen binding protein in yeast. Surprisingly, they found estrogenic activity with yeast conditioned culture medium in the absence of 17β-estradiol, where the culture medium was prepared using distilled water autoclaved in polycarbonate flasks. Later they concluded that BPA was responsible for the estrogenic activity that was observed with yeast conditioned culture medium and that BPA was released from the polycarbonate culture flasks during autoclaving into the distilled water. In the same study, they confirmed the estrogenic activity of BPA using different methods such as competitive binding studies to ERs, induction of progesterone receptors and proliferation of MCF-7 cells. These estrogenic actions were not observed in the presence of tamoxifen, a partial ER antagonist. In another study (Ashby and Tinwell, 1998), increased uterus weight was observed in immature rats after treatment with BPA at 400, 600 and 800 mg/kg per day for 3 days. Many later studies confirmed the estrogenic activity of BPA using

the uterotrophic assay as an *in vivo* marker (Markey et al., 2001; Nagel et al., 2001; reviewed in Richter et al., 2007). More recently, a study reported that BPA at a dose of 750 μg/mouse stimulates uterine proliferation in an ERα dependent manner (Hewitt and Korach, 2011). It has also been reported that BPA produced adverse effects on testis, epididymis, sperm and seminal vesicles (reviewed in Richter et al., 2007).

The types of effect described above are mostly restricted to the reproductive system, but BPA also has diverse effects on various other systems (Figure 1.8) including changes in epigenetic modification that lead to the development of prostate cancer; perturbation of thyroid hormone function, altered development and function of the central nervous system, changes in body composition and body weight, altered glucose homeostasis and alteration in immune system response (reviewed in Rubin, 2011).

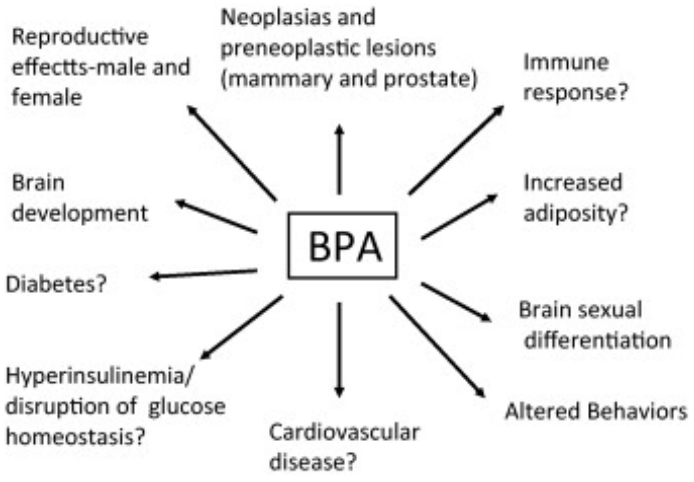


Figure 1.9 Overview of various putative effects of bisphenol A exposure (taken from Rubin, 2011).

1.5.1.3 Effects on Xenobiotic-Metabolizing Enzymes

Results from studies with experimental animals suggest that BPA is an estrogenic chemical and that can alter the serum hormonal levels by perturbing the hypothalamo-pituitary

axis and can directly bind to ERs in the target organs such as testis and ovaries. For example, administration of BPA at 2.4, 10, 100 and 200 mg/kg/day for 15 days decreased serum LH, testosterone and estradiol levels in male rats (Akingbemi et al., 2004). In another study, exposure of male rats to BPA at 200 mg/kg/day for 4 weeks decreased serum LH, FSH, and testosterone levels compared to vehicle-treated rats (Nakamura et al., 2010). Thoei et al. (2001) reported that administration of BPA at 1mg/kg/day for 14 days increased serum prolactin and LH hormones and decreased testosterone levels in male rats. The three studies suggest that BPA has a suppressive effect on steroidogenesis *in vivo*. Suppression of steroidogenesis can be attributed to decreased expression of steroidogenic enzymes including CYP17A1, CYP11A1 and HSD. It is known that expression of testicular xenobiotic-metabolizing CYP enzymes is influenced by pituitary and gonadal hormones (Lee et al., 1980, Leung et al., 2009), so we hypothesize that BPA, as an endocrine-disrupting chemical, can alter the expression of testicular xenobiotic-metabolizing CYP enzymes. No reports are available on this aspect at present.

1.6 Rationale

Suppression or induction of testicular xenobiotic-metabolizing CYP enzymes, in response to various exogenous foreign compounds such as therapeutic drugs, environmental pollutants and hormones could adversely affect the physiological function of the testis. In light of this, it is essential to know about the expression of CYP enzymes and their regulation in testis. To date, the presence of CYP1B1, CYP2A1 and CYP2E1 enzymes (at the protein level) and their regulation in rat testis have been reported (Seng et al., 1991; Jiang et al., 1998; Lee et al., 2007; Otto et al., 2001, Leung et al., 2009; Sonderfan et al., 1989; Deb et al., 2010). However, no comprehensive or systematic study of the expression and regulation of CYP and other xenobiotic-metabolizing enzymes, at the protein level, in rat testis has been reported.

A previous study (Leung et al., 2009) had shown that testicular expression of CYP1B1 was regulated by pituitary and gonadal hormones in adult male rats. In the same study, testicular expression of CYP1B1 was suppressed by more than 90% in adult male rats after administration of 17β-estradiol benzoate at a dosage of 1.5 mg/kg body weight once daily for 14 consecutive days. Results of this study indicate that circulating estrogens have a potential role in the regulation of testicular CYP expression in adult male rats. As an extension of the study by Leung et al.,(2009), I used a dose-response study with 17β-estradiol benzoate and assessed the testicular expression of CYP and other xenobiotic-metabolizing enzymes to determine the minimum dosage of 17β-estradiol benzoate that could produce a significant effect.

Knowing the suppressive effect of estrogens on the testicular expression of CYP enzymes in adult male rats (Leung et al., 2009), I hypothesize that a similar risk could be associated with the synthetic xenoestrogens such as BPA. BPA is ubiquitously present in the environment and one of the chemicals that humans are highly exposed, to an extent where 95% of the US

populations have detected BPA in their urine sample (Calafat et al., 2005). Based on results of the various *in vitro* and *in vivo* studies (Krishnan et al., 1993; Thoei et al., 2001), it is accepted that BPA could act as an estrogenic chemical and exerts some of its actions through estrogenic receptors and also has suppressive control on the expression of steroidogenic enzymes in the rat testis and adrenal (Akingbemi et al., 2004). However, it is not known, if BPA has the similar effects as 17β-estradiol benzoate on the expression of xenobiotic-metabolizing enzymes in adult male rat testis.

1.7 Hypotheses

1. Xenobiotic-metabolizing CYP enzymes, mEH and POR are expressed in rat testis and their localization within the testis is cell type specific.
2. Administration of exogenous 17β-estradiol benzoate or an estrogenic chemical such as bisphenol A will suppress expression of xenobiotic-metabolizing CYP, mEH and POR enzymes in adult rat testes.

1.8 Specific Aims

1. To detect, quantify and determine the cellular localization of various CYP and other xenobiotic-metabolizing enzymes in adult Sprague-Dawley rat testis.
2. To determine the effect of 17β-estradiol benzoate on testicular expression of xenobiotic-metabolizing CYP enzymes, mEH and POR in adult male Sprague-Dawley rats.
3. To determine the effect of bisphenol A on the expression of testicular xenobiotic-metabolizing CYP enzymes, mEH and POR in adult male Sprague-Dawley rats.

2. Materials and Methods

2.1 Chemicals

Chemicals and reagents were obtained from the following sources:

BD Gentest (Woburn, MA, USA)

Rabbit anti-rat CYP1B1 serum; baculovirus-insect cell microsomes containing expressed rat CYP enzyme (CYP1A1, CYP1A2, CYP1B1, CYP2C6, CYP2D1 and CYP2D2) coexpressed with rat CYP oxidoreductase (BD Supersomes) were purchased from BD Biosciences (Oakville, Ontario, Canada) (BD-Supersomes^{TM})

Bio-Rad Laboratories Inc. (Hercules, CA, USA)

Precision Plus Protein^{TM} prestained dual color standards. A mixture of 10 recombinant proteins (10–250 kDa)

Chemicon International Inc. (Temecula, CA, USA)

Rabbit anti-human/rat CYP2E1 serum; rabbit anti-rat CYP4A1/2/3 serum; rabbit anti-human/rat P450-reductase serum

Daiichi Pure Chemicals Co., LTD (Tokyo, Japan)

Rabbit anti-human CYP 2D6 serum (cross react with rat species)

Fisher Scientific (Vancouver, BC, Canada)

Acrylamide 99.9%; *N*,*N*´-methylene-bis-acrylamide (Bis); sodium dodecyl sulphate, 2-mercaptoethanol; *N*,*N*,*N*´,*N*´-tetramethylethylenediamine (TEMED); tris (hydroxymethyl) aminoethane (Tris base); *p*-nitro-blue tetrazolium chloride (NBT); 5-bromo-4-chloro-3-

indolyphosphate p-toluidine salt (BCIP); bovine serum albumin (BSA); sodium chloride, sodium phosphate; potassium dihydrogen phosphate; ACS grade methanol; dimethyl sufoxide (DMSO); propylene glycol; concentrated hydrochloric acid; bromophenol blue; sodium chloride; sodium phosphate; potassium dihydrogen phosphate; sucrose; sodium carbonate; sodium bicarbonate and sucrose

Invitrogen Corporation (Camarillo, CA, USA)

Normal goat serum (protein concentration is not given in the product data sheet); normal goat IgG (IgG concentration 1mg/ml)

Jakson Immuno Research Laboratory Inc. (West Grove, PA, USA)

Alkaline phosphatase-conjugated affinipure $F(ab´)_2$ fragment goat anti-rabbit IgG (H+L) (antibody concentration 0.6 mg/ml); alkaline phosphatase-conjugated donkey anti-sheep IgG (protein concentration 6.9 mg/ml); normal rabbit IgG (IgG concentration 11 mg/ml); normal rabbit serum (protein concentration 60 mg/ml)

Pacific Milk Division (Vancouver, BC, Canada)

Skim milk powder

Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA)

Rabbit anti-human 3β-HSD polyclonal IgG (cross react with 3β-HSD isoforms 1-4 of rat origin); goat anti-mouse CYP17A1 polyclonal IgG (cross react with CYP17A1 of rat origin); alkaline phosphatase-conjugated swine anti-goat IgG (IgG concentration 0.6 mg/ml)

Sigma-Aldrich Corporation (St. Louis, MO, USA)

Bisphenol A (white crystalline powder, assay purity ≥99%); 17β-estradiol 3-benzoate (white crystalline powder, assay purity ≥97%); alkaline phosphatase-conjugated swine anti-goat IgG; rabbit anti-human calnexin, polyclonal IgG

VWR Scientific products (West Chester, PA, USA) and ***Pall Corporation (Pensacola, FL, USA)***

Nitrocellulose membrane (Bio Trace^{TM} NT), pore size 0.2 μm, thickness 101.6 μm

Professor S.M Bandiera (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, BC, Canada)

Purified rat EH protein; rabbit anti-rat CYP1A1 polyclonal IgG; rabbit anti-rat CYP1A2 serum; rabbit anti-rat EH polyclonal IgG; rabbit anti-rat CYP2B1 polyclonal IgG (cross react with CYP2B1, CYP2B2 and CYP2B3 of rat origin); purified rat CYP2B1 protein; rabbit anti-rat CYP2C11 polyclonal IgG (cross react with CYP2C6, CYP2C7, CYP2C11, CYP2C12 and CYP13 of rat origin); rabbit anti-rat CYP3A polyclonal IgG (cross react with CYP3A1 and CYP3A2); purified rat CYP2C11 protein; testis microsomes prepared from individual untreated adult Sprague-Dawely rats (Prepared by Grace Leung, June 2005, stored at -80°C); liver microsomes prepared from individual untreated adult Long-Evans rats (Prepared by Claudio Erratico, May 2009, stored at -80°C); pooled liver microsomes prepared from clofibrate (300 mg/kg/day for 7 days) treated Long-Evans rats (prepared by Stelvio Bandiera, December 1988, stored at -80); pooled adrenal microsomes prepared from adult male Sprague-Dawely rats (prepared by Subrata Deb, July 2006, stored at -80°C)

Professor Paul E. Thomas (Rutgers, The State University of New Jersey, NJ, USA)

Purified rat liver cytochrome P450-oxidoreductase protein; sheep anti-rat CYP2A1 polyclonal IgG (cross react with CYP2A2, Anderson et al., 1998); rabbit anti-human CYP2E1 serum

Professor A. Parkinson (University of Kansas Medical Center, KS, USA)

Purified rat liver CYP2A1 protein

Professor A. Wayne Vogl (Life sciences institute, University of British Columbia, Vancouver, BC, Canada)

Alexa fluor™ 488 goat anti-rabbit IgG (H+L) conjugate (concentration 2 mg/ml); Alexa fluor™ 568 goat anti-rabbit IgG (H+L) conjugate (concentration 2 mg/ml); Alexa fluor™ 568 donkey anti-goat IgG (H+L) conjugate (concentration 2 mg/ml); Alexa fluor™ 568 donkey anti-sheep IgG (H+L) conjugate (concentration 2 mg/ml). These chemicals are procured from Molecular Probes Inc (Eugene, OR, USA)

2.2 Animals

Adult male Sprague-Dawley rats, 8-9 weeks of age, 250-300g of body weight, were purchased from Charles River (Montreal, Canada). They were housed at the Animal Resource Unit of the University of British Columbia (British Columbia, Canada). Rats were housed in polycarbonate cages with corn-cob bedding, and had ad libitum access to food (Rodent laboratory diet, No. 5001, PMI Feeds Inc., Richmond, IN) and water. The room was maintained at 21°C with 35 - 45% relative humidity, and on a 12 h light dark cycle (6 a.m on and 6 p.m off). Animals were allowed to acclimatize to these conditions for one week before starting the

treatment. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. Another set of adult male Sprague-Dawely rats for immunohistochemical analysis were provided by Dr. Wayne Vogl. The animals were purchased from Charles River (Montreal, Canada). Animals were housed and used in accordance with guidelines established by the Canadian Council on Animal Care and according to protocols approved by the Animal Care Committee of the University of British Columbia.

2.3 Animal Treatment and Tissue Collection

17β-Estradiol benzoate (EB) Study

Following one week of acclimatization, rats were randomly divided into 5 groups with 4 rats in each group, except for the vehicle group (n = 5). Rats were injected subcutaneously with EB (0.004, 0.04, 0.4 or 4 μmol/kg body weight) or an equal volume (1ml/kg body weight) of vehicle (propylene glycol), once daily for 14 days. Rats were terminated by decapitation on the morning following the last dose. Testes, adrenal and liver were harvested from each rat.

Propylene glycol was used as the solvent for preparing the EB solutions. A clear solution was observed after mixing EB solutions at 4 μmol/ml with propylene glycol for one hour on a magnetic stirrer. A large volume (30 ml) of EB solutions at 4 μmol/ml was prepared initially and the remaining EB solutions at 0.004, 0.04 and 0.4 μmol/ml were made by diluting the higher concentration with propylene glycol. All stock solutions were stored at room temperature in a dark cabinet. All stock solutions were thoroughly mixed and preheated at 37°C for 30 min before injecting to the animals.

BPA Study

Rats were randomly divided into six groups with four rats in each group. Animals were subcutaneously injected saline (0.9% NaCl), vehicle (propylene glycol) or BPA at 400, 800 or 1600 μmol/kg body weight, once daily for 14 days. Rats were terminated by decapitation on the morning following the last dose. Testes, adrenal and liver were harvested from each rat.

BPA was dissolved in propylene glycol. A clear solution was observed after mixing thoroughly for four hours on magnetic stirrer. A large volume (6 ml) of BPA solutions was prepared at higher concentration (1600 μmol/ml) initially and the remaining BPA solutions (800 and 1600 μmol/ml) were made from the higher concentration (1600 μmol/ml) by diluting with propylene glycol. All the stock solutions were prepared 16-18 hr before dosing and stored at room temperature in a dark cabinet. The solutions were thoroughly mixed and preheated at 37°C for 30 min before injecting the animals. Solubility limitations were observed at BPA concentration beyond 1600 μmol/ml. For this reason, 1600 μmol/kg of BPA was chosen as the highest BPA dosage for the present study.

2.4 Preparation of Testes Microsomes

Immediately after decapitation of animals, testes (both right and left testis of each animal) were excised and homogenized with 20 ml of ice-cold 0.05M Tris buffer (pH 7.4) containing 1.15% KCl, using a Potter-Elvehjem glass mortar (Talboys Engineering Corp., Emerson NJ, USA). The tissue homogenates were spun at 9,000g for 20 min at 5°C in a Beckman J2-21 centrifuge (Beckman Instrument, Palo Alto, CA, USA). The supernatants were collected and further spun at 105,000g for 60 min at 5°C in Beckman LE-80 ultracentrifuge (Lu and Levin,

1972). The resulting pellet was suspended in freshly prepared, ice-cold 0.25 M sucrose and stored at -80°C.

2.5 Determination of Total Protein Concentration

Total protein was measured by the method of Lowry et al., (1951) using bovine serum albumin as the protein calibration standards. Testicular microsomal samples were diluted (1:20) using distilled water and 100 µl was used for protein determination. Absorbance was measured at 750 nm using a microplate reader (model EL 309, BIO-TEK Instruments Inc.). Protein concentrations were calculated from the slope and intercept values generated from the calibration curve (absorbance versus protein concentrations) of the calibration standards.

2.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli, (1970) using a Hoefer SE 600 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The stacking gel is composed of 3% acrylamide: *N*,*N*´-methylene-bis-acrylamide (Bis) (22.2%:0.6% w/w), 0.125 M Tris-HC1 (pH 6.8), 0.1% (w/v) SDS, 0.08% (w/v) ammonium persulphate, and 0.05% (v/v) *N*,*N*,*N*´,*N*´-tetramethylethylenediamine (TEMED). The separating gel was composed of 7.5% acrylamide:Bis (22.2%:0.6% w/w), 0.375 Tris-HC1 (pH 8.8), 0.1% (w/v) SDS, 0.042% (w/v) ammonium persulphate, and 0.03% (v/v) TEMED. The electrophoresis buffer contained 0.1 M Tris base, 0.767M glycine and 0.4% (w/v) SDS. Microsomal and standard protein samples were diluted to appropriate concentrations with sample dilution buffer containing 0.062 M Tris-HC1 (pH 6.8), 1% (w/v) SDS, 0.001% (w/v) bromophenol blue, 10% (v/v) glycerol, and 5% (v/v)

mercaptoethanol. The diluted samples were boiled for 2 min. A 20 µl of aliquot of each sample and molecular weight marker were loaded (for the amount of protein, refer to Table 2.1) in to each well in the stacking gel and subjected to electrophoresis under constant current of 12 mA/gel for approximately 1 h or until the dye front entered the stacking gel. The current was then increased to 23 mA/gel for approximately 2 h.

Table 2.1 Amount of testicular microsomal protein loaded per lane for SDS-PAGE

Treatment group	Protein of interest						
	CYP1B1	**CYP2A1**	**CYP17A1**	**EH**	**POR**	**Calnexin**	**3β-HSD**
Study 2 (EB study)							
Propylene glycol	10	20	20	10	40	20	80
0.004 μmol/kg	10	20	20	10	40	20	80
0.04 μmol/kg	20	80	80	20	80	20	80
0.4 μmol/kg	20	80	80	20	80	20	80
4 μmol/kg	20	80	80	20	80	20	80
Study 3 (BPA study)							
Saline	10	20	20	10	40	20	80
Propylene glycol	10	20	20	10	40	20	80
400 μmol/kg	20	80	80	20	80	20	80
800 μmol/kg	20	80	80	20	80	20	80
1600 μmol/kg	20	80	80	20	80	20	80

Values are presented as μg of total testicular microsomal protein

Note 1: In Study 1, for all the immunoblot experiments, unless otherwise mentioned, the amount of testicular microsomal protein loaded was 20 μg/lane. In search for each protein of interest, a total of two immunoblot experiments were performed on two different days.

Note 2: In Study 2 and 3, for each protein of interest, immunoblot experiments were performed three times on different days. In the first immunoblot experiment, 20 μg of testicular microsomal protein per lane was loaded, after considering the results the amount of testicular microsomal protein per lane was changed to values as reported in Table 2.1 for the remaining experiments.

2.7 Immunoblot Analysis

Testicular microsomal proteins that were resolved by SDS-PAGE were transferred electrophoretically onto nitrocellulose membranes at a constant current of 0.4 A for 2 hr using a Hoefer Transphor Apparatus (Model TE 52) (Towbin et al., 1979). After transfer, nitrocellulose membranes were incubated overnight at 4°C with blocking solution consisting of 1% BSA, 3% skim milk powder in PBS. The membranes were washed three times (10 min/wash) with wash buffer (0.05% Tween in PBS). Then membranes were incubated at 37°C for a period of 2 h with appropriate primary antibodies that were prepared in antibody dilution buffer (1% BSA, 3% skim milk powder, 0.05% Tween in PBS) (For antibodies and dilution factors refer to Tables 2.1 and 2.2). For detection of CYP17A1 and calnexin proteins, the nitrocellulose membranes were incubated with appropriate primary antibodies at 4°C for overnight followed by incubation at for 2 h. Membranes were washed with wash buffer and then incubated with respective alkaline phosphatase-conjugated secondary antibody at 37°C for 2h (For antibodies and dilution factors refer to Tables 2.1 and 2.2). Membranes were washed with wash buffer for three times (10 min/wash). Finally, membranes washed with distilled water to remove excess wash buffer contents and exposed to chromogenic substrate solution. Substrate solution (0.01% NBT, 0.005% BCIP in 0.1M Tris –HCl, 0.5 mM $MgCl_2$, pH 9.5) was freshly prepared under dim light and added to membranes as a substrate to alkaline phosphatase.

We used colorimetric detection to visualize the protein bands. Alkaline phosphatse (conjugated to secondary antibodies) produced a dark purple color bands when it come in contact with its chromogenic substrate (BCIP and NBT). The intensity of the color is dependent on amount of protein loaded and incubation time (time allowed for color development) (Table 2.4). Based on experience from several previous studies, our laboratory established that color

development time for most of the CYP proteins is linear between 3-5 min. Immunoreactive protein bands were quantified using a pdi 420oe scanning densitometer (Bio-Rad Laboratories, Hercules, CA) as described previously (Ickenstein and Bandiera, 2002). In brief, the immunoreactive band intensity was measured as contour quantity (CQ), calculated by the software program as optical density × contour area (OD × mm^2). The CQ values were divided by CQ value of a purified standard that was included in each gel. CYP1B1, CYP2A1, mEH and POR protein concentration in testicular microsomes were quantified by using their respective recombinant or purified proteins as calibration standards (described in Results section).

2.8 Immunohistochemical Analysis

Tissue sections preparation

All the immumohistochemical experiments were performed in the laboratory of Dr. Wayne Vogl, Life Sciences Institute, UBC. Rats were deeply anaesthetized with isofluorane and then testis was excised immediately. Initially, testes were perfused with PBS (150 mM NaCl, 5 mM KCl, 0.8 mM KH_2PO_4, and 3.2 mM Na_2HPO_4, pH 7.4) for 2 min through the spermatic arteries using a 26-gauge needle and then perfused with a fixative consisting of 3% paraformaldehyde in PBS. Finally, testes were perfused with PBS for 30 min to remove excess fixative. The perfusion-fixed testis was placed in a small amount of Optimal Cutting Temperature compound (Sakura Finetek USA, Torrance, CA) and then frozen using liquid nitrogen. Sections (5 μm thick) were cut from each testis using a cryostat. These tissue sections were immediately placed onto ploy-L-lysine (poly-L-Lysine (Sigma-Aldrich, Oakville, ON, Canada) coated glass slides and then submerged in cold acetone (-20°C) for 5 min. Slides were

removed from acetone and allowed to air-dry. A water-repellent circle was drawn around each tissue section with a Liquid Blocker Super Pap Pen (Cedarlane, Burlington, ON, Canada) to retain liquid over the sections. Then tissue sections were allowed to air dry for 15 min and used them for immunostaining as described below.

2.9 Immunostaining Procedure

Immunostaining and examination of tissue sections was performed as described in Young et al., (2009). In brief, tissue sections were blocked with 5% normal goat serum prepared in TPBS-BSA (0.5% of Tween-20 and 0.1% BSA dissolved in PBS) for 20 min. Blocking solution was decanted and tissue sections were incubated at 4°C for overnight with appropriate primary antibodies (Table 2.5) prepared in antibody dilution buffer containing 1% normal goat serum in TPBS-BSA. Tissue sections were washed three times for 30 min with TPBS-BSA and then incubated for 1 h at 37°C with secondary antibodies conjugated to fluorescent dyes (Alexa Fluor™ 488 or 568), which were diluted to 1:100 in TPBS-BSA (Table 2.5). Following incubation, tissue sections were washed with TPBS-BSA three times, 30 min/wash and then coverslips were mounted on the tissue sections using vectashield (Vector Labs, Burlington, ON, Canada) containing 4′,6′-diamidino-2-phenylindole (DAPI). Procedures involved with secondary antibody were performed under dim light to minimize the bleaching of fluorescent label from the tissue sections. Immunostaining was examined and photographs were captured by using Zeiss Axiophot microscope (Carls Zeiss Microimaging GmbH, Gottingen, Germany), using appropriate fluorescence filters. As a staining control for the primary antibody, the primary antibody was replaced with normal immunoglobulin or serum at the same concentration as

shown in Table 2.5. All experiments were done at least in duplicate using tissue from different animals.

2.10 Immunohistochemical Images Collection Procedure

Immunohistochemical images were collected using a computer that was connected to a fluorescent microscope. Using appropriate wavelength filters, tissues sections were exposed to fluorescent light for specific period of time, and separate images were collected for DAPI staining and secondary antibody flurescent staining using Northern Eclipse (version 8) software. The exposure time was kept constant for both experimental and control tissue sections. Collected images (DAPI and antibody fluorescent staining) were merged using ImageJ 1.43m software (National Institute of Health, USA).

2.11 Statistical Analysis

The differences in means of the different groups were analyzed by one-way or two-way ANOVA and followed by the Newman-Keuls multiple comparison test (SigmaStat software program, SPSS Inc., Chicago, IL). Mean differences with a p-value < 0.05 were considered to be statistically significant.

Table 2.2 Amount of primary and secondary antibodies used for immunoblot analysis

Protein (to be detected)	Primary antibody		Secondary antibody	
	Name of the antibody	Dilution/concentrations used for incubations	Name of the antibody	Dilution used for incubations
CYP1A1	Rabbit anti-rat CYP1A1 polyclonal IgG	2 μg/ml	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000
CYP1A2	Rabbit anti-rat CYP1A2 serum	1:500	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000
CYP1B1	Rabbit anti-rat CYP1B1 serum	1:1000	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000
CYP2A1	Sheep anti-rat CYP2A1 polyclonal IgG	20 μg/ml	Alkaline phosphatase-conjugated-donkey anti-sheep IgG	1:3000
CYP2B1	Rabbit anti-rat CYP2B1 polyclonal IgG	4 μg/ml	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000
CYP2C6/7/11/12/13	Rabbit anti -rat CYP2C polyclonal IgG	25 μg/ml	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000
CYP2D1/CYP2D2	Rabbit anti-human CYP 2D6 serum	1:500	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000
CYP2E1	Rabbit anti-human/rat CYP2E1 serum	1:1000	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000

Table 2.3 Amount of primary and secondary antibodies used for immunoblot analysis

Protein (to be detected)	Primary antibody		Secondary antibody	
	Name of the antibody	Dilutions/concentrations used for incubation	Name of the antibody	Dilutions used for incubation
CYP3A1/2	Rabbit anti- rat CYP 3A polyclonal IgG	50 μg/ml	Alkaline phosphatase-conjugated -goat anti-rabbit IgG	1:3000
CYP4A1/A2/A3	Rabbit anti-rat CYP4A1/2/3 serum	1:500	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000
CYP17A1	Goat anti-mouse CYP17 purified polyclonal IgG	2 μg/ml	Alkaline phosphatase-conjugated-swine anti-goat IgG	1:3000
POR	Rabbit anti-human/rat POR serum	1:5000	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000
EH	Rabbit anti- rat epoxide hydrolase purified IgG	20 μg/ml	Alkaline phosphatase-conjugated -goat anti-rabbit IgG	1:3000
3β-HSD	Rabbit anti-human 3β-HSD polyclonal IgG	2 μg/ml	Alkaline phosphatase-conjugated -goat anti-rabbit IgG	1:3000
Calnexin	Rabbit anti-human calnexin serum	1:10,000	Alkaline phosphatase-conjugated-goat anti-rabbit IgG	1:3000

Table 2.4 Details of the experimental conditions for the immunoblot assay in Study 1

Protein	Standard protein (amount loaded/lane)	Testicular microsomal protein (amount loaded/lane) (n = 4)	Color development time (min)	
			Time at which immunnoreactive band (color) for standard protein visualized	Maximum time allowed for the color development
CYP1A1	cDNA-expressed rat CYP1A1 protein (0.25 pmol)	20 µg	2	10
CYP1A2	cDNA-expressed rat CYP1A2 protein (0.1 pmol)	20 µg	3	15
CYP1B1	cDNA-expressed rat CYP1B1 protein (0.2 pmol)	20 µg	30 sec	3
CYP2A1	Purified rat liver CYP2A1 protein (0.125 pmol)	20 µg	2	4
CYP2B1	Purified rat liver CYP2B1 protein (0.125 pmol)	20 µg	3	10
CYP2C6/7/11/12/13	cDNA-expressed rat CYP2C6 protein (0.25 pmol), CYP2C7 protein (0.125 pmol), CYP2C12 protein (0.1 pmol), CYP2C13 protein (0.25 pmol) and purified rat liver CYP2C11 (0.2 pmol)	20 µg	2	7
CYP2D1/2D2	cDNA-expressed rat CYP2D1 protein (0.2 pmol), CYP2D2 (0.1 pmol)	20 µg	1	5
CYP2E1	Hepatic microsomes prepared from untreated rats (20 µg)	20 µg	5	30
CYP3A1/2	cDNA-expressed rat CYP3A1 and CYP3A2 protein (0.25 pmol)	20 µg	1	5
CYP4A1/A2/A3	Hepatic microsomes (5 µg) prepared from clofibrate treated (300 mg/kg for 7days) Long-Evans rats	20-60 µg	30 sec	15
CYP17A1	Adrenal microsomes prepared from untreated rats (20 µg)	20 µg	3	5
POR	Purified rat liver P450-reductase protein (25 ng)	20-80 µg	3	5
mEH	Purified rat liver epoxide hydrolase protein (0.001 µg)	20-80 µg	30 sec	3

Table 2.5 Amount of primary and secondary antibodies used for immunohistochemical analysis

Protein (to be detected)	Primary antibody		Secondary antibody	
	Name of the antibody (experimental/control)	Concentration (or) Dilution used for incubation	Name of the antibody	Dilution used for incubation
CYP1B1	Rabbit anti-rat CYP1B1 serum.	1:100	Alexa Fluor™ 488 goat anti-rabbit IgG	1:100
	Normal rabbit serum	1:100		1:100
CYP2A1	Sheep anti-rat CYP2A1 polyclonal IgG	80 μg/ml	Alexa Fluor™ 568 donkey anti-sheep IgG	1:100
	Normal sheep IgG	80 μg/ml		1:100
CYP17A1	Goat anti-mouse CYP17 purified polyclonal IgG	0.5 μg/ml	Alexa Fluor™ 568 donkey anti-goat IgG	1:100
	Normal goat IgG	0.5 μg/ml		1:100
EH	Rabbit anti- rat EH purified IgG	60 μg/ml	Alexa Fluor™ 488 goat anti-rabbit IgG	1:100
	Normal rabbit IgG	60 μg/ml		1:100
POR	Rabbit anti human /rat POR serum	1:500	Alexa Fluor™ 568 goat anti-rabbit IgG	1:100
	Normal rabbit serum	1:500		1:100
3β-HSD	Rabbit anti human 3β-HSD polyclonal IgG	8 μg/ml	Alexa Fluor™ 488 goat anti-rabbit IgG	1:100
	Rabbit polyclonal IgG	8 μg/ml		1:100

3. Results

The results of three studies (Study 1, 2 and 3) are presented. The aim of Study 1 is to characterize the expression of various CYP and other xenobiotic-metabolizing enzymes, using immunoblot and immunohistochemical analyses, in testes collected from untreated adult rats. The results of this study provide information about the xenobiotic-metabolizing enzymes that are constitutively expressed in rat testis and their cellular localization. The aim of Study 2 is to determine the effect of treatment with 17β-estradiol benzoate (EB) on the protein expression of xenobiotic-metabolizing enzymes (detected in Study 1) in adult rat testis. The aim of Study 3 is to determine the effect of treatment with bisphenol A (BPA) on the protein expression of xenobiotic-metabolizing enzymes (detected in Study 1) in adult rat testis.

3.1 Study 1: Immunochemical Characterization of CYP and Other Xenobiotic-Metabolizing Enzymes in Rat Testis

3.1.1 Immunoblot Analysis

Immunoblot analysis was used to detect the protein expression of xenobiotic-metabolizing enzymes (at basal levels) in rat testicular microsomes prepared from adult, male untreated rats (n = 4). Testicular microsomal samples for these experiments were obtained from a previous study by Leung et al. (2009) and were stored at -80°C in our laboratory. The expression of following xenobiotic-metabolizing enzymes CYP1A1, CYP1A2, CYP1B1, CYP2A1, CYP2B1, CYP2E1, CYP2D1, CYP2D2, CYP2C6, CYP2C7, CYP2C12 CYP2C13, CYP3A1, CYP3A2, CYP4A1, CYP4A2, CYP4A3, POR and mEH was investigated. The expression of two steroidogenic enzymes (CYP17A1 and 3β-HSD) was also investigated and these two proteins

were served as reference proteins for the microsomes prepared from testis. As this is an explorative study, the immunoblot experimental conditions (Table 3.1), such as the amount of testicular microsomal protein, specificity and amount of antibodies used and color development time (Table 3.1) were varied from one protein to another. Most of these experimental conditions were drawn from previous studies from our laboratory (Leung et al., 2009; Deb et al., 2010; Anderson et al., 1998).

Testicular microsomal samples were probed with rabbit anti-rat CYP1A1 polyclonal IgG to determine if CYP1A1 was expressed in rat testis. No immunoreactive protein band having the similar electrophoretic mobility as CYP1A1 standard (~55 kDa) was detected in lanes containing testicular microsomal samples. The protein band for the CYP1A1 standard was observed within 2 min after the immunoblot membrane was exposed to chromogenic substrate solution. The protein band for CYP1A1 was not detected in testicular microsomes even when color reaction time was extended up to 10 min to try to detect weak signals (Figure 3.1A).

To determine if CYP1A2 was expressed in rat testis, testicular microsomes were probed with CYP1A2 specific antibody. Protein band having similar electrophoretic mobility as the CYP1A2 standard with molecular size of ~48 kDa was not detected in the lanes containing testicular microsomal samples (Figure 3.1B).

To determine if CYP1B1 was expressed in rat testis, testicular microsomes were probed with rabbit anti-rat CYP1B1 serum. A single protein band was reasily detected having similar electrophoretic mobility as CYP1B1 standard (~55 kDa) in the lanes containing testicular microsomal samples prepared from untreated rats (Figure 3.1C).

To determine if CYP2A1 was expressed in rat testis, testicular microsomes were probed with sheep anti-rat CYP2A1 polyclonal IgG. A single protein band having the similar

electrophoretic mobility as the CYP2A1 standard (~48 kDa) was detected (Figure 3.1D) in the lanes containing testicular microsomal samples prepared from untreated rats.

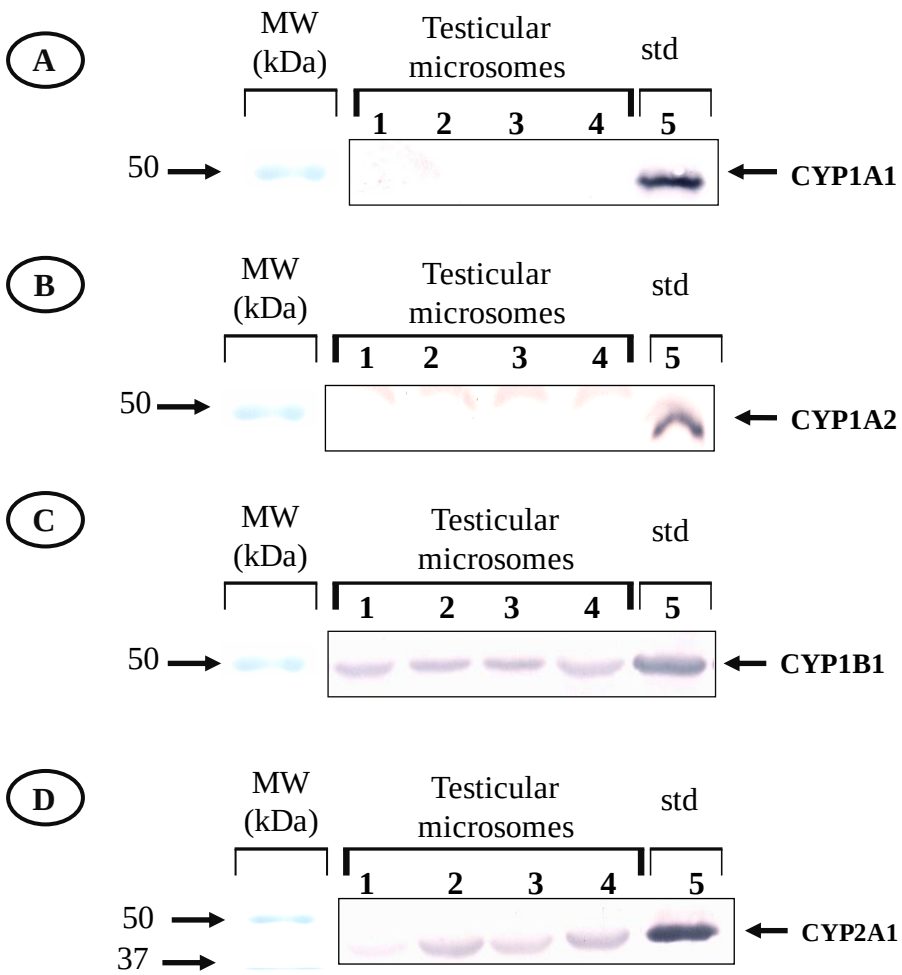


Figure 3.1 Representative immunoblots of rat testicular microsomes probed with antibodies against CYP1A1, CYP1A2, CYP1B1 and CYP2A1 in rat testicular microsomes. The amount of testicular microsomal protein used was 20 μg/lane for all the immunoblots. For antibody details refer to Table 2.1 and 2.2. (A). Immunoblot of testicular microsomes probed with rabbit anti-rat CYP1A1 polyclonal IgG. Lanes 1-4 contained testicular microsomes. Lane 5 contained cDNA-expressed rat CYP1A1 protein (0.25 pmol/lane). (B). Immunoblot of testicular microsomes probed with rabbit anti-rat CYP1A2 serum. Lanes 1-4 contained testicular microsomes. Lane 5 contained cDNA-expressed rat CYP1A2 protein (0.1 pmol/lane). (C). Immunoblot of testicular microsomes probed with rabbit anti-rat CYP1B1 serum. Lanes 1-4 contained testicular microsomes. Lane 5 contained cDNA-expressed rat CYP1B1 protein (0.2 pmol/lane). (D). Immunoblot of testicular microsomes probed with sheep anti-rat CYP2A1 polyclonal IgG. Lanes 1-4 contained testicular microsomes. Lane 5 contained purified rat liver CYP2A1 protein (0.125 pmol/lane).

Rabbit anti-rat CYP2B1 polyclonal IgG, which react with CYP2B1, CYP2B2 and CYP2B3 (Ickenstein et al., 2001), was used to detect if CYP2B enzymes were expressed in rat testis. The protein bands specific for any of the CYP2B isoforms were not detected in lanes containing testicular microsomal samples, but strong protein bands (at molecular size ~50 kDa) were detected in lanes containing standard CYP2B1 and rat liver microsomal samples. A non-specific protein band, which has higher mobility than CYP2B1, was also detected in lane containing liver microsomes. This band could be either CYP2B2 or CYP2B3 (Figure 3.2A).

For CYP2C proteins, the immunoblot contained testicular microsomes was probed with rabbit anti-rat CYP2C polyclonal IgG, which cross react with CYP2C6, CYP2C7, CYP2C11, CYP2C12 and CYP2C13 (Bandiera et al., 1986). The protein bands specific for any of the CYP2C isoforms were not detected in lanes containing testicular microsomal samples, but clear bands were observed with the standard proteins with molecular size of ~50 kDa (Figure 3.2B). The protein bands for standard CYP2C proteins were detected readily within 3 min of color reaction time, but no protein bands were detecte for CYP2C proteins in testicular microsomes ever after color reaction time was extended to 7 min.

Rabbit anti-human CYP2D6 serum, which can detect rat CYP2D1 and CYP2D2 (Anand and Bandiera, 2008), was used to detect if CYP2D enzymes were expressed in rat testis. The protein bands specific for CYP2D1 and CYP2D2 isoforms were not detected in lanes containing testicular microsomal samples, but clear protein bands (at molecular size ~50 kDa) were detected in lanes containing standard CYP2D and rat liver microsomal samples (Figure 3.2C).

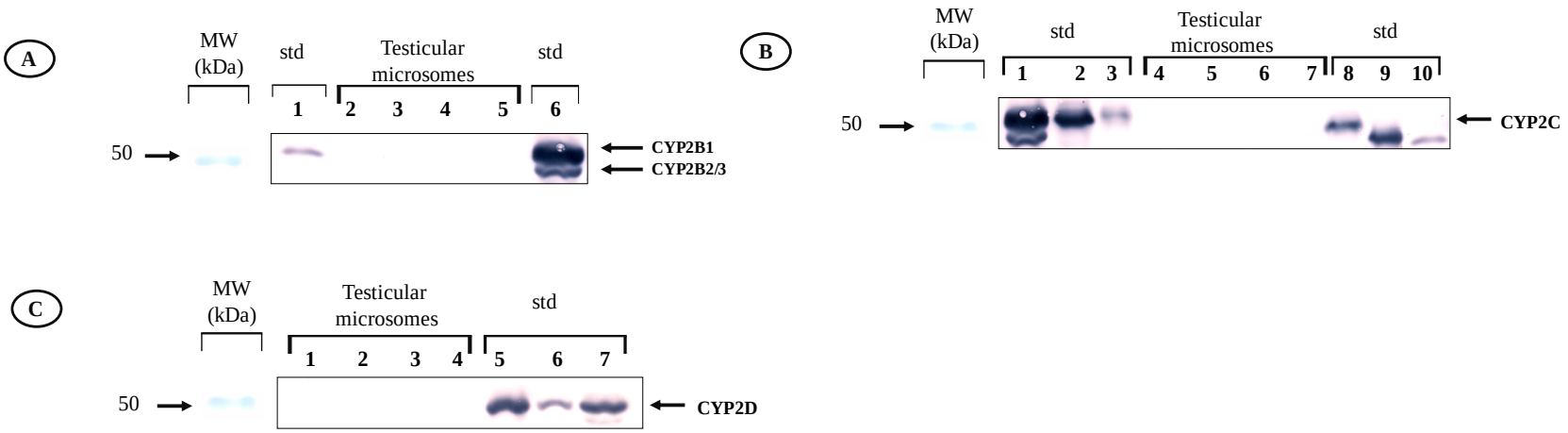


Figure 3.2 Representative immunoblots of rat testicular microsomes probed with antibodies against CYP2B, CYP2C and CYP2D in rat testicular microsomes. The amount of testicular microsomal protein used was 20 μg/lane for all the immunoblots. For antibody details refer to Table 2.1 and 2.2. (A). Immunoblot of testicular microsomes probed with rabbit anti-rabbit CYP2B1 polyclonal IgG. Lanes 2-5 contained testicular microsomes. Lane 1 contained purified rat liver CYP2B1 protein. Lane 6 contained rat liver microsomes 20 μg/lane. (0.125 pmol/lane) (B). Immunoblot of testicular microsomes probed with rabbit anti-rat CYP2C polyclonal IgG. Lane 1 contained untreated rat liver microsomes 20 μg/lane. Lane 2 contained cDNA-expressed rat CYP2C6 protein (0.25 pmol/lane). Lane 3 contained CYP2C7 protein (0.125 pmol/lane). Lane 8 contained purified rat liver CYP2C11 protein (0.2 pmol/lane). Lane 9 contained CYP2C12 protein (0.1 pmol/lane). Lane 10 contained purified rat liver CYP2C13 protein (0.25 pmol/lane). Lanes 4-7 contained testicular microsomes. (C). Immunoblot of testicular microsomes probed with rabbit anti-human CYP2D6 serum. Lanes 1-4 contained testicular microsomes. Lanes 5 and 6 contained cDNA-expressed rat CYP2D1 protein (0.2 pmol/lane) and CYP2D2 (0.1 pmol/lane), respectively. Lane 7 contained rat liver microsomes 20 μg/lane.

Testicular microsomal samples were probed with rabbit anti-rat CYP2E1 serum to determine if CYP2E1 was expressed in rat testis. The protein band for CYP2E1 (~55 kDa) was not detected in lanes containing testicular microsomal samples, but clear protein band was detected with the expected molecular size in the lanes containing rat liver microsomes, which served as a positive control for the CYP2E1 protein (Figure 3.3A).

To determine if CYP3A1 and CYP3A2 proteins are expressed in rat testis, testicular microsomes were probed with rabbit anti-rat CYP3A polyclonal IgG, which react with CYP3A1 and CYP3A2 proteins. The protein band specific for any of the CYP3A isoforms at ~55 kDa was not detected in lanes containing testicular microsomes, but clear bands with molecular size of ~55 kDa were detected in lanes containing standard CYP3A1, CYP3A2 and liver microsomal samples (Figure 3.3B).

The CYP4A subfamily consists of three isoforms, CYP4A1, CYP4A2 and CYP4A3 in rats. To detect these isoforms, liver microsomes prepared from clofibrate treated rats were loaded as a positive control because clofibrate is a strong CYP4A inducer in rats (Aoyama et al., 1990). Since these isoforms have different molecular sizes (CYP4A1 molecular size ~51 kDa, 4A2 molecular size ~53 kDa and 4A3 molecular size ~55 kDa) they can be resolved by SDS-PAGE. Immunoblots were incubated with rabbit anti-rat CYP4A1/2/3 serum for 2 hr at 37°C or 16 hr at 4°C. In both experiments, immunoreactive bands specific for any of the CYP4A enzymes were not detected in the lanes containing testicular microsomal samples prepared from the untreated rats. But the lanes containing liver microsomes prepared from rats treated with clofibrate were shown three protein bands with the expected molecular size, which served as a positive control (Figure 3.3C).

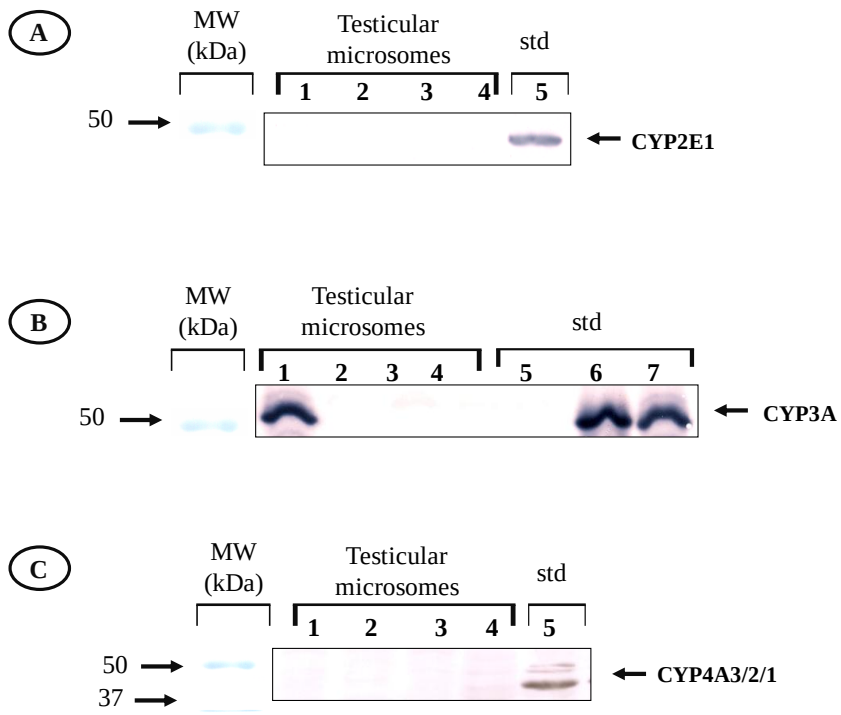


Figure 3.3 Representative immunoblots of rat testicular microsomes probed with antibodies against CYP2E1, CYP3A and CYP4A in rat testicular microsomes. For all the immunoblot experiments, 20 µg of testicular microsomal protein was loaded per lane. For antibody details refer to Table 2.1 and 2.2. (A). Immunoblot of testicular microsomes probed with rabbit anti-rat CYP2E1 serum. Lanes 1-4 contained testicular microsomes. Lane 5 contained rat liver microsomes 20 µg/lane. (B). Immunoblot of testicular microsomes probed with polyclonal antibody against rat CYP3A proteins. Lane 1 contained untreated rat liver microsomes 20 µg/lane. Lanes 2-5 contained testicular microsomes. Lanes 6 and 7 contained cDNA-expressed rat CYP3A1 and CYP3A2 protein (0.25 pmol/lane), respectively. (C). Immunoblot of testicular microsomes probed with polyclonal antibody against rat CYP4A1/2/3. Lanes 1-4 contained testicular microsomes. Lane 5 contained liver microsomes (5 µg/lane) prepared from clofibrate-treated (300 mg/kg for 7 days) Long-Evans rats.

To determine CYP17A1 expression in rat testis, testicular microsomes prepared from adult rats were probed with goat anti-mouse CYP17A1 IgG. According to the information provided by vendor (Santa Cruz biotechnology) this antibody also reacts with CYP17A1 of rat origin. A single protein band with molecular size of ~55 kDa was detected in the testicular microsmal samples. This band has similar electrophoretic mobility as protein band detected in adrenal microsomal samples prepared from adult male rats, which served as positive control for CYP17A1 (Figure 3.4C).

To determine if POR was expressed in rat testis, testicular microsomes were probed with rabbit anti-rat POR serum. A single protein band having similar electrophoretic mobility as standard POR with molecular size of ~72 kDa was detected in the lanes containing testicular microsomal samples (Figure 3.4B).

Rabbit anti-human 3β-HSD polyclonal IgG was used to detect if 3β-HSD enzymes were expressed in rat testis. A single protein band with molecular size of ~55 kDa was detected in the testicular microsmal samples. This band has similar electrophoretic mobility as 3β-HSD protein band observed in positive control sample (adrenal microsomes prepared from adult male rats) (Figure 3.4D).

To determine if mEH was expressed in rat testis, testicular microsomes were probed with rabbit anti-rat EH IgG. A single protein band having similar electrophoretic mobility as standard EH with molecular size of ~47 kDa was detected in the lanes containing testicular microsomal samples prepared from untreated rats (Figure 3.4A).

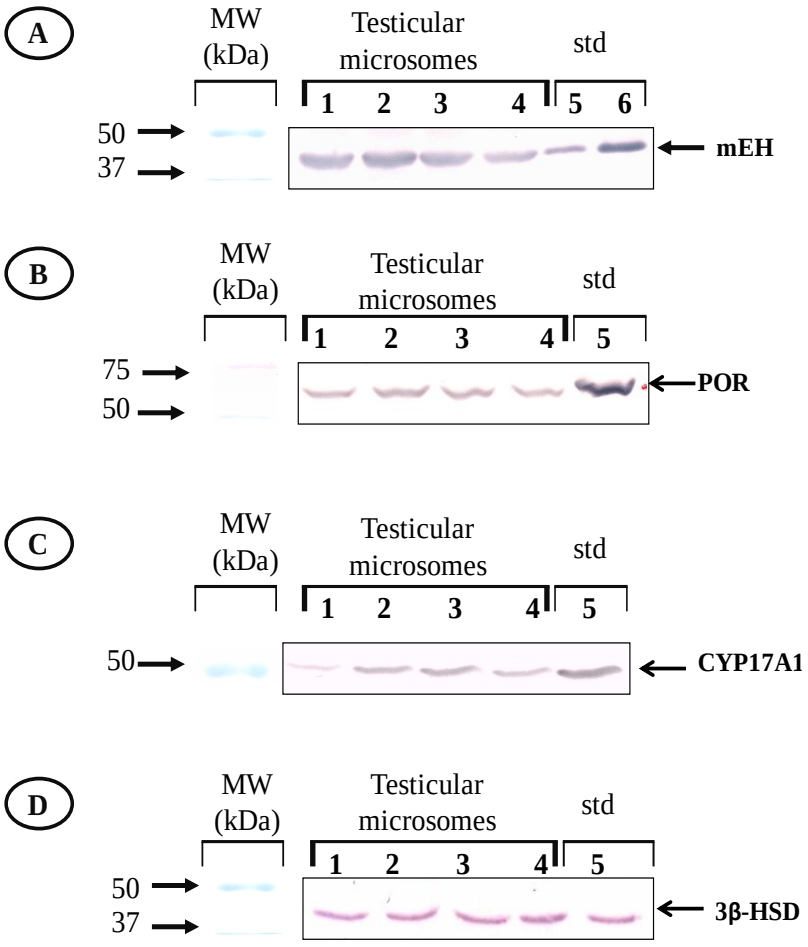


Figure 3.4 Representative immunoblots of rat testicular microsomes probed with antibodies against mEH, POR and CYP17A1 in rat testicular microsomes. For all the immunoblot experiments, 20 µg of testicular microsomal protein was loaded per lane. For antibody details refer to Table 2.1 and 2.2. (A). Immunoblot of testicular microsomes probed with rabit anti-rat EH IgG. Lanes 1-4 contained testicular microsomes. Lane 5 contained purified rat liver epoxide hydrolase protein (0.001 µg/lane). Lane 6 contained rat liver microsomes 20 µg/lane. (B). Immunoblot of testicular microsomes probed with rabbit anti-rat POR serum. Lanes 1-4 contained testicular microsomes. Lane 5 contained purified rat liver POR protein (25 ng/lane). (C). Immunoblot of testicular microsomes probed with goat anti-mouse CYP17A1 polyclonal IgG. Lanes 1-4 contained testicular microsomes. Lane 5 contained adrenal microsomes (20 µg/lane) prepared from adult male rats. D). Immunoblot of testicular microsomes probed with rabbit anti-human 3β-HSD polyclonal IgG. Lanes 1-4 contained testicular microsomes. Lane 5 contained adrenal microsomes (20 µg/lane) prepared from adult male rats.

In summary, CYP1B1, CYP2A1, POR, CYP17A1, 3β-HSD and mEH were detected in testicular microsomes isolated from adult rats. By comparison, CYP1A1, CYP1A2, CYP2B1, CYP2E1, CYP2D1, CYP2D2, CYP2C6, CYP2C7, CYP2C12 CYP2C13, CYP3A1, CYP3A2, CYP4A1, CYP4A2 and CYP4A3 were not detectable in the same microsomal samples.

Basal expression levels of CYP1B1, CYP2A1, POR and mEH were measured by immunoquantification using the respective recombinant or purified proteins as calibration standards. As shown in Figure 3.5, the estimated ranges of basal protein levels in testicular microsomes prepared from saline-treated rats (n = 4) were as follows, for CYP1B1, 52-76 pmol/mg; for CYP2A1, 3-5 pmol/mg; for POR, 6-7 μg/mg; and for mEH, 0.2-0.17 μg/mg of testicular microsomal protein. Similarly, the estimated ranges of basal protein levels in testicular microsomes prepared from propylene glycol-treated rats (n = 9) were as follows; for CYP1B1, 57-70 pmol/mg; for CYP2A1, 4-9 pmol/mg; for POR, 6-7 μg/mg; and for mEH, 0.18-0.28 μg/mg of testicular microsomal protein. No difference was observed in basal protein expression of CYP1B1, CYP2A1, POR and mEH between the saline and propylene glycol-treated groups.

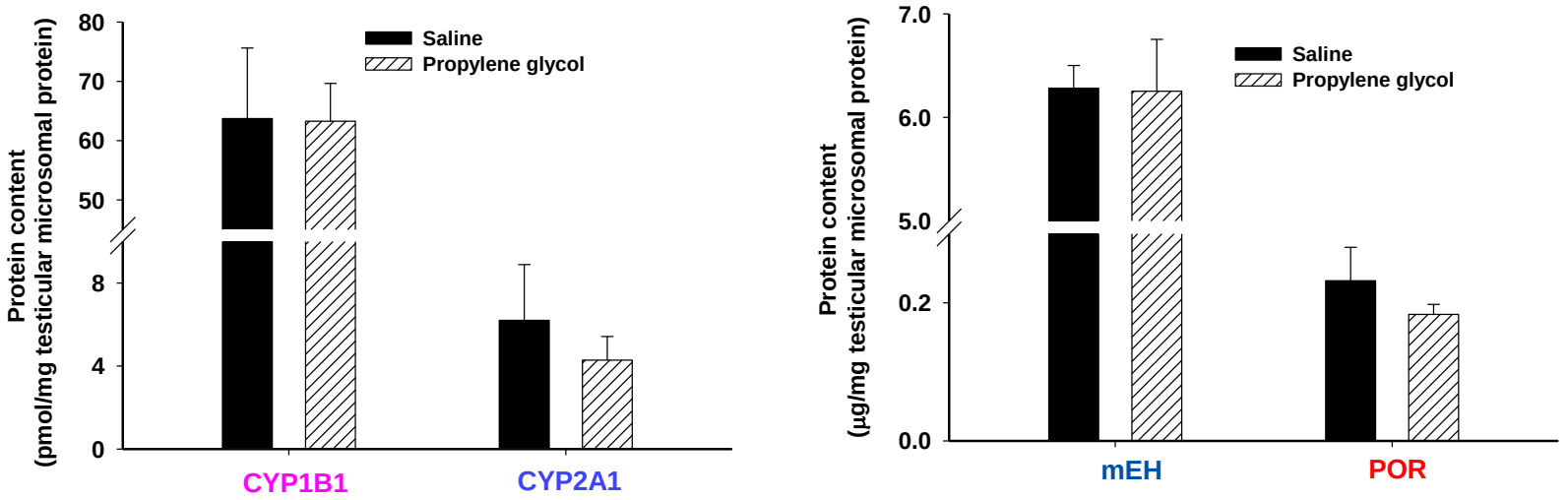


Figure 3.5 Immunoquantification of CYP1B1, CYP2A1, mEH and POR protein levels in testicular microsomes prepared from adult male rats treated with either propylene glycol or saline. Data are expressed as mean ± SD of three independent experiments (n = 9 for the propylene glycol-treated group and n = 4 for the saline-treated group).

3.1.2 Immunohistochemical Analysis

Immunohistochemical analysis was performed to corroborate immunoblot results and to determine the localization of CYP1B1, CYP2A1, mEH, POR, CYP17A1 and 3β-HSD expression in rat testis. Immunohistochemical analysis was carried out as described in the Materials and Methods section. CYP17A1 and 3β-HSD are steroidogenic enzymes that are abundantly expressed in Leydig cells (Sakaue et al., 2002) and were used, in this study, as Leydig cell markers. A strong immunoreactive fluorescent signal was observed for CYP1B1, CY2A1, CYP17A1 and 3β-HSD in interstitial cells, which are comprised mainly of Leydig cells, when compared with their respective controls (Figure 3.6 and 3.7). Fluorescent signal was not detected in seminiferous tubules, which contain Sertoli and spermatogenic cells. Whereas for mEH and POR, an immunoreactive fluorescent signal was detected across the testis including the interstitial and seminiferous tubule portions, when compared to control tissue sections (Figure 3.8). Within the seminiferous tubule, a strong signal was observed in spermatogenic cells, but a relatively low signal was observed in Sertoli cells. Summary of the results were shown in Table 3.1.

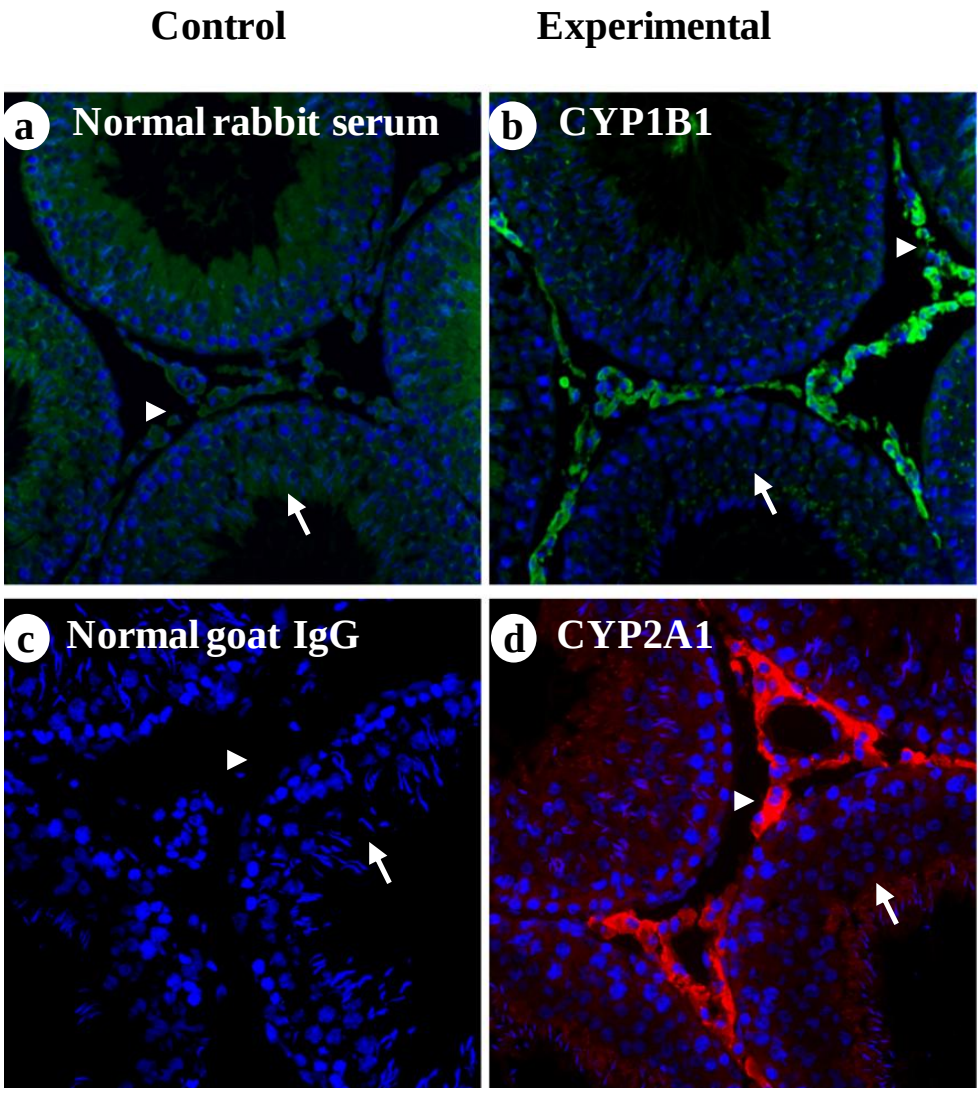


Figure 3.6 Immunohistochemical detection of CYP1B1 and CYP2A1 expression in adult rat testis. Testis tissue cryosections were prepared and subjected to immunostaining as described in the *Materials and Methods* section. Panels a and c are representative cryosections probed with normal rabbit serum and normal goat IgG, respectively. Panels b and d are representative cryosections that were probed with anti-rat CYP1B1 and CYP2A1, respectively. For the antibody concentrations refer to Table 2.5. Note that the above images are merged images of antibody (either red or green fluorescent stain to show protein expression) and DAPI (blue stain to show nuclei) treated cryosections. Arrowheads represent interstitial cell compartment, arrows represent seminiferous tubules.

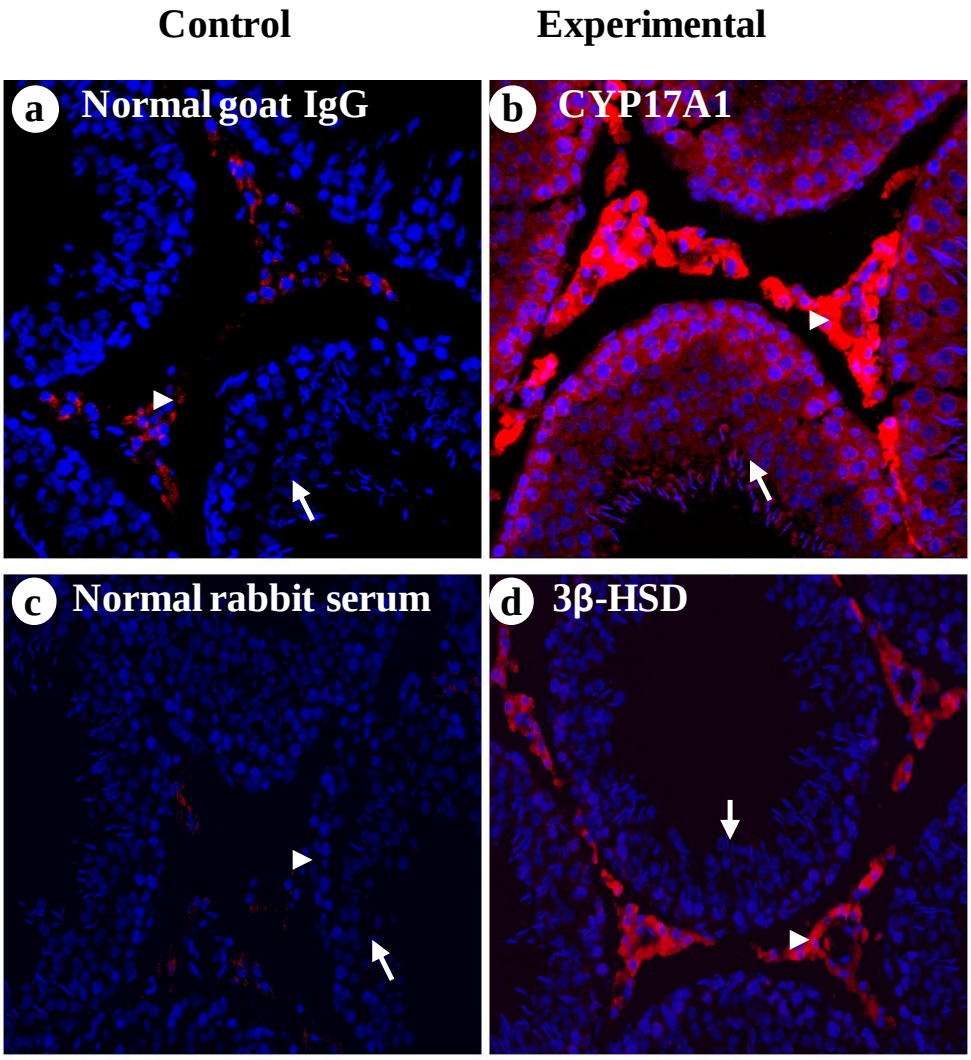


Figure 3.7 Immunohistochemical detection of CYP17A1 and 3β-HSD expression in adult rat testis. Testis tissue cryosections were prepared and subjected to immunostaining as described in the *Materials and Methods* section. Panels a and c are representative cryosections probed with normal goat IgG and normal rabbit serum, respectively. Panels b and d are representative cryosections that were probed with anti-rat CYP17A1 and 3β-HSD respectively. For the antibody concentrations refer to Table 2.5. Note that the above images are merged images of antibody (pink fluorescent stain to show protein expression) and DAPI (blue stain to show nuclei) treated cryosections. Arrowheads represent interstitial cell compartment, arrows represent seminiferous tubules.

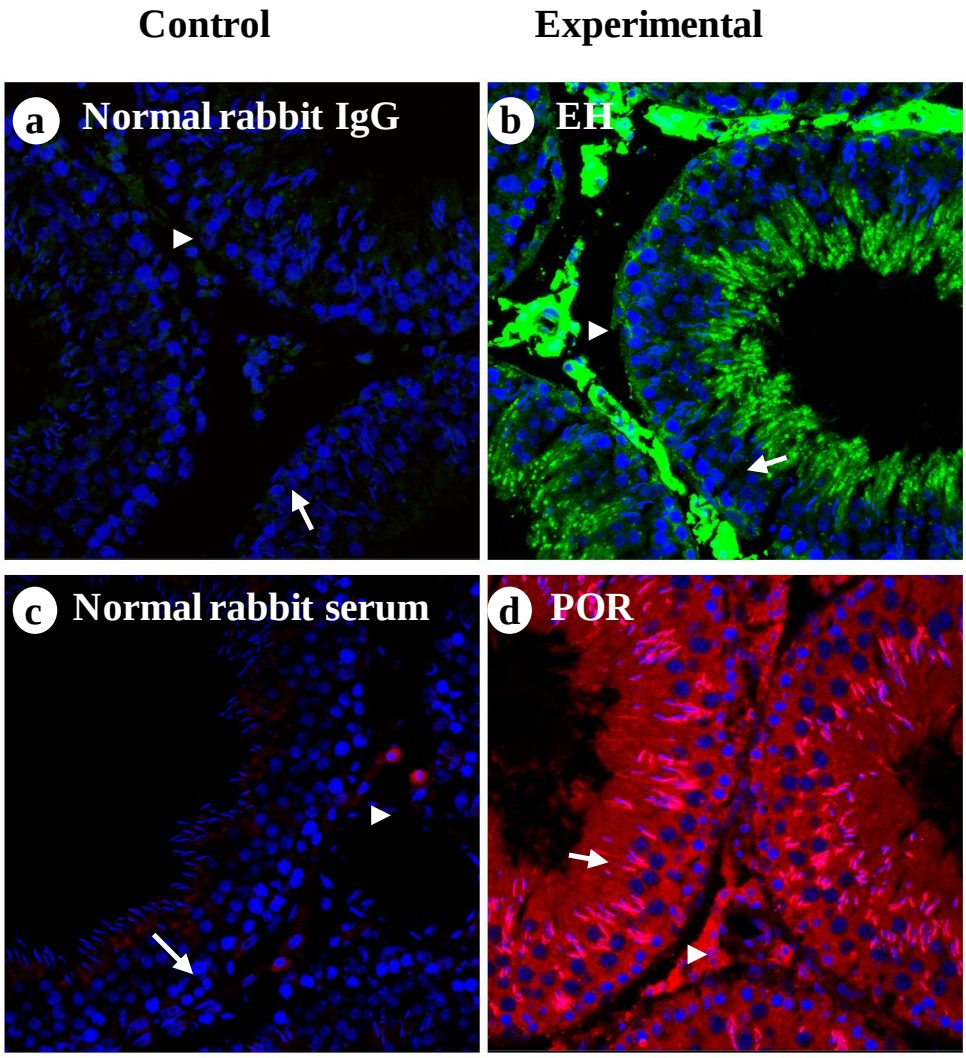


Figure 3.8 Immunohistochemical detection of EH and POR expression in adult rat testis. Testis tissue cryosections were prepared and subjected to immunostaining as described in the *Materials and Methods* section. Panels a and c are representative cryosections probed with normal rabbit IgG and normal rabbit serum, respectively. Panels b and d are representative cryosections that were probed with anti-rat EH and POR respectively. For the antibody concentrations refer to Table 2.5. Note that the above images are merged images of antibody (pink or green fluorescent stain to show protein expression) and DAPI (blue stain to show nuclei) treated tissue sections. Arrowheads represent interstitial cell compartment, arrows represent seminiferous tubules.

Table 3.1 Summary of immunohistochemical analyses of CYP1B1, CYP2A1, CYP17A1, mEH, 3β-HSD and POR in adult male rat testis

Protein	Immunohistochemistry outcome	
	Presence of immunoreactive fluorescent signal in interstitial cells	**Presence of immunoreactive fluorescent signal in seminiferous tubules/ spermatogenic cells**
CYP1B1	+	-
CYP2A1	+	-
CYP17A1	+	-
mEH	+	+
POR	+	+
3β-HSD	+	-

+, positive; -, negative

3.2 Study 2: 17β-Estradiol Benzoate Study

This study examined the effect of treatment with 17β-estradiol benzoate (EB) on various parameters such as body weight, organ weights and testicular expression of CYP1B1, CYP2A1, CYP17A1, mEH, POR and 3β-HSD. Rats were treated with varying dosages of EB (0.004, 0.04, 0.4 and 4 μmol/kg) or an equal volume (1 ml/kg) of vehicle (propylene glycol), once daily for 14 days and were killed 24 hr after the last dose.

3.2.1 Effect of 17β-Estradiol Benzoate on Body and Organ Weights

Based on daily physical observation of the rats, all of the rats appeared to be healthy during the entire study period. Animal body weights were recorded every morning before treatment and on the day they were killed. The effect of treatment with EB on body weight from day 1 to day 15 is presented in Figure 3.9A. EB decreased body weight in dose-dependent manner. EB at highest dosage (4 μmol/kg) decreased body weight on day 7 and the effect was continued till day 15 when compared with the propylene glycol-treated group. EB at 0.04 μmol/kg, decreased body weight on day 11 and the effect was continued till day 15. EB at a dosage of 0.004 μmol/kg decreased body weight on day 15 when compared with the propylene glycol-treated group. As shown in Figure 3.9B, the body weight gain was decreased by 25%, 50%, 65% and 119% at 0.004, 0.04, 0.4 and 4 μmol of EB/kg, respectively, when compared with the propylene glycol-treated group.

No difference was found when liver, adrenal and testis weights (as a percent of body weight) of EB-treated groups were compared with those of the control group (Figure 3.10A). EB had no effect on absolute testis weight at any of the tested dosages, except at the highest dosage (4 μmol/kg), where EB decreased absolute testis weight by 20% when compared with propylene

glycol-treated group (Figure 3.10B). EB decreased absolute liver weight in dose-dependent manner. EB decreased absolute liver weight by 15%, 20% and 17% at 0.04, 0.4 and 4 μmol/kg dosages, respectively. No difference was found when absolute adrenal weight of EB-treated group compared with propylene glycol-treated group.

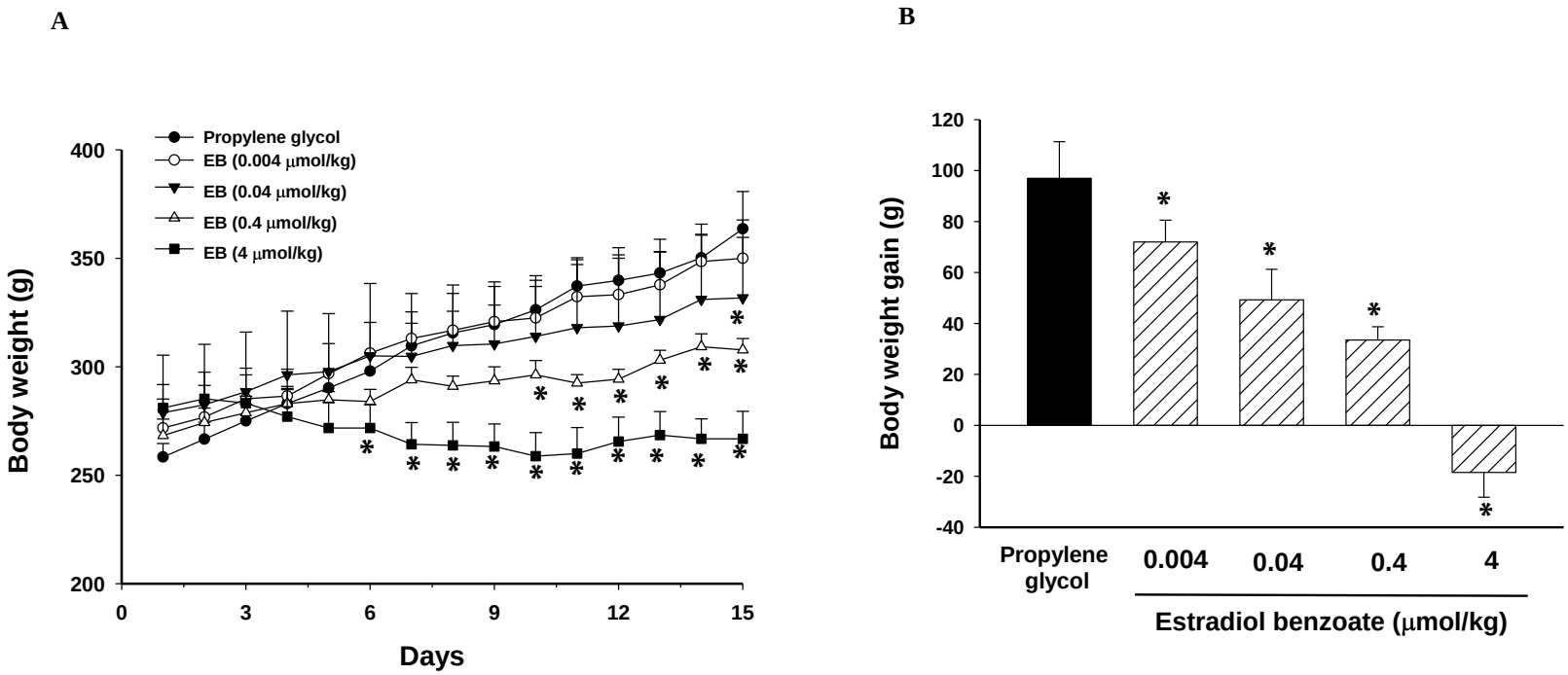


Figure 3.9 Effect of varying dosages of EB on (A) body weight during the treatment period and on (B) body weight gain. Body weight gain was calculated by subtracting the body weight on day 15 from the body weight on day 1 for each rat. Data are expressed as mean ± SD of the 4 rats per group, except for the propylene glycol-treated group (n = 5). *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

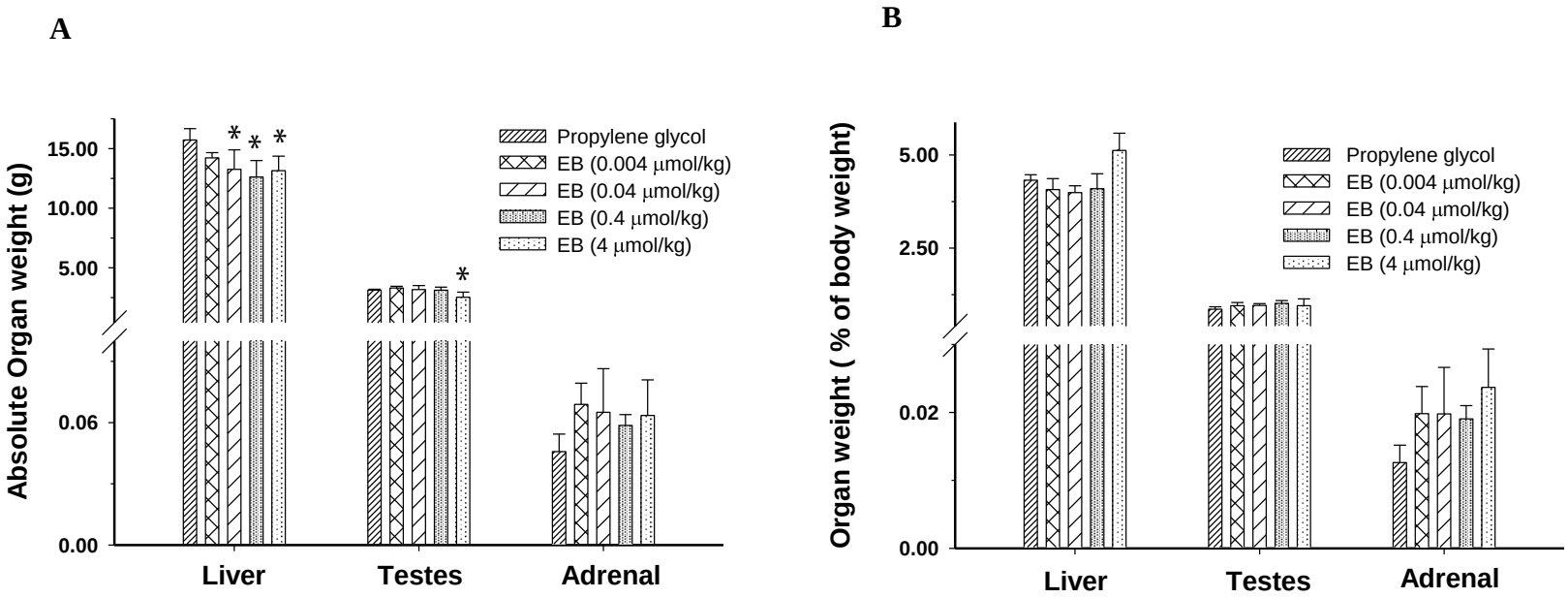


Figure 3.10 Effect of treatment with varying dosages of EB on (A) absolute organ weights and on (B) organ weights as percent of body weight. Data are expressed as mean ± SD with n = 4 per group, except for the propylene glycol-treated group (n = 5). *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

3.2.2 Effect of 17β-Estradiol Benzoate Treatment on Testicular Expression of CYP1B1, CYP2A1, CYP17A1, mEH, POR and 3β-HSD proteins in Adult Male Rat Testis

3.2.2.1 Immunoblot Analysis of Testicular Microsomes Probed for CYP1B1

Immunoblots containing testicular microsomes from treatment groups, and standard CYP1B1 (cDNA expressed protein) were probed with rabbit anti-rat CYP1B1 serum. A scanned image of the immunoblot is shown in Figure 3.11. Darkly stained CYP1B1 protein bands were detected in lanes containing testicular microsomal samples prepared from propylene glycol-treated rats and rats treated with lowest dosage of EB (0.004 μmol/kg). The staining intensity of CYP1B1 protein bands was gradually decreased with increasing dosages of EB.

A standard curve was constructed from three different concentrations of standard CYP1B1 proteins to quantify the CYP1B1 protein levels in testicular microsomes. The calculated mean slope from 3 experiments was 4.52 with a limit of quantitation of 0.01 pmol/lane. The interday variation (coefficient of variation) of slopes obtained from three experiments was 4.84% (Table 3.3).

A bar graph summarizing the mean testicular CYP1B1 levels for each treatment group is presented in Figure 3.12. Treatment with EB decreased testicular CYP1B1 protein levels by 15, 56, 70 and 80% at 0.004, 0.04, 0.4 and 4 μmol of EB/kg dosages, respectively, when compared with the propylene glycol-treated group (63 ± 6.2 pmol of CYP1B1/ mg of microsomal protein).

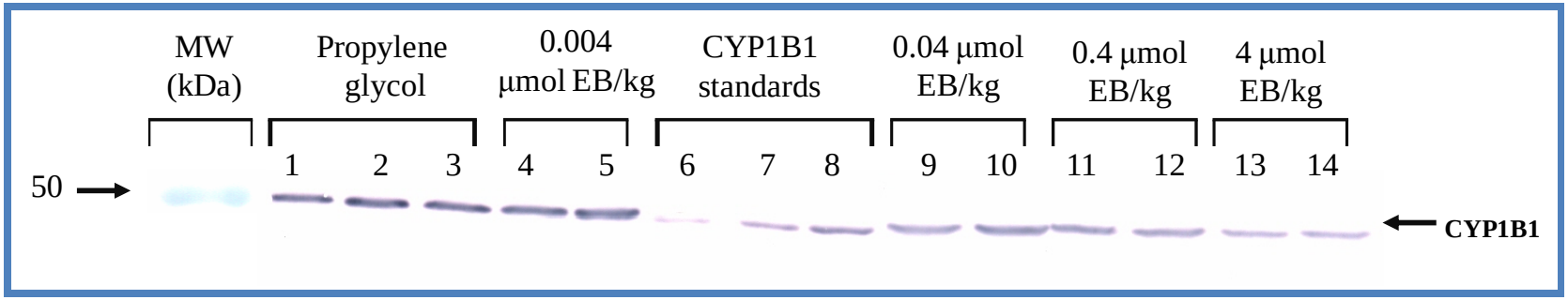


Figure 3.11 A representative immunoblot of rat testicular microsomes probed with rabbit anti-rat CYP1B1 serum. For antibody details refer to Table 2.1. The color development reaction time was 3 min. The amount of testicular microsomal protein loaded was 20 µg /lane. Lanes 1-3 contained testicular microsomal samples prepared from propylene glycol-treated rats. Lanes 4-5 contained testicular microsomal samples prepared from rats treated with 0.004 µmol/kg of EB. Lanes 9-10 contained testicular microsomal samples prepared from rats treated with 0.04 µmol/kg of EB. Lanes 11-12 contained testicular microsomal samples prepared from rats treated with 0.4 µmol/kg of EB. Lanes 13-14 contained testicular microsomal samples prepared from rats treated with 4 µmol/kg of EB. Lanes 6, 7 and 8 contained cDNA expressed rat CYP1B1 protein at 0.1, 0.2 and 0.4 pmol/lane, respectively.

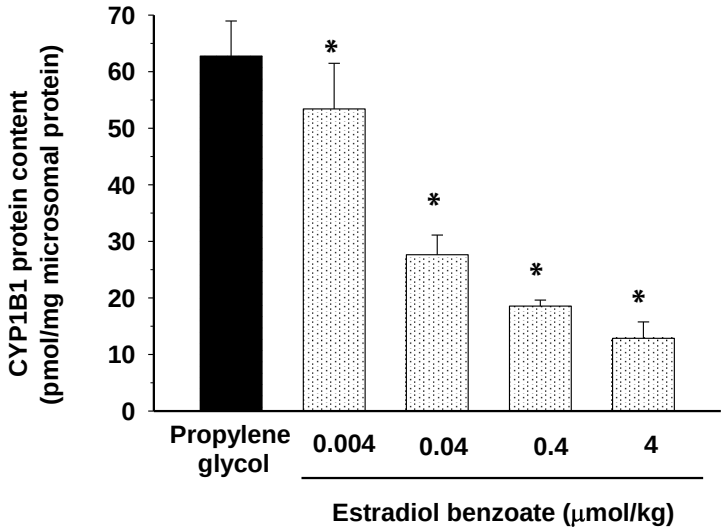


Figure 3.12 Graphical representation of CYP1B1 protein content in testicular microsomes prepared from adult male rats treated with EB or propylene glycol (vehicle). Data are expressed as mean ± SD of three individual experiments with 4 rats per group, except for the propylene glycol-treated group (n = 5). *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

Table 3.2 Details of the calibration curve used for immunoquantitation of CYP1B1 protein in rat testicular microsomes

Immunoblot experiment	CYP1B1 standards (pmol/lane)	Calibration curve	Slope	r^2
1	0.1, 0.2 or 0.4	a	4.8	0.97
		b	4.2	0.95
2	0.1, 0.2 or 0.4	a	4.4	0.98
		b	4.6	0.97
3	0.1, 0.2 or 0.4	a	4.6	0.99
		b	4.6	0.99
		Mean	4.5	
		SD	0.22	
		% CV	4.8	

Note: Three independent experiments were performed on different days. Each experiment included two immunoblots with two calibration curves (a, b) to analyze all the samples in a single attempt. Abbreviations: SD, standard deviation; % CV, coefficient of variation; r^2, coefficient of determination.

3.2.2.2 Immunoblot Analysis of Testicular Microsomes Probed for CYP2A1

Immunoblots containing testicular microsomes from treatment groups, and purified rat liver CYP2A1 proteins were probed with sheep anti-rat CYP2A1 polyclonal IgG. A scanned image of the immunoblot is shown in Figure 3.13. The staining intensity of CYP2A1 protein bands for microsomal samples prepared from propylene glycol-treated group was relatively stronger compared to EB treated groups. Within EB treated groups, the staining intensity of protein bands was decreased gradually with increasing EB dosage and lowest staining intensity (below the limit of quantitation) was observed in lanes containing microsomes prepared from 0.4 and 4 EB μmol/kg dosage groups.

A standard curve was constructed from four different concentrations of standard CYP2A1 proteins to quantify the CYP2A1 protein levels in testicular microsomes. The calculated mean slope from 3 experiments was 4.0 with a limit of quantitation of 0.0625 pmol/lane. The interday variation (coefficient of variation) of slopes obtained from three experiments was 9.3% (Table 3.4).

A bar graph summarizing the mean testicular CYP2A1 levels for each treatment group is presented in Figure 3.14. EB treatment decreased testicular CYP2A1 protein levels by 48, 79, 97 and 98% at 0.004, 0.04, 0.4 and 4 μmol/kg dosages of EB, respectively, when compared with the propylene glycol-treated group (8.3 ± 1.3 pmol/mg microsomal protein).

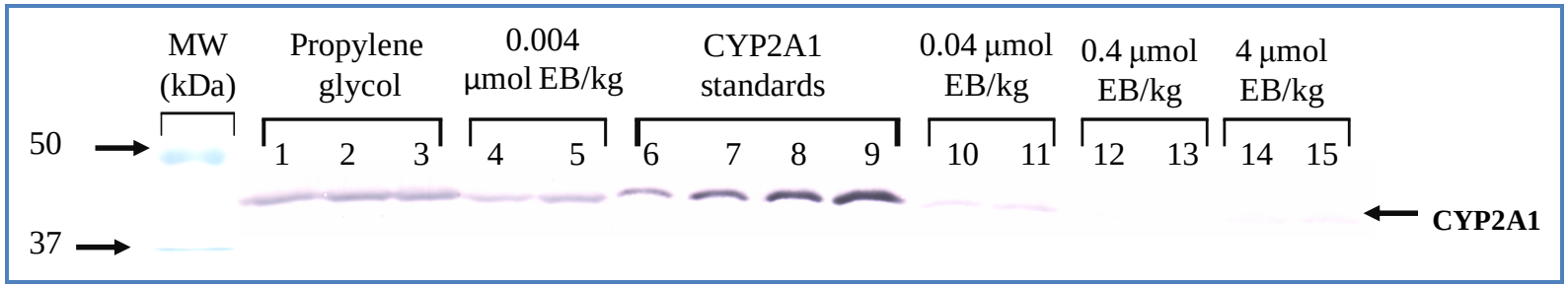


Figure 3.13 A representative immunoblot of rat testicular microsomes probed with sheep anti-rat CYP2A1 polyclonal IgG. For antibody concentrations refer to Table 2.1. The amount of testicular microsomal protein loaded was 20 µg/lane. The color development reaction time was 4 min. Lanes 1-3 contained testicular microsomes prepared from propylene glycol-treated rats. Lanes 4-5 contained testicular microsomes prepared from rats treated with 0.004 µmol of EB /kg. Lanes 10-11 contained testicular microsomes prepared from rats treated with 0.04 µmol of EB/kg. Lanes 12-13 contained testicular microsomes prepared from rats treated with 0.4 µmol/kg of EB. Lanes 14-15 contained testicular microsomes prepared from rats treated with 4 µmol of EB/kg. Lanes 6, 7, 8 and 9 contained purified rat liver CYP2A1 protein at 0.0625, 0.125, 0.25 and 0.35 pmol/lane, respectively.

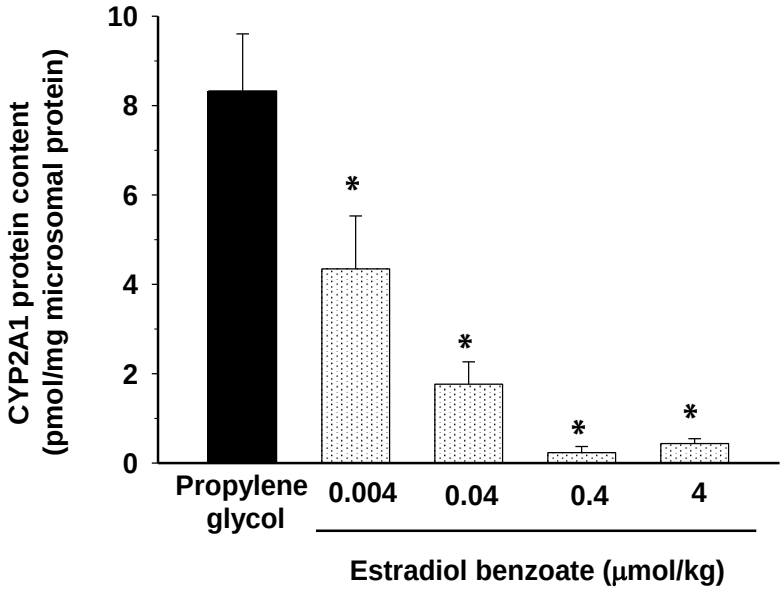


Figure 3.14 Graphical representation of CYP2A1 protein content in testicular microsomes prepared from adult male rats treated with EB or propylene glycol (vehicle). Data are expressed as mean ± SD of three individual experiments with 4 rats per group, except for the propylene glycol-treated group (n = 5). *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

Table 3.3 Details of the calibration curve used for immunoquantitation of CYP2A1 protein in rat testicular microsomes

Immunoblot experiment	CYP2A1 standards (pmol/lane)	Calibration curve	Slope	r^2
1	0.0625, 0.125, 0.25 or 0.35	a	4.7	0.95
		b	4.0	0.88
2	0.0625, 0.125, 0.25 or 0.35	a	4.3	0.96
		b	3.6	0.98
3	0.0625, 0.125, 0.25 or 0.35	a	3.9	0.91
		b	3.8	0.92
		Mean	4.0	
		SD	0.38	
		% CV	9.3	

Note: Three independent experiments were performed on different days. Each experiment included two immunoblots with two calibration curves (a, b) to analyze all the samples in a single attempt. Abbreviations: SD, standard deviation; % CV, coefficient of variation; r^2, coefficient of determination.

3.2.2.3 Immunoblot Analysis of Testicular Microsomes Probed for CYP17A1

Immunoblots containing testicular microsomes from different treatment groups were probed with goat anti-mouse CYP17A1 polyclonal IgG. A scanned image of the immunoblot is shown in Figure 3.15. Due to lack of purified rat CYP17A1 protein, adrenal microsomes prepared from untreated rats were loaded on to the gel to serve as a positive control. The staining intensity of CYP17A1 protein bands for microsomal samples prepared from propylene glycol-treated group was much darker when compared to those of EB treated groups. Within EB treated groups, the staining intensity of CYP17A1 protein bands was decreased gradually with increasing EB dosages and weakly stained protein bands were detected in lanes containing microsomes prepared from rat treated with highest dosage (4 μmol/kg) of EB.

A standard curve for CYP17A1 was not generated due to lack of purified or recombinant CYP17A1 protein. Hence, the relative level of CYP17A1 protein was presented as protein band intensity per milligram of microsomal protein (Figure 3.16). Administration of EB suppressed testicular CYP17A1 protein expression in a dose-dependent manner (Figure 3.14). EB decreased the CYP17A1 protein levels by 44, 94 and 97% at 0.04, 0.4 and 4 μmol of EB/kg dosages, respectively, when compared with propylene glycol-treated rats. However, there was no difference in CYP17A1 protein expression between rats treated with the lowest dosage of EB and the propylene glycol-treated group.

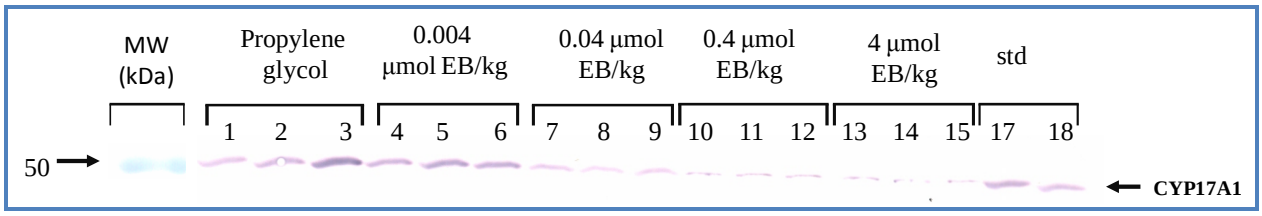


Figure 3.15 A representative immunoblot of rat testicular microsomes probed with polyclonal goat anti-mouse CYP17A1 IgG. For antibody concentrations refer to Table 2.3. The color development reaction time was 4 min. The amount of testicular microsomal protein loaded was 20 µg/lane. Lanes 1-3 contained testicular microsomal samples prepared from propylene glycol-treated rats. Lanes 4-6 contained testicular microsomal samples prepared from rats treated with 0.004 µmol of EB/kg. Lanes 7-9 contained testicular microsomal samples prepared from rats treated with 0.04 µmol of EB/kg. Lanes 10-12 contained testicular microsomal samples prepared from rats treated with 0.4 µmol of EB/kg. Lanes 13-15 contained testicular microsomal samples prepared from rats treated with 4 µmol of EB/kg. Lanes 16-17 contained adrenal microsomal samples prepared from untreated rats.

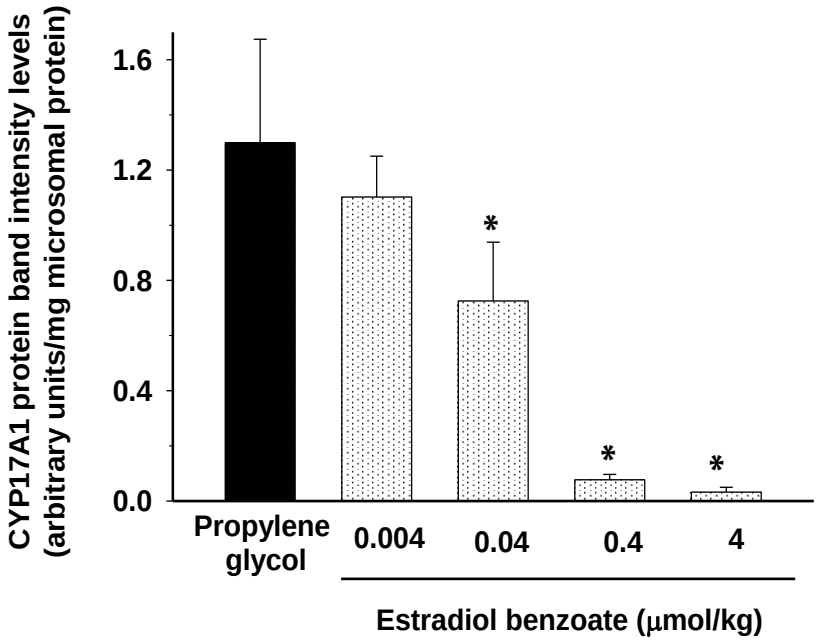


Figure 3.16 Graphical representation of relative levels of CYP17A1 protein content in testicular microsomes prepared from adult male rats treated with EB or propylene glycol (vehicle). Data are expressed as mean ± SD of three individual experiments with 4 rats per group, except for the propylene glycol-treated group (n = 5). *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

3.2.2.4 Immunoblot Analysis of Testicular Microsomes Probed for mEH

Immunoblots containing testicular microsomes from treatment groups, and purified rat liver EH protein were probed with rabbit anti-rat EH polyclonal IgG. A scanned image of the immunoblot is shown in Figure 3.17. Darker protein bands were observed with lanes containing microsomal samples prepared from vehicle and lowest EB dosage group. The staining intensity of protein bands was slightly decreased in lanes containing microsomal samples prepared from groups treated with 0.04, 0.4 and 4 μmol of EB/kg dosages, when compared with lowest EB dosage or vehicle group.

A standard curve was constructed from three different concentrations of purified rat liver EH proteins to quantify the mEH levels in testicular microsomes. The calculated mean slope from 3 experiments was 15.2 with a limit of quantitation of 0.01 μg/lane. The interday variation (coefficient of variation) of slopes obtained from three experiments was 3.32%.

A bar graph summarizing the mean testicular mEH levels for each treatment group is presented in Figure 3.18. Treatment with EB decreased testicular mEH protein levels by 49, 64 and 67% at 0.04, 0.4 and 4 μmol of EB/kg dosages, respectively, when compared with the propylene glycol-treated group (6.4 ± 0.39 μg of mEH/ mg microsomal protein). However, there was no difference in mEH protein expression between rats treated with the lowest dosage of EB and the propylene glycol-treated group.

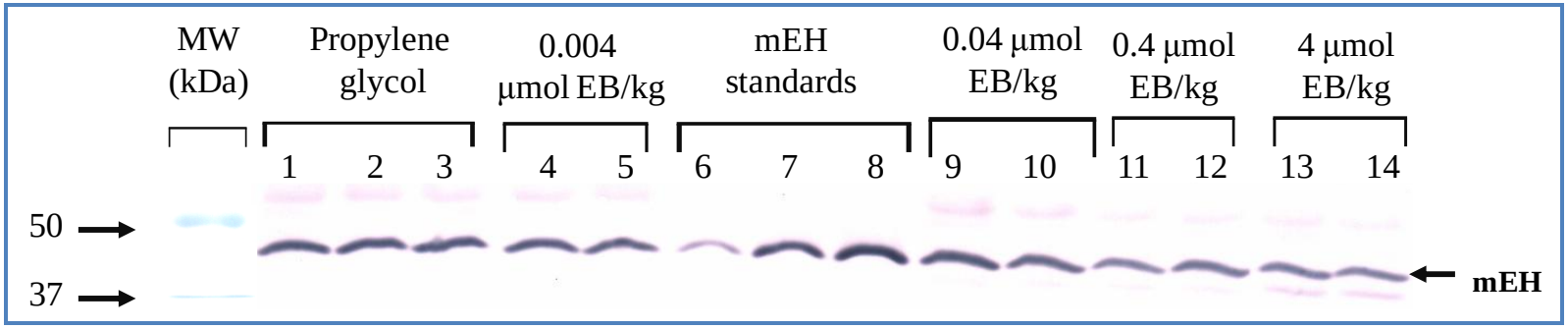


Figure 3.17 A representative immunoblot of rat testicular microsomes probed with rabbit anti-rat EH polyclonal IgG. For antibody concentrations refer to Table 2.3. The amount of testicular microsomal protein loaded was 20 µg/lane. The color development reaction time was 2 min. Lanes 1-3 contained testicular microsomes prepared from propylene glycol-treated rats. Lanes 4-5 contained testicular microsomes prepared from rats treated with 0.004 µmol of EB /kg. Lanes 9-10 contained testicular microsomes prepared from rats treated with 0.04 µmol of EB/kg. Lanes 11-12 contained testicular microsomes prepared from rats treated with 0.4 µmol/kg of EB. Lanes 13-14 contained testicular microsomes prepared from rats treated with 4 µmol of EB/kg. Lanes 6, 7, and 8 contained purified rat liver EH protein at 0.01, 0.05, and 0.1 pmol/lane, respectively.

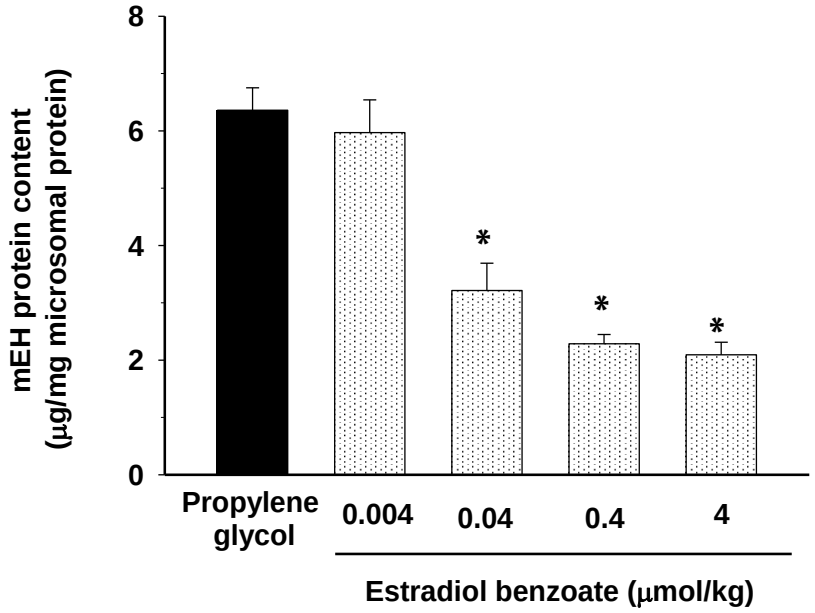


Figure 3.18 Graphical representation of mEH protein content in testicular microsomes prepared from adult male rats treated with EB or propylene glycol (vehicle). Data are expressed as mean ± SD of three individual experiments with 4 rats per group, except for the propylene glycol-treated group (n = 5). *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

Table 3.4 Details of the calibration curve used for immunoquantitation of mEH protein in rat testicular microsomes

Immunoblot experiment	EH standards (μg/lane)	Calibration Curve	Slope	r^2
1	0.01, 0.05 or 0.1	A	16.0	0.93
		B	15.5	0.92
2	0.01, 0.05 or 0.1	A	14.7	0.86
		B	15.2	0.90
3	0.01, 0.05 or 0.1	A	15.2	0.91
		B	14.6	0.82
		Mean	15.2	
		SD	0.50	
		% CV	3.32	

Note: Three independent experiments were performed on different days. Each experiment included two immunoblots with two calibration curves (a, b) to analyze all the samples in a single attempt. Abbreviations: SD, standard deviation; % CV, coefficient of variation; r^2, coefficient of determination.

3.2.2.5 Immunoblot Analysis of Testicular Microsomes Probed for POR

Immunoblots containing testicular microsomes from treatment groups, and purified rat liver POR protein were probed with rabbit anti-rat POR serum. A scanned image of the immunoblot is shown in Figure 3.19. Darkly stained POR protein bands were detected with lanes containing microsomal samples prepared from vehicle and lowest EB dosage group. The staining intensity of POR protein bands was slightly decreased in lanes containing microsomal samples prepared from rat groups treated with 0.04, 0.4 and 4 μmol of EB/kg dosage, when compared with lowest EB dosage or vehicle group.

A standard curve was constructed from three different concentrations of purified rat liver POR proteins to quantify the POR level in testicular microsomes. The calculated mean slope from 3 experiments was 0.036 with a limit of quantitation of 6.25 ng/lane. The interday variation (coefficient of variation) of slopes obtained from three experiments was 14% (Table 3.5).

A bar graph summarizing the mean testicular mEH levels for each treatment group is presented in Figure 3.20. Exogenous administration of EB reduced POR protein levels by 53, 58 and 48% at 0.04, 0.4 and 4 μmolof EB/kg dosages, respectively, when compared with the propylene glycol-treated group (270 ± 2.5 ng/mg microsomal protein). However, there was no difference in POR protein expression between rats treated with the lowest dosage of EB and the propylene glycol-treated group.

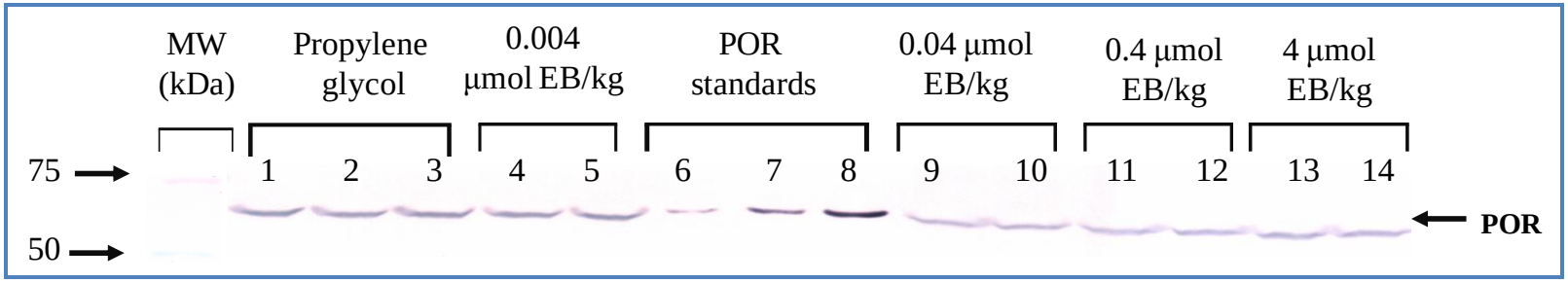


Figure 3.19 A representative immunoblot of rat testicular microsomes probed with rabbit anti-rat POR serum. For antibody concentrations refer to Table 2.3. The color development reaction time was 5 min. Lanes 1-3 and 4-5 contained testicular microsomal samples (40 µg of microsomal protein/lane) prepared from vehicle or 0.004 µmol of EB/kg treated rats, respectively. Lanes 9-10 contained testicular microsomal samples (80 µg of microsomal protein/lane) prepared from rats treated with 0.04 µmol of EB/kg. Lanes 11-12 contained testicular microsomal samples (80 µg of microsomal protein/lane) prepared from rats treated with 0.4 µmol of EB/kg. Lanes 13-14 contained testicular microsomal samples (80 µg of microsomal protein/lane) prepared from rats treated with 4 µmol of EB/kg. Lanes 6, 7, and 8 contained purified rat liver POR proteins at 6.25, 12.5, and 25 ng/lane, respectively.

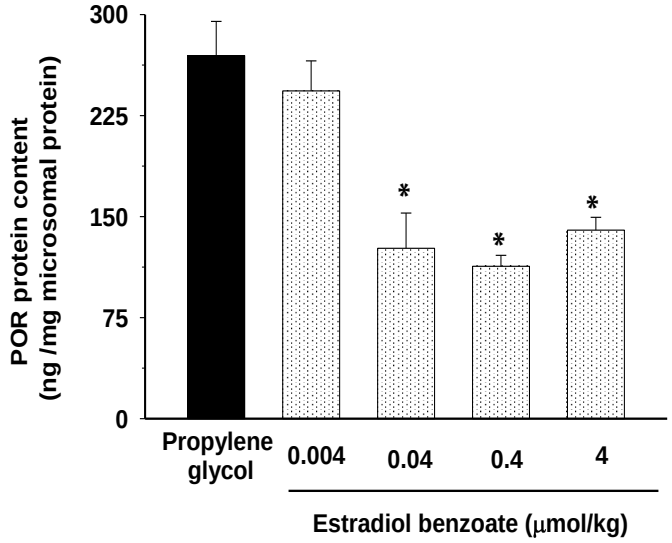


Figure 3.20 Graphical representation of POR protein content in testicular microsomes prepared from adult male rats treated with EB or propylene glycol (vehicle). Data are expressed as mean ± SD of three individual experiments with 4 rats per group, except for the propylene glycol-treated group (n = 5). *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

Table 3.5 Details of the calibration curve used for immunoquantitation of POR protein in rat testicular microsomes

Immunoblot experiment	POR standards (ng/lane)	Calibration Curve	Slope	r^2
1	6.25, 12.5 or 25	A	0.029	0.87
		B	0.031	0.96
2	6.25, 12.5 or 25	A	0.042	0.97
		B	0.039	0.99
3	6.25, 12.5 or 25	A	0.037	0.94
		B	0.038	0.96
		Mean	0.036	
		SD	0.005	
		% CV	14	

Note: Three independent experiments were performed on different days. Each experiment included two immunoblots with two calibration curves (a, b) to analyze all the samples in a single attempt. Abbreviations: SD, standard deviation; % CV, coefficient of variation; r^2, coefficient of determination.

3.2.2.6 Immunoblot Analysis of Testicular Microsomes Probed for 3β-HSD

Immunoblots containing testicular microsomes from different treatment groups were probed with rabbit anti-human 3β-HSD polyclonal IgG. A scanned image of the immunoblot is shown in Figure 3.21. Adrenal microsomes prepared from untreated rats were loaded on to the gel to serve as a positive control. The staining intensity of the 3β-HSD protein bands was similar in all lanes containing microsomes prepared from propylene glycol- and EB-treated rats.

A standard curve for 3β-HSD was not generated due to lack of purified or recombinant 3β-HSD protein. Hence, the relative level of 3β-HSD protein was presented as protein band intensity per milligram of microsomal protein (Figure 3.22). The results indicate that there was no difference in relative levels of 3β-HSD protein between the propylene glycol- and each of the EB-treated groups.

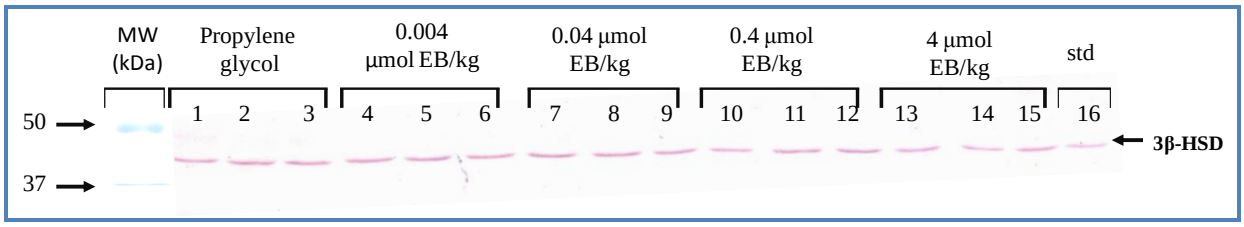


Figure 3.21 A representative immunoblot of rat testicular microsomes probed with rabbit anti-human 3β-HSD polyclonal IgG. For antibody concentrations refer to Table 2.3. The color development reaction time was 7 min. The amount of testicular microsomal protein loaded was 20 µg/lane). Lanes 1-3 contained testicular microsomes prepared propylene glycol-treated rats. Lanes 4-6 contained testicular microsomes prepared from rats treated with 0.004 µmol of EB/kg. Lanes 7-9 contained testicular microsomes prepared from rats treated with 0.04 µmol of EB/kg. Lanes 10-12 contained testicular microsomes prepared from rats treated with 0.4 µmol of EB/kg. Lanes 13-15 contained testicular microsomes prepared from rats treated with 4 µmol of EB/kg. Lane 16 contained rat adrenal microsomes 20 µg/lane, used as a positive control for the 3β-HSD protein.

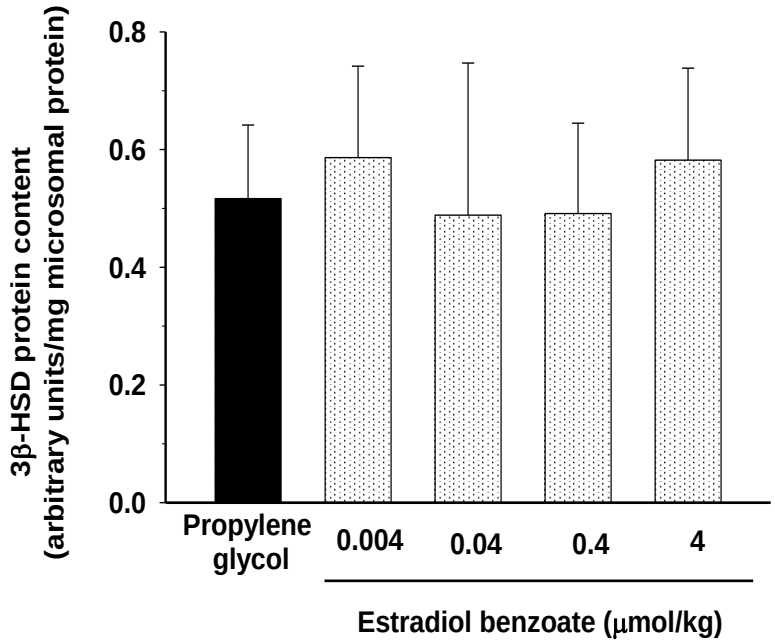


Figure 3.22 Graphical representation of relative levels of 3β-HSD protein content in testicular microsomes prepared from adult male rats treated with EB or propylene glycol (vehicle). Data are expressed as mean ± SD of three individual experiments with 4 rats per group, except for the propylene glycol-treated group (n = 5).

3.2.2.7 Immunoblot Analysis of Testicular Microsomes Probed for Calnexin

Immunoblots containing testicular microsomes from different treatment groups were probed with rabbit anti-human calnexin serum. A scanned image of the immunoblot is shown in Figure 3.23. Liver microsomes prepared from untreated rats were loaded on to the gel to serve as a positive control. The staining intensity of the immunoreactive protein bands was similar in all lanes containing microsomes prepared from propylene glycol- and EB-treated rats.

A standard curve for calnexin was not generated due to lack of purified calnexin protein. Hence, the relative level of calnexin protein was presented as protein band intensity per milligram of microsomal protein (Figure 3.24). No significant difference was observed in calnexin protein levels between the vehicle and each of the EB-treated groups.

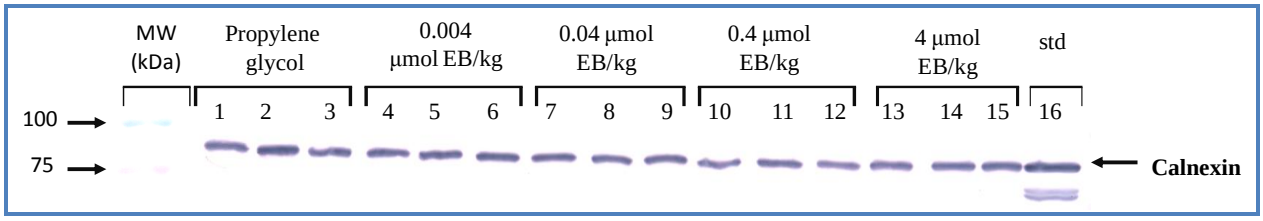


Figure 3.23 A representative immunoblot of rat testicular microsomes probed with rabbit anti-human calnexin serum. The color development reaction time was 90 sec. For antibody concentrations refer to Table 2.3. Lanes 1-3 contained testicular microsomes prepared propylene glycol-treated rats. Lanes 4-6 contained testicular microsomes prepared from rats treated with 0.004 µmol of EB/kg. Lanes 7-9 contained testicular microsomes prepared from rats treated with 0.04 µmol of EB/kg. Lanes 10-12 contained testicular microsomes prepared from rats treated with 0.4 µmol of EB/kg. Lanes 13-15 contained testicular microsomes prepared from rats treated with 4 µmol of EB/kg. Lane 16 contained rat liver microsomes 20 µg/lane, used as a positive control for the calnexin protein

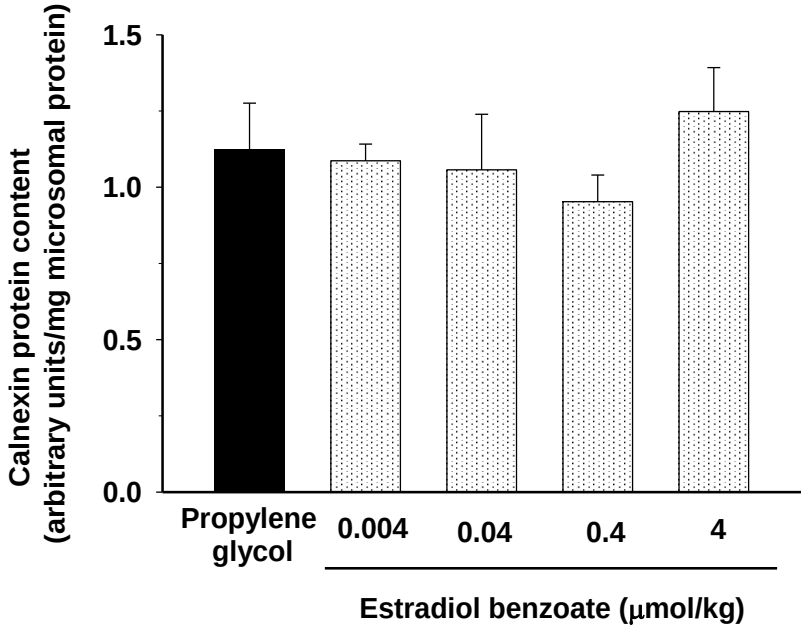


Figure 3.24 Graphical representation of relative levels of calnexin protein content in testicular microsomes prepared from adult male rats treated with EB or propylene glycol (vehicle). Data are expressed as mean ± SD of three individual experiments with 4 rats per group, except for the propylene glycol-treated group (n = 5).

3.3 Study 3: BPA Study

This study examined the effect of treatment with BPA on various parameters such as body weight, organ weights and testicular expression of CYP1B1, CYP2A1, CYP17A1, mEH, POR and 3β-HSD is presented after the rats were treated with varying dosages of BPA (400, 800 and 1600 μmol/kg) or an equal volume (1 ml/kg) of propylene glycol or saline (0.9% NaCl), once daily for 14 days and were killed 24 hr after the last dose.

3.3.1 Effect of BPA on Body and Organ Weights

Based on daily physical observation of the rats, all of the rats appeared to be healthy during the entire study period. Animal body weights were recorded every morning before treatment and on the day they were killed. The effect of treatment with BPA on body weight from day 1 to day 15 was presented in Figure 3.25A. Rats treated with BPA at 800 and 1600 μmol/kg decreased body weight at day 15 when compared with the corresponding day body weight of propylene glycol-treated rats. As shown in Figure 3.25B, BPA decreased body weight gain by 54, 74 and 94% at 400, 800 and 1600 μmol/kg, respectively, when compared with propylene glycol-treated rats.

Administration of BPA at 800 and 1600 μmol/kg dosages decreased absolute testis weight by 27% and 38%, respectively, when compared with propylene glycol-treated rats (Figure 3.26B). Similarly, BPA decreased testis weight (as percent of body weight) by 20%, and 30% at 800 and 1600 μmol/kg dosages, respectively, when compared with propylene glycol-treated rats (Figure 3.26A). No difference was found when relative and absolute liver and adrenal weights of BPA-treated groups were compared to those of the propylene glycol-treated group (Figure 3.26).

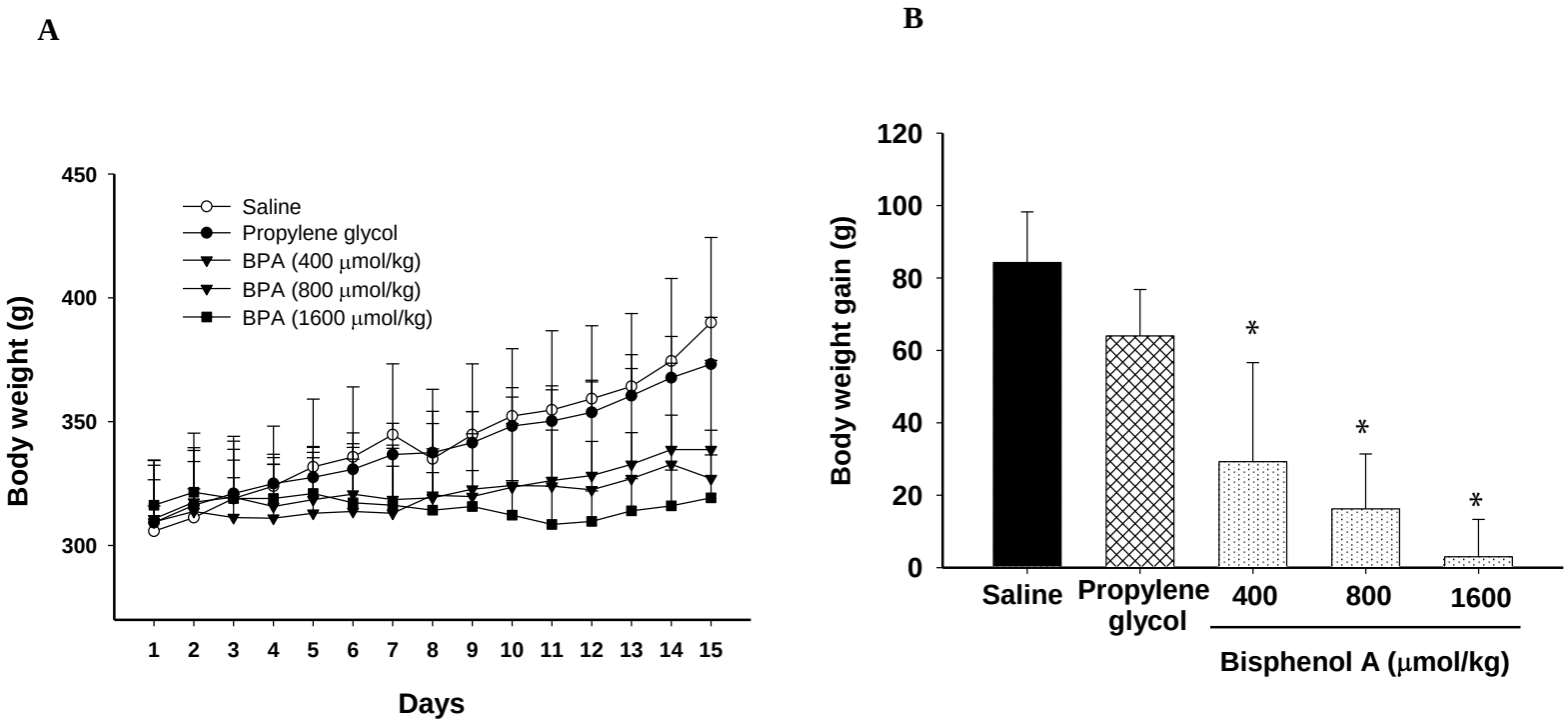


Figure 3.25 Effect of varying dosages of BPA treatment on (A) body weight during the treatment period and on (B) body weight gain. Data are expressed as mean ± SD for 4 rats per group. *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

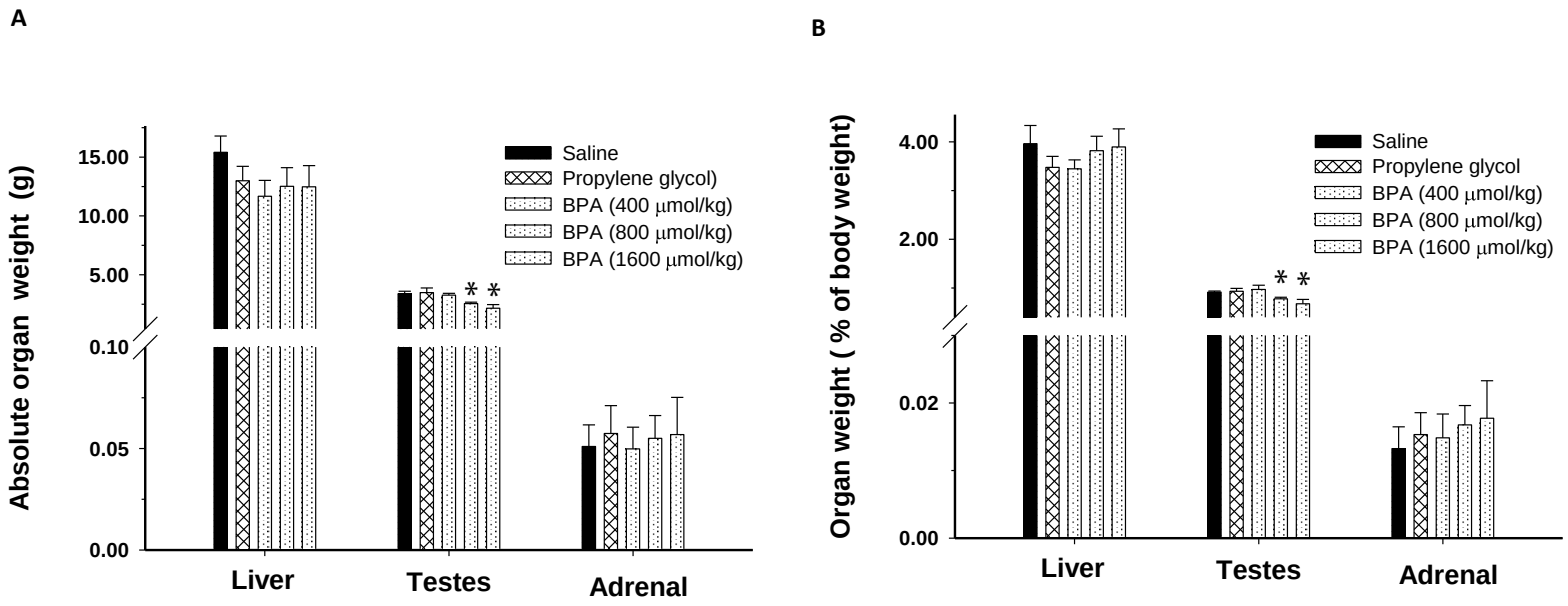


Figure 3.26 Effect of treatment with varying dosages of BPA on (A) absolute weights of liver, testis and adrenal and on (B) relative weights of liver, testis and adrenal (as percent of bodyweight). Data are expressed as mean ± SD for 4 rats per group. *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

3.3.2 Effect of BPA Treatment on Testicular Expression of CYP1B1, CYP2A1, CYP17A1, mEH, POR and 3β-HSD in Adult Male Rats

3.3.2.1 Immunoblot Analysis of Testicular Microsomes Probed for CYP1B1

Immunoblots containing testicular microsomes from different treatment groups, and standard CYP1B1 (cDNA expressed proteins) was probed with rabbit anti-rat CYP1B1 serum. A scanned image of immunoblot is shown in Figure 3.27. Relatively darker color protein bands were detected in lanes containing microsomal samples prepared from saline- or propylene glycol-treated rats than those of lanes containing microsomal samples of BPA. Within BPA treated groups, the staining intensity of the CYP1B1 bands was decreased with increasing dosages of BPA.

A standard curve was constructed from three different concentrations of standard CYP1B1 proteins to quantify the CYP1B1 level in testicular microsomes (Table 3.7). The calculated mean slope from 3 experiments was 4.9 with a limit of quantitation of 0.01 pmol/lane. The interday variation [coefficient of variation (CV)] of slopes obtained from three experiments was 11%.

A bar graph summarizing the mean testicular CYP1B1 levels for each treatment group is presented in Figure 3.28. Administration of BPA to adult male rats had shown a decrease in CYP1B1 protein levels by 51, 87, and 89% at 400, 800 and 1600 μmol/kg dosages, respectively, when compared with the propylene glycol-treated (64 ± 12 pmol of CYP1B1/mg microsomal protein) and saline-treated rats (64 ± 7.5 pmol of CYP1B1/mg microsomal protein). There was no difference in CYP1B1 expression between the propylene glycol- and saline-treated groups.

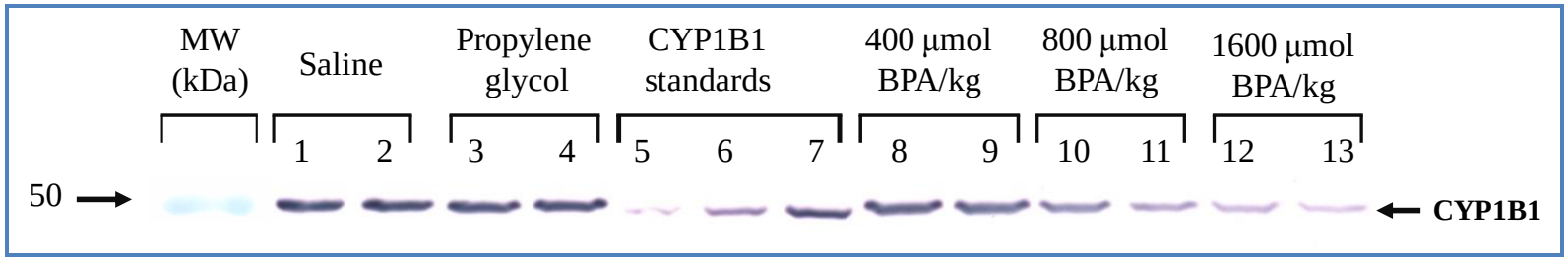


Figure 3.27 A representative immunoblot of rat testicular microsomes probed with rabbit anti-rat CYP1B1 serum. For antibody details refer to Table 2.1. The color development reaction time was 3 min. The amount of testicular microsomal protein loaded was 20 µg/lane. Lanes 1-2 contained testicular microsomal samples prepared from saline-treated rats. Lanes 3-4 contained testicular microsomal samples prepared from rats treated with propylene glycol-treated rats. Lanes 8-9 contained testicular microsomal samples prepared from rats treated with 400 µmol/kg of BPA. Lanes 10-11 contained testicular microsomal samples prepared from rats treated with 800 µmol/kg of BPA. Lanes 12-13 contained testicular microsomal samples prepared from rats treated with 1600 µmol/kg of BPA. Lanes 5, 6, and 7 contained cDNA expressed rat CYP1B1 protein at 0.1, 0.2 and 0.4 pmol/lane, respectively.

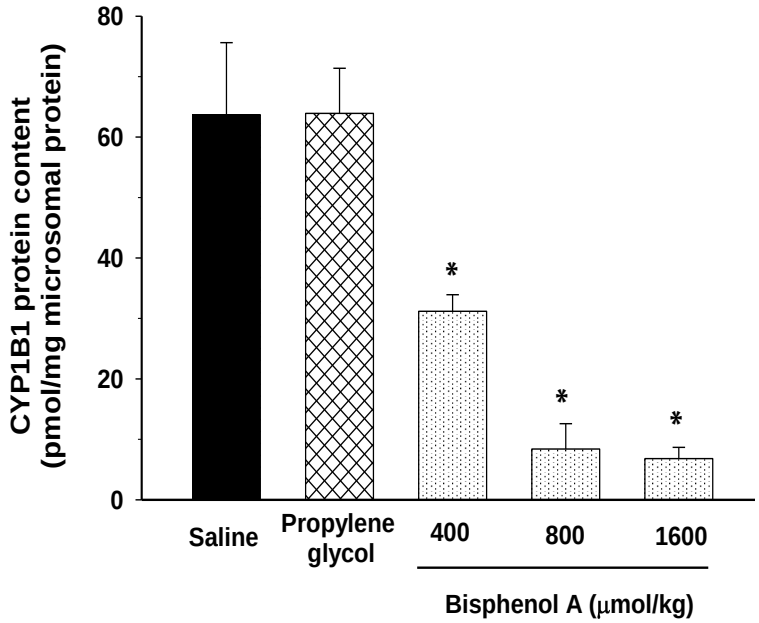


Figure 3.28 Graphical representation of CYP1B1 protein content in testicular microsomes prepared from adult male rats treated with BPA, propylene glycol (vehicle) or saline. Data are expressed as mean ± SD for 4 rats per group. *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

Table 3.6 Details of the calibration curve used for immunoquantitation of CYP1B1 protein in rat testicular microsomes

Immunoblot experiment	CYP1B1 standards (pmol/lane)	Calibration Curve	Slope	r^2
1	0.1, 0.2 or 0.4	A	5.2	0.97
		B	5.1	0.97
2	0.1, 0.2 or 0.4	A	5.2	0.98
		B	4.4	0.98
3	0.1, 0.2 or 0.4	A	4.2	0.95
		B	5.6	0.94
		Mean	4.9	
		SD	0.52	
		% CV	11	

Note: Three independent experiments were performed on different days to analyze each sample. Each experiment included two immunoblots with two calibration curves (a, b) to analyze all the samples in a single attempt. Abbreviations: SD, standard deviation; % CV, coefficient of variation; r^2, coefficient of determination..

3.3.2.2 Immunoblot Analysis of Testicular Microsomes Probed for CYP2A1

Immunoblots containing testicular microsomes from treatment groups, and purified rat liver CYP2A1 proteins were probed with sheep anti-rat CYP2A1 polyclonal IgG. A scanned image of immunoblot is shown in Figure 3.29. Relatively darker color immunoreactive bands were detected in lanes containing microsomes prepared from saline- or propylene glycol-treated rats than those of prepared from rats treated with varying BPA dosages. Within BPA treated groups, the staining intensity of CYP2A1 bands was decreased gradually with from lanes containing microsomal samples prepared from rats treated with lowest BPA dosage (400 μmol/kg) to highest BPA dosage (1600 μmol/kg).

A standard curve was constructed from four different concentrations of standard CYP2A1 proteins to quantify the CYP2A1 protein levels in testicular microsomes (Table 3.8). The calculated mean slope from 3 experiments was 4.2 with a limit of quantitation of 0.0625 pmol/lane. The interday variation [coefficient of variation (CV)] of slopes obtained from three experiments was 5.1%.

A bar graph summarizing the mean testicular CYP2A1 levels for each treatment group is presented in Figure 3.30. Treatment with BPA decreased CYP2A1 protein levels by 72, 92 and 92% at of 400, 800 and 1600 μmol/kg dose levels, respectively, when compared with the vehicle (3.5 ± 0.36 pmol/mg microsomal protein) or saline-treated rats (4.5 ± 1.1 pmol/mg microsomal protein). No difference was observed in CYP2A1 expression between the propylene glycol- and saline-treated groups.

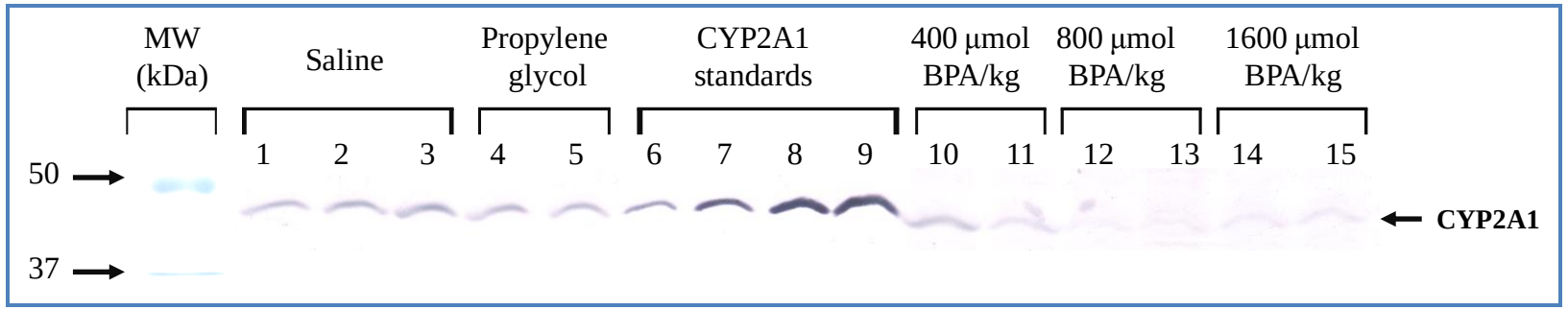


Figure 3.29 A representative immunoblot of rat testicular microsomes probed with sheep anti-rat CYP2A1 polyclonal IgG. For antibody details refer to Table 2.1. The color development reaction time was 4 min. Lanes 1-3 and 4-5 contained testicular microsomal samples (20 µg of microsomal protein per lane) prepared from saline or propylene glycol-treated rats, respectively. Lanes 10-11 contained testicular microsomal samples (80 µg of microsomal protein per lane) prepared from rats treated with BPA at 400 µmol/kg. Lanes 12-13 and 13-14 contained testicular microsomal samples (80 µg of microsomal protein per lane) prepared from rats treated with BPA at 800 µmol/kg. Lanes 13-14 contained testicular microsomal samples (80 µg of microsomal protein per lane) prepared from rats treated with BPA at 1600 µmol/kg. Lanes 6, 7, 8 and 9 contained purified rat liver CYP2A1 protein at 0.0625, 0.125, 0.25 and 0.35 pmol/lane, respectively.

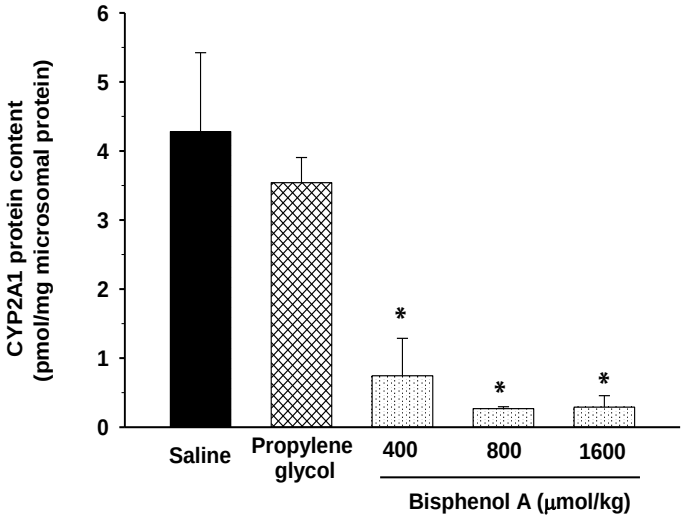


Figure 3.30 Graphical representation of CYP2A1 protein content in testicular microsomes prepared from adult male rats treated with BPA, propylene glycol (vehicle) or saline. Data are expressed as mean ± SD for 4 rats per group. *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

Table 3.7 Details of the calibration curve used for immunoquantitation of CYP2A1 protein in rat testicular microsomes

Immunoblot experiment	CYP2A1 standards (pmol/lane)	Calibration curve	Slope	r^2
1	0.0625, 0.125, 0.25 or 0.35	a	3.9	0.98
		b	4.3	0.92
2	0.0625, 0.125, 0.25 or 0.35	a	4.4	0.84
		b	4.2	0.94
3	0.0625, 0.125, 0.25 or 0.35	a	4.4	0.78
		b	4.2	0.79
		Mean	4.2	
		SD	0.21	
		% CV	5.1	

Note: Three independent experiments were performed on different days to analyze each sample. Each experiment included two immunoblots with two calibration curves (a, b) to analyze all the samples in a single attempt. Abbreviations: SD, standard deviation; % CV, coefficient of variation; r^2, coefficient of determination.

3.3.2.3 Immunoblot Analysis of Testicular Microsomes Probed for CYP17A1

Immunoblots containing testicular microsomes from different treatment groups were probed with goat anti-mouse CYP17A1 polyclonal IgG. A scanned image of the immunoblot is shown in Figure 3.31. Adrenal microsomes prepared from untreated rats were loaded on to the gel to serve as a positive control. Relatively darker color CYP17A1 immunoreactive bands were detected in lanes containing microsomes prepared from saline- or propylene glycol-treated rats than those of lanes containing testicular microsomal samples prepared from rats treated with BPA at lowest dosage. There was no CYP17A1 protein bands were detected in the lanes containing microsomal samples prepared from rats treated with BPA at 400 μmol/kg and 1600 μmol/kg.

A standard curve for CYP17A1 was not generated because of a lack of purified or recombinant CYP17A1 proteins. Hence relative levels of testicular CYP17A1 protein was presented as protein band intensity per milligram of testicular microsomal protein (Figure 3.32). BPA decreased CYP17A1 protein levels by 49% at 400 μmol/kg dosage, but CYP17A1 levels were completely abolished and not detectable at 800 and 1600 μmol/kg dosages, when compared with propylene glycol- and saline-treated groups. There was no difference in CYP17A1 protein expression between the propylene glycol- and saline-treated groups.

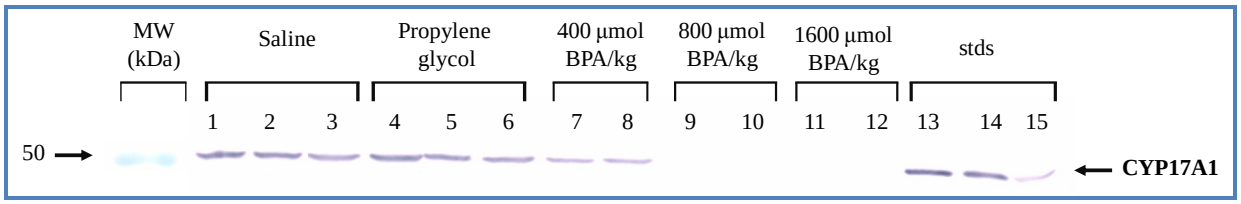


Figure 3.31 A representative immunoblot of rat testicular microsomes probed with polyclonal goat anti-mouse CYP17A1 IgG. For antibody details refer to Table 2.3. The color development reaction time was 4 min. Lanes 1-3 and 4-6 contained testicular microsomal samples (20 µg of microsomal protein per lane) prepared from rats treated with saline or vehicle, respectively. Lanes 7-8 contained testicular microsomal samples (20 µg of microsomal protein per lane) prepared from rats treated with 400 µmol of BPA/kg. Lanes 9-10 contained testicular microsomal samples (80 µg of microsomal protein per lane) prepared from rats treated with 800 µmol of BPA/kg. Lanes 11-12 contained testicular microsomal samples (80 µg of microsomal protein per lane) prepared from rats treated with 1600 µmol of BPA/kg. Lanes 13 and 14 contained rat adrenal microsomes 20 µg/lane, used as a positive control for the CYP17A1 protein detection. Lane 15 contained testicular microsomes prepared from pooled testis of untreated rats.

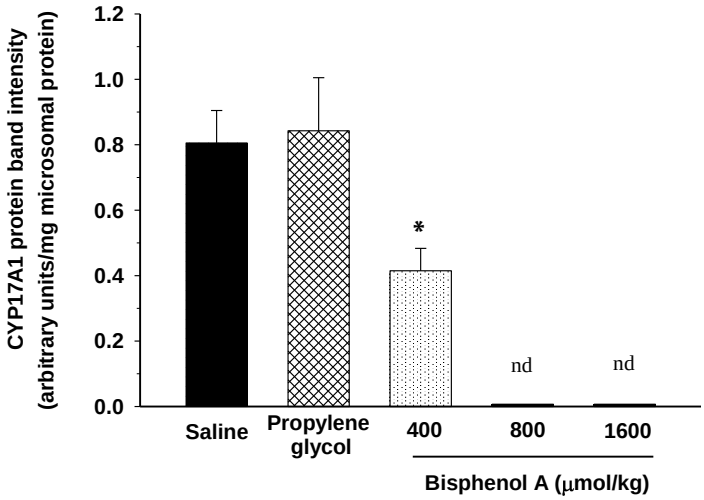


Figure 3.32 Graphical representation of relative levels of CYP17A1 protein content in testicular microsomes prepared from adult male rats treated with BPA, propylene glycol (vehicle) or saline. Data are expressed as mean ± SD for 4 rats per group. *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

3.3.2.4 Immunoblot Analysis of Testicular Microsomes Probed for mEH

Immunoblots containing testicular microsomes from treatment groups, and purified rat liver EH protein were probed with rabbit anti-rat EH polyclonal IgG. A scanned image of the immunoblot is shown in Figure 3.33. Relatively darker color protein bands were detected in lanes containing microsomal samples prepared from saline- or propylene glycol-treated rats than those of lane containing microsomal samples prepared from rats treated with varying BPA dosages. Within BPA treated groups, the staining intensity of the immunoreactive bands was decreased gradually from lanes containing microsomes prepared from rats treated with lowest BPA dosage (400 μmol/kg) to highest BPA dosage (1600 μmol/kg).

A standard curve was constructed from three different concentrations of purified rat liver EH proteins to quantify the mEH level in testicular microsomes (Table 3.9). The calculated mean slope from 3 experiments was 15 with a limit of quantitation of 0.01 μg/lane. The interday variation [coefficient of variation (CV)] of slopes obtained from three experiments was 6.4%

A bar graph summarizing the mean testicular mEH levels for each treatment group is presented in Figure 3.36. BPA decreased the testicular mEH protein levels by 50, 67 and 67% at 400, 800 and 1600 μmol/kg dosages, respectively, when compared with vehicle (6.1 ± 0.65 μg/mg microsomal protein) or saline (6.3 ± 0.22 μg//mg microsomal protein) treated group. There was no difference in mEH expression between the propylene glycol- and saline-treated rats.

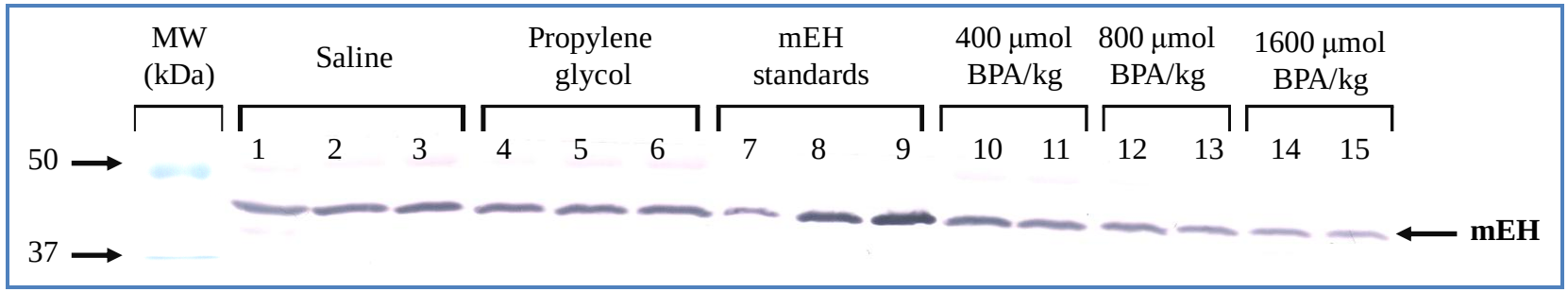


Figure 3.33 A representative immunoblot of rat testicular microsomes probed with rabbit anti-rat EH polyclonal IgG. For antibody concentrations refer to Table 2.3. The color development reaction time was 2 min. Lanes 1-3 and 4-6 contained testicular microsomal samples (10 µg of microsomal protein per lane) prepared from propylene glycol- and saline treated rats, respectively. Lanes 10-11 contained testicular microsomal samples (20 µg of microsomal protein per lane) prepared from rats treated with 400 µmol of BPA/kg. Lanes 12-13 contained testicular microsomal samples (20 µg of microsomal protein per lane) prepared from rats treated with 800 µmol of BPA/kg. Lanes 14-15 contained testicular microsomal samples (20 µg of microsomal protein per lane) prepared from rats treated with 1600 µmol of BPA/kg. Lanes 7, 8 and 9 contained purified rat liver EH protein at 0.01, 0.05, and 0.1 pmol/lane, respectively.

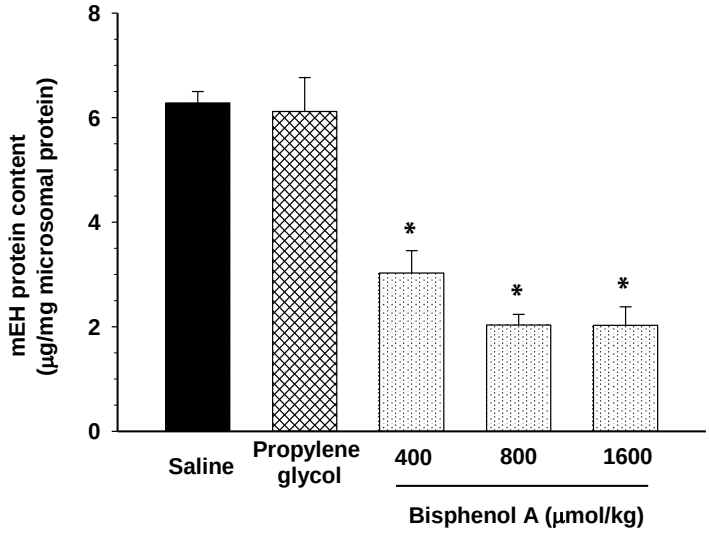


Figure 3.34 Graphical representation of mEH protein content in testicular microsomes prepared from adult male rats treated with BPA, propylene glycol (vehicle) or saline. Data are expressed as mean ± SD for 4 rats per group. *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

Table 3.8 Details of the calibration curve used for immunoquantitation of mEH protein in rat testicular microsomes

Immunoblot experiment	EH standards (µg/lane)	Calibration curve	Slope	r^2
1	0.01, 0.05 or 0.1	a	15	0.82
		b	16	0.85
2	0.01, 0.05 or 0.1	a	15	0.59
		b	16	0.71
3	0.01, 0.05 or 0.1	a	15	0.91
		b	13	0.72
		Mean	15	
		SD	0.96	
		% CV	6.4	

Note: Three independent experiments were performed on different days to analyze each sample. Each experiment included two immunoblots with two calibration curves (a, b) to analyze all the samples in a single attempt. Abbreviations: SD, standard deviation; % CV, coefficient of variation; r^2, coefficient of determination.

3.3.2.5 Immunoblot Analysis of Testicular Microsomes Probed for POR

Immunoblots containing testicular microsomes from treatment groups, and purified rat liver POR protein were probed with rabbit anti-rat POR serum. A scanned image of the immunoblot is shown in Figure 3.35. Relatively darker color protein bands were detected in lanes containing microsomal samples prepared from saline- or propylene glycol-treated rats than those of lanes containing microsomal samples prepared from rats treated with varying BPA dosages. Within BPA treated groups, the staining intensity of POR protein bands was decreased gradually from lanes containing microsomes prepared from rats treated with lowest BPA dosage (400 μmol/kg) to highest BPA dosage (1600 μmol/kg).

A standard curve was constructed from three different concentrations of purified rat liver POR proteins to quantify the POR level in testicular microsomes (Table 3.10). The calculated mean slope from 3 experiments was 0.037 with a limit of quantitation of 6.25 ng/lane. The interday variation [coefficient of variation (CV)] of slopes obtained from three experiments was 22%

A bar graph summarizing the mean testicular POR levels for each treatment group is presented in Figure 3.36. Administration of BPA decreased POR protein levels by 43, 66 and 67% at 400, 800 and 1600 μmol/kg dosages, respectively, when compared with propylene glycol-treated (185 ± 11 ng/mg microsomal protein) and saline-treated (183 ± 15 ng/mg microsomal protein) groups. There was no difference in POR protein levels between the propylene glycol- and saline-treated rats.

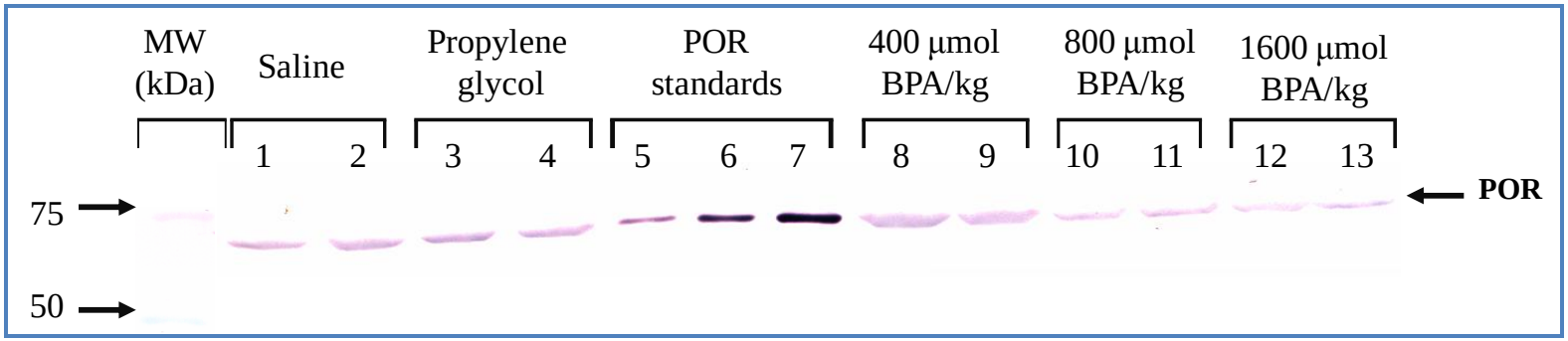


Figure 3.35 A representative immunoblot of rat testicular microsomes probed with rabbit anti-rat POR serum. For antibody details refer to Table 2.3. The color development reaction time was 5 min. Lanes 1-2 and 3-4 contained testicular microsomal samples (40 µg of microsomal protein/lane) prepared from propylene glycol- or saline-treated rats. Lanes 8-9 contained testicular microsomal samples (80 µg of microsomal protein per lane) prepared from rats treated with 400 µmol of BPA/kg. Lanes 10-11 contained testicular microsomal samples (80 µg of microsomal protein per lane) prepared from rats treated with 800 µmol of BPA/kg. Lanes 12-13 contained testicular microsomal samples (80 µg of microsomal protein per lane) prepared from rats treated with 1600 µmol of BPA/kg, respectively. Lanes 5, 6, and 7 contained purified rat liver P450-reductase protein at 6.25, 12.5, and 25 ng/lane, respectively.

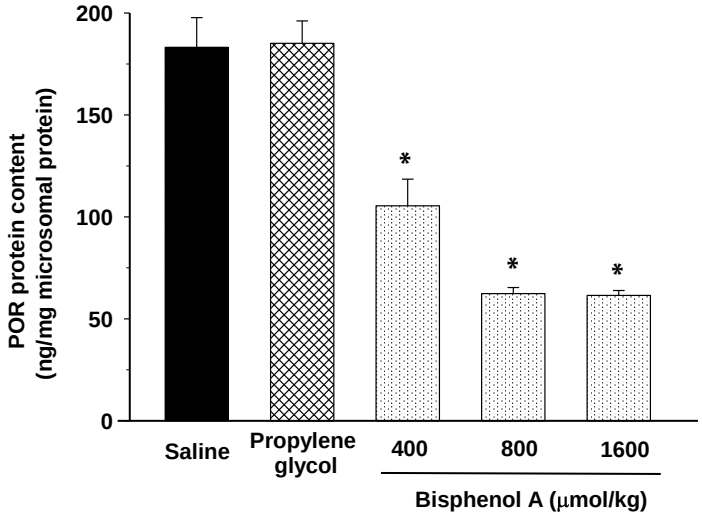


Figure 3.36 Graphical representation of POR protein content in testicular microsomes prepared from adult male rats treated with BPA, propylene glycol (vehicle) or saline. Data are expressed as mean ± SD for 4 rats per group. *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

Table 3.9 Details of the calibration curve used for immunoquantitation of POR protein in rat testicular microsomes

Immunoblot experiment	POR standards (ng/lane)	Calibration curve	Slope	r^2
1	6.25, 12.5 or 25	a	0.038	0.96
		b	0.037	0.93
2	6.25, 12.5 or 25	a	0.041	0.98
		b	0.021	0.94
3	6.25, 12.5 or 25	a	0.0403	0.98
		b	0.042	0.95
		Mean	0.037	
		SD	0.0079	
		% CV	22	

Note: Three independent experiments were performed on different days to analyze each sample. Each experiment included two immunoblots with two calibration curves (a, b) to analyze all the samples in a single attempt. Abbreviations: SD, standard deviation; % CV, coefficient of variation; r^2, coefficient of determination.

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3.3.2.6 Immunoblot Analysis of Testicular Microsomes Probed for 3β-HSD

Immunoblots containing testicular microsomes from different treatment groups were probed with rabbit anti-human 3β-HSD polyclonal IgG. A scanned image of the immunoblot is shown in Figure 3.37. Adrenal microsomes prepared from untreated rats were loaded on to the gel to serve as a positive control. The staining intensity of protein bands was not affected in lanes containing microsomal samples prepared from saline or vehicle or BPA treated (at 800 μmol/kg) groups, but the staining intensity was decreased in lanes containing microsomes prepared from rats treated with highest BPA dosage (at 1600 μmol/kg).

A standard curve for 3β-HSD was not generated due to lack of purified or recombinant 3β-HSD protein. Hence the relative level of 3β-HSD protein was presented as protein band intensity per milligram of microsomal protein (Figure 3.38). Administration of BPA decreased 3β-HSD protein expression by 48% at highest dosage (1600 μmol/kg), but not at the remaining dosages, when compared with propylene glycol- and saline-treated rats. There was no differenrce in 3β-HSD expression between the propylene glycol- and saline-treated rats.

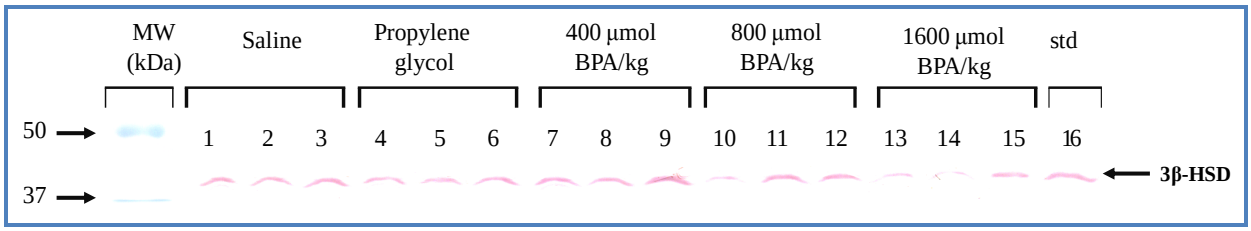

Figure 3.37 A representative immunoblot of rat testicular microsomes probed with rabbit anti-human 3β-HSD polyclonal IgG. The amount of testicular microsomal protein loaded was 20 µg/lane. For antibody details refer to Table 2.3. The color development reaction time was 7 min. Lanes 1-3 and 4-6 contained testicular microsomes prepared from rats treated with saline or vehicle, respectively. Lanes 7-9 contained testicular microsomes prepared from rats treated with 400 µmol of BPA/kg. Lanes 10-12 contained testicular microsomes prepared from rats treated with 800 µmol of BPA/kg. Lanes 13-15 contained testicular microsomes prepared from rats treated with 1600 µmol of BPA/kg. Lanes 16 were contained rat adrenal microsomes 20 µg/lane, used as a positive control for the 3β-HSD protein.

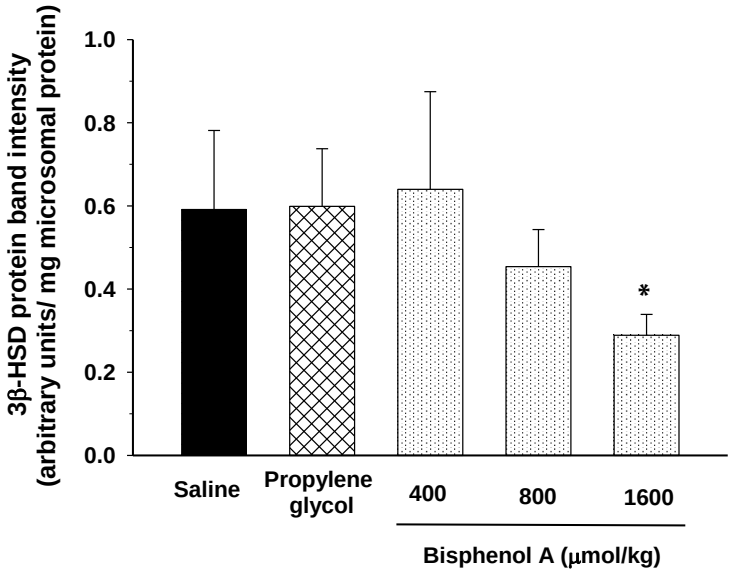

Figure 3.38 Graphical representation of relative levels of 3β-HSD protein content in testicular microsomes prepared from adult male rats treated with BPA, propylene glycol (vehicle) or saline. Data are expressed as mean ± SD for 4 rats per group. *, significantly different ($p < 0.05$) from propylene glycol-treated rats.

3.3.2.7 Immunoblot Analysis of Testicular Microsomes Probed for Calnexin

Immunoblots containing testicular microsomes from different treatment groups were probed with rabbit anti-human calnexin serum. A scanned image of the immunoblot is shown in Figure 3.39. Liver microsomal samples prepared from untreated rats were loaded on to the gel to serve as a positive control. The staining intensity of protein bands was similar in all the lanes containing microsomal samples prepared from propylene glycol- or saline- or BPA-treated groups

A standard curve for calnexin was not generated due to lack of recombinant or purified calnexin protein. Hence, the relative level of calnexin protein was presented as protein band intensity per milligram of microsomal protein (Figure 3.40). Administration of BPA had shown no effect on protein expression of calnexin in testicular microsomes prepared from varying dosages of BPA-treated rats. No difference was observed in calnexin protein levels bitween any of the treatment groups.

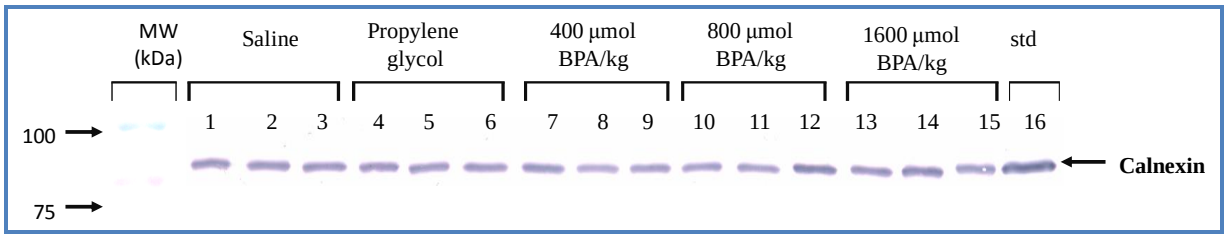

Figure 3.39 A representative immunoblot of rat testicular microsomes probed with rabbit anti-human calnexin serum. The color development reaction time was 90 sec. For the antibody details refer to Table 2.3. Amount of testicular microsomal protein loaded was 20 µg/lane Lanes 1-3 and 4-6 contained testicular microsomes prepared from rats treated with saline or vehicle, respectively. Lanes 7-9 contained testicular microsomes prepared from rats treated with 400 µmol of BPA/kg. Lanes 10-12 contained testicular microsomes prepared from rats treated with 800 µmol of BPA/kg. Lanes 13-14 contained testicular microsomes prepared from rats treated with 1600 µmol of BPA/kg. Lane 15 containing testicular microsomes prepared from pooled testis of untreated rats. Lane 16 contained rat liver microsomes 20 µg/lane, used as a positive control for the calnexin protein.

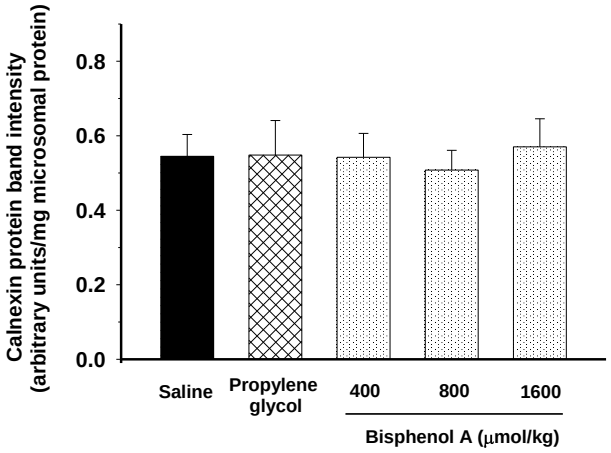

Figure 3.40 Graphical representation of relative levels of calnexin protein content in testicular microsomes prepared from adult male rats treated with BPA, propylene glycol (vehicle) or saline. Data are expressed as mean ± SD for 4 rats per group.

4. Discussion

4.1 Study 1: Immunochemical Detection of Various CYP and Other Xenobiotic-Metabolizing Enzymes in Rat Testicular Microsomes

The focus of the Study 1 was to characterize the expression of CYP and other xenobiotic-metabolizing enzymes at the protein level in rat testis.

CYP1B1

Previous studies reported that CYP1B1 protein was expressed in steroidogenic tissues including adrenal cortex, ovary and testis (Bhattacharyya et al., 1995, Otto et al., 1992; Walker et al., 1995; Leung et al., 2009; Deb et al., 2010). CYP1B1 was readily detected, by immunoblot analysis, in rat testis microsomes in the present study. Basal expression of CYP1B1 in testicular microsomes prepared from propylene glycol- or saline-treated rats was determined to be 63 ± 6 pmol or 64 ± 12 pmol per mg of total testicular microsomal protein (mean ± SD), respectively. Basal expression of testicular CYP1B1 protein was previously reported to be 57 and 60 pmol per mg of microsomal protein (Leung et al 2009; Deb et al., 2011). Although total CYP content in testicular microsomes was not measured in this study, one can assume a total testicular CYP content of 80 pmol per mg of microsomal protein (Leung et al., 2009). Thus, the basal level of CYP1B1 (64 pmol) that determined in the present study represents 80% of the total CYP content (as determined by CO difference spectrum) in the adult rat testis. This suggests that CYP1B1 is abundantly expressed in adult rat testis.

CYP1B1 immunoreactivity was confined to interstitial cells, which are comprised mainly of Leydig cells. No immunoreactivity was detected within the seminiferous tubules, which

include spermatogenic and Sertoli cells. These results suggest that CYP1B1 expression in rat testis is cell type specific and primarily localized in Leydig cells.

The abundant basal expression of CYP1B1 in testis may be of toxicological significance. CYP1B1 is actively involved in the bioactivation of various toxic chemicals, *in vitro* and *in vivo*. For example, human recombinant CYP1B1 is able to convert polycyclic aromatic hydrocarbons and various heterocyclic and aryl amines into toxic metabolites (Shimada et al., 1996). For instance, CYP1B1 converted 7,12-dimethylbenz[*a*]anthracene (DMBA) to 1,2-epoxide-3,4-diol-DMBA, which is a carcinogenic metabolite, in rat testicular microsomes, *in vitro* (Otto et al., 1992). Moreover, formation of the carcinogenic metabolite of DMBA and formation of DNA-DMBA adducts was observed in mouse testis, when CYP1B1 knockout mice and wild-type mice were treated with DMBA at 200 μg/mice/day for 3 weeks. The levels of DNA adducts found in testicular homogenate of CYP1B1 knockout mice were seven times lower than those observed in wild-type mice (Buter et al., 2002). These studies suggest that CYP1B1 plays a role in the development of testicular toxicity via bioactivation of environmental chemicals such as DMBA and BaP (Georgellis et al., 1989). Because, CYP1B1 expression in rat testis is cell specific, interstitial cells may be highly vulnerable to toxic insult mediated by CYP1B1 in testis.

CYP2A1

Using CYP2A1-specific polyclonal antibody, an immunoreactive protein band with molecular size of approximately 48 kDa and similar electrophoretic mobility as the CYP2A1 protein standard was detected in testicular microsomes of adult rats. These results agree with previous studies (Sonderfan et al., 1989 and Seng et al., 1991), in which CYP2A1 was detected in rat Leydig cell homogenate by immunoblot analysis or measurement of CYP2A1-mediated

activity namely, conversion of testosterone to 7-α hydroxytestosterone. In rats, the CYP2A subfamily consists of three enzymes, CYP2A1, CYP2A2 and CYP2A3 (Martignoni et al., 2006), which can be resolved by SDS-PAGE (Anderson et al., 1998). In the present study, we did not detect any immunoreactive bands other than CYP2A1 in the testicular microsomal samples. This indicates that CYP2A2 and CYP2A3 are either not constitutively expressed or are expressed at a very low level in rat testis. Further studies are required to thoroughly investigate the possible expression of CYP2A2 and CYP2A3 in rat testis.

Our study is the first to quantify basal expression of CYP2A1 protein, which was estimated to be 6 ± 3 pmol or 4 ± 1 pmol per mg of total testicular microsomal protein (mean ± SD) in testicular microsomes of rats treated with propylene glycol or saline, respectively. In comparison to testicular CYP1B1 protein levels, CYP2A1 basal levels are very low. If one assumes the total CYP content of testicular microsomes is 80 pmol /mg microsomal protein (Leung et al., 2009), then the CYP2A1 level (5 pmol) determined in the present study represents approximately 6% of the total CYP content in adult rat testis.

Immunohistochemical analysis revealed that the cellular localization of CYP2A1 is similar to that of CYP1B1. CYP2A1 immunoreactivity was confined to interstitial cells, which are comprised mainly of Leydig cells. Immunoreactivity for CYP2A1 was not detected in seminiferous tubules. These results are in agreement with a previous study (Seng et al., 1996), in which CYP2A1 immunoreactivity was detected in Leydig cells after immunohostochemical analyses of tissue sections prepared from rat testis.

CYP2A1 may play a role in the maintenance of hormonal homeostasis in the testis. CYP2A1 is catalytically active in the metabolism of testosterone to 7α-hydroxytestosterone in rat liver and testicular microsomes, *in vitro* (Sonderfan et al., 1989; Seng et al., 1996). Thus,

changes in CYP2A1 expression levels could affect testosterone levels and the physiological function mediated by testosterone in testis.

CYP1A

Unlike CYP1B1 and CYP2A1, immunoreactive bands corresponding to CYP1A1 and CYP1A2 were not detected in testicular microsomes from adult male rats. This result confirms earlier reports of little or no expression of CYP1A1 mRNA or protein in homogenates of rat primary Leydig cells or TM3 Leydig cells (immortalized mouse Leydig cells) or in rat testicular microsomes (Chung et al., 2007; Korkalainen et al., 2004). A recent study (Deb et al., 2010) from our laboratory also reported that CYP1A1 and CYP1A2 are not expressed constitutively in rat testis or after treatment of rats with typical CYP1A inducers such as BaP and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

CYP2B

In the present study, immunoreactive bands corresponding to CYP2B1 or CYP2B2 or CYP2B3 proteins were not detected in microsomes prepared from adult rat testis. Conflicting information is available about the expression of CYP2B enzymes in rat testis. Andric et al. (2006) reported the presence of CYP2B1 mRNA in interstitial cells isolated from pooled rat testis, whereas, Imaoka et al. (2005) did not detect CYP2B1 mRNA in rat testis. On the basis of these two studies, it is possible that CYP2B1 mRNA is expressed at very low levels in rat testis, but translation of mRNA into detectable protein is unlikely.

CYP2D

Immunoreactive bands corresponding to CYP2D1 and CYP2D2 proteins were not detected in testis microsomes from adult rats. These results are in agreement with a previous study by Hirori et al. (1998), in which CYP2D2 and CYP2D3 mRNA was not detected in rat testis. In the same study, CYP2D1, CYP2D4, CYP2D5 and CYP2D18 mRNAs were detected in rat testis but the expression levels were relatively very low compared to liver, kidney and small intestine. Although the primary antibody used in the present study is polyspecific and detected recombinant CYP2D1 and CYP2D2 protein standards, it is not known whether the antibody was able to detect CYP2D3, CYP2D4, CYP2D5 and CYP2D18 enzymes. Possible reasons for not detecting any of the rat CYP2D enzymes are 1) CYP2D proteins are not expressed in rat testis, 2) lack of specific antibodies and purified standards for CYP2D3, CYP2D4, CYP2D5 and CYP2D18 and 3) the immunoblot method may not be sensitive enough to detect low levels of CYP2D proteins.

CYP2E1

An immunoreactive protein band corresponding to CYP2E1 was not found in microsomes prepared from adult rat testis. These results contradict two previous reports, in which trace levels of CYP2E1 protein and mRNA were detected in testis from untreated rats or from rats that were treated with pyridine or halothane (Jiang et al., 1998; Oropeza-Hernandez et al., 2003). The amount of testicular microsomal protein (20 μg/lane) analyzed in the present study was less than that used in the previous studies (40 μg/lane) (Jiang et al., 1998; Oropeza-Hernandez et al., 2003). In addition, the authors used chemiluminesence detection, which might be more sensitive for detecting a weak signal than the colorometric method used in the present

study. It is also possible that the antibody used in the previous studies was more sensitive than the antibody used in the present study. Further studies are required to confirm the expression or lack of expression of CYP2E1 in rat testis.

CYP2C

In the present study, immunoreactive bands specific for CYP2C6, CYP2C7, CYP2C11, CYP2C12 or CYP2C13 were not detected in the testis microsomes obtained from adult rats. These results are in agreement with a previous study, in which no CYP2C11 protein or functional activity was observed in the rat testicular microsomes (Seng et al., 1996). Thus, it is less likely that CYP2C enzymes play a role in the detoxification or bioactivation of toxicants in rat testis.

CYP3A and CYP4A

Immunoreactive bands corresponding to CYP3A1 and CYP3A2 were not detected in microsomes prepared from adult male rat testis. Similarly, immunoreactive CYP4A proteins (CYP4A1, CYP4A2 and CYP4A3) were not detected in rat testicular microsomes. To my knowledge, there are no previous reports on the expression of CYP3A and CYP4A proteins in rat testis.

CYP17A1 and 3β-HSD

CYP17A1 and 3β-HSD are key enzymes responsible for the synthesis of androgens in steroidogenic tissues such as adrenal, testis and ovary (Payne and Hales, 2004). In the present study, these two enzymes were used as reference proteins for testicular microsomes and

immunoreactive bands corresponding to CYP17A1 and 3β-HSD were detected in testicular microsomes from adult rats. The expression of these enzymes in rat testis has been well studied (Payne and Hales, 2004). Immunohistochemical analysis using specific antibodies for CYP17A1 and 3β-HSD suggests that CYP17A1 and 3β-HSD expression was confined to interstitial cells, which are mainly comprised of Leydig cells. Our results confirm the results of previous studies (Fevold et al., 1989; Lee goasconge et al., 1991; Pelletier et al., 2001; Payne and Hales, 2004; Simard et al., 1993), in which CYP17A1 expression was detected at the mRNA and protein level in rat liver, stomach, testis, adrenal gland and ovary. It has been also reported that CYP17A1 was developmentally regulated in these tissues (Vianello et al., 1997). 3β-HSD expression was observed in the smooth endoplasmic reticulum of Leydig cells of rat testis (Majdic et al., 1995, Haider and Servos, 1998). Because, CYP17A1 and 3β-HSD expression is Leydig cell specific, most of the studies have used these two proteins as Leydig cell markers.

NADPH-cytochrome P450 oxidoreductase (POR)

POR is an obligatory partner for microsomal CYP enzymes and is involved in the transfer of electrons from NADPH, which is a critical step in the catalytic cycle of CYP enzymes (Ram and Waxman 1992). In the present study, POR protein was detected in testis microsomes from adult male rats. These results are in agreement with previous studies (Waxman et al., 1989b; Ram and Waxman 1992), in which POR was detected in rat testicular microsomes using immunoblot analysis. Our study is the first to quantify basal expression of POR protein, which was estimated to be 232 ± 48.4 μg or 183 ± 14.5 pmol per mg of total testicular microsomal protein (mean ± SD) in testicular microsomes of rats treated with propylene glycol or saline, respectively.

The present study is the first to report the localization and cellular distribution of POR in rat testis. Unlike CYP1B1 and CYP2A1, immunoreactivity for POR was distributed across the testis including interstitial cell and seminiferous tubules. In tubules, immunoreactivity was observed in spermatogenic and Sertoli cells. This result is not surprising because CYP enzymes are expressed in Leydig cells (CYP17A1, CYP1B1 and CYP2A1), Sertoli cells (CYP19A1) and spermatogenic cells (CYP19A1). As an obligatory partner of CYP enzymes, it was expected that POR would be expressed in all cells in the testis.

POR plays an important role in testicular functions such as steroidogenesis and spermatogenesis as a partner for CYP enzymes. Alteration in POR expression could cause reproductive abnormalities by affecting the catalytic activities of CYP enzymes. It has been reported that hepatic expression of POR is regulated by pituitary and thyroid hormones and induced by various xenobiotics such as phenobarbital or pregnenolone-16α-carbonitrile (Taira et al., 1980). However, it is unknown if the testicular POR expression is inducible by drugs such as phenobarbital or pregnenolone-16α-carbonitrile.

Microsomal epoxide hydrolase (mEH)

Immunoreactive band corresponding to mEH was readily detected and the basal expression of mEH protein levels were quantified to be 6.3 ± 0.5 μg or 6.2 ± 0.2 pmol per mg of total testicular microsomal protein (mean ± SD) in testicular microsomes of rats treated with propylene glycol or saline, respectively. Immunoreactivity for mEH was detected across the testis including interstitial cells and seminiferous tubules. In tubules, mEH immunoreactivity was observed in spermatogenic and Sertoli cells. In spermatogenic cells, mEH immunoreactivity was observed to be stage specific. The predominant flurescence was observed in later-stage

spermatids. Based on the intensity of immunoreactive fluorescence, mEH appeared to be more strongly expressed in seminiferous tubules than in the interstitium. Mukhtar et al. (1978) reported that mEH activity in the spermatogenic cell fraction was two-fold greater than that of similar fractions prepared from interstitial cells. Our results are in agreement with previous studies, which found that mEH immunoreactivity was distributed across the testis in Wistar and Holtzmn rats (Ishii-Ohba et al., 1984).

Summary

In summary, CYP1B1, CYP2A1, POR, and EH were detected in testicular microsomes isolated from adult rats. By comparison, CYP1A1, CYP1A2, CYP2B1, CYP2E1, CYP2D1, CYP2D2, CYP2C6, CYP2C7, CYP2C12 CYP2C13, CYP3A1, CYP3A2, CYP4A1, CYP4A2 and CYP4A3 were not detected in the same microsomal samples. CYP17A1 and 3β-HSD were detected as reference proteins in testicular microsomes. Immunohistochemical results suggest that the protein expression pattern for CYP1B1, CYP2A1 and CYP17A1 is cell specific and is primarily confined to interstitial cells, whereas POR and mEH expression occurred in interstitial cells and in cells of the seminiferous epithelium.

4.2 Study 2: 17β-Estradiol Benzoate Study

4.2.1 Effect of 17β-Estradiol Benzoate on Body and Organ Weights

Treatment with 17β-estradiol benzoate **(**EB**)** for 14 days decreased body weight gain in a dose-dependent manner, but had no effect on relative liver weight, testes weight or adrenal weight (as percent of body weight). Similarly, EB had no effect on absolute testes weight, except at the highest dosage (4 μmol/kg), where BPA decreased testes weight by 20%, as compared with the propylene glycol-treated rats. These results are in agreement with a previous study (Nakamura et al., 2010), in which male rats were treated with EB at 10 and 100 μg/kg/day for 4 days/week for 6 weeks and decreased body weight was observed. The same study reported that EB at 100 μg/kg decreased absolute testis weight, when compared with the control group. A previous study from our laboratory also found a change in testis weight (unpublished data). Male rats treated with EB at 1.5 mg/kg/day (4 μmol/kg) for 14 days had decreased body weight and decreased absolute testes weight.

Earlier studies reported that 17β-estradiol exerts a suppressive effect on body weight (Nunez et al., 1980; Sivelle et al., 1978; Palmer and Grey et al., 1986). This effect was attributed to suppression of feeding behavior and was thought to be due to the action of 17β-estradiol on periventricular or ventromedial nuclei in the hypothalamus (Nunez et al., 1980; Sivelle et al., 1978; Palmer and Grey et al., 1986). A decrease in food intake (by 30% compared to day 1) was observed in male rats that were injected with EB at 2 μg/animal for 3 days (Palmer and Grey et al., 1986). Several studies proposed that the decrease in food intake is a consequence of 17β-estradiol-produced anorexia (loss of appetite), which is attributed to different pathways. For example, Rivera et al. (2012) found that 17β-estradiol increased the release of a specific

neurotransmitter, 5-hydroxytryptamine, in the brain. Normally, 5-hydroxytryptamine secretion during meals functions to control the amount of food ingested and decrease appetite. Therefore, an increase in 5-HT levels may reduce appetite and food intake. Another pathway that leads to loss of appetite is suppression of lipoprotein lipase activity. Lipoprotein lipase is highly expressed in heart, muscle and adipose tissues (Mead et al., 2002). Lipoprotein lipase normally converts triglyceride into free fatty acids and promotes the uptake of these fatty acids into tissues (Mead et al., 2002). 17β-Estradiol is reported to suppress lipoprotein lipase activity in adipose tissue (Gray and Greenwood, 1982; Nunez et al., 1980). This effect leads to increased availability of triglycerides in the circulation, which serve as a metabolic energy source for various physiological functions, thereby decreasing appetite and food intake.

4.2.2 Effect of 17β-Estradiol Benzoate on Testicular Expression of CYP1B1, CYP2A1, CYP17A1, 3β-HSD, mEH and POR in Sprague-Dawley Rats

The present study is the first to report a dose-response suppression of CYP1B1, CYP2A1 and CYP17A1 protein expression in adult rat testis by exogenous administration of EB. EB also reduced testicular expression of mEH and POR, but not in a dose-dependent manner. In contrast, EB had no effect on 3β-HSD and calnexin protein expression. This suggests that the suppressive effect of EB on the expression of CYP1B1, CYP2A1, CYP17A1, mEH and POR was specific and was not a result of a generalized decrease in testicular protein expression.

In the present study, maximum suppression of testicular CYP1B1 protein expression (by 80%) was observed at the highest dosage (4 μmol/kg). This finding is in agreement with a previous study by Leung et al. (2009), in which a decrease in testicular CYP1B1 protein expression (by more than 90%) was observed after adult male rats were treated with EB at a

single dosage of 1.5 mg/kg (or 4 μmol/kg) for 14 days. In another study (Deb et al., 2011), male rats were treated with EB at 1.5 mg/kg for 3 days during the neonatal period or 11 days during pubertal period. A decrease in expression of testicular CYP1B1 (by more than 90%) was observed, when compared with propylene glycol-treated rats. It has also been reported that 17β-estradiol elicited dose- and time-dependent suppression of CYP1B1 expression at the mRNA level in MA-10 mouse Leydig cells *in vitro* (Deb et al., 2011). The results of these *in vitro* and *in vivo* studies suggest that the suppressive effect of EB on CYP1B1 protein expression in testis may be regulated at the gene transcription level.

In the present study, testicular expression of CYP17A1 was suppressed by EB at each of the dosages tested. Previous studies reported that EB had a suppressive effect on testicular CYP17A1 mRNA and protein levels. For instance, Sakaue et al. (2001) reported that intramuscular treatment with EB at various dosages ranging from 0.2 to 200 μg/kg for one day decreased CYP17A1 mRNA expression in adult male rat testis. In another study, male rats were treated with EB at 10 and 100 μg/kg/day for 4 days/week for 6 weeks, and a decrease in CYP17A1 mRNA and protein levels was observed, when compared with the control group (Nakamura et al., 2010). Taken together, the results suggest that the suppressive effect EB on testicular CYP17A1 expression could be regulated at the transcriptional level, similar to CYP1B1.

The constitutive expression of testicular CYP1B1, CYP2A1, CYP17A1, mEH and POR exhibited differential sensitivity to the suppressive effect of EB. Among the enzymes tested, CYP2A1 seemed to be highly sensitive because its expression was decreased by 48% at the lowest dosage (0.004 μmol/kg) of EB, and the suppressive effect is continued with increasing EB dosage. Maximum suppression (greater than 97%) of CYP2A1 was observed at 0.4 μmol/kg.

CYP1B1 and CYP17A1 are the second most sensitive enzymes to the effects of EB. Maximum suppression of CYP1B1 and CYP17A1 (by 80-95%) was observed at the highest EB dosage (4 μmol/kg). Constitutive expression of mEH and POR was suppressed by 49-50% at a dosage of 0.4 μmol/kg, but there was no further increase in suppression with increasing dosages of EB, suggesting that mEH and POR are relatively less sensitive to the suppressive effect of EB, when compared to CYP1B1, CYP2A1, and CYP17A1.

Possible reasons for the differential sensitivity of the CYP enzymes, mEH and POR to the effects of EB include the cellular distribution pattern of the enzymes within testis and their regulation by multiple hormones. CYP enzymes including CYP1B1, CYP2A1 and CYP17A1 are expressed in Leydig cells, whereas mEH and POR are expressed in all cell types including Leydig, Sertoli and spermatogenic cells. It is well-established that steroid synthesis in Leydig cells is under the control of testosterone, 17β-estradiol and the hypothalamo-pituitary axis (Payne and Hales, 2004). Thus, Leydig cells may be more vulnerable than other cell types to exogenously administered EB. CYP enzymes may be more sensitive than mEH and POR, as their expression is confined to Leydig cells only. Another possible explanation is that testicular expression of CYP1B1, CYP17A1 is regulated by LH, FSH, prolactin and 17β-estradiol in rats, whereas hepatic expression of POR is regulated by thyroid and ACTH hormones. Thus, exogenous administration of EB may not alter the serum levels of these regulatory hormones equally, resulting in differential suppressive effects on different enzymes (Waxman et al., 1989b; Leung et al., 2009; Denlinger and Vesell, 1989; Horsfield et al., 1992; Lee et al., 1980; Mukhtar et al., 1978; Ram and Waxman, 1992; Waxman et al., 1989).

It is well known that testicular development and testicular functions are under the control of complex endrocrine regulation, called the hypothalamo-pituitary-gonadal axis. Studies with

hypophysectomized animals have shown that testicular expression of CYP enzymes, mEH and POR is regulated by the pituitary hormones (Mukhtar et al., 1978; Ram and Waxman, 1992; Waxman et al., 1989). The suppressive effect of 17β-estradiol on testicular expression of CYP1B1, CYP2A1, CYP17A1, mEH and POR could be direct or indirect.

Indirect effects could be attributed to perturbing the hypothalamo-pituitary axis or to producing anorexia. Exogenous administration of 17β-estradiol could cause stimulatory or inhibitory (feedback) effects on the hypothalamo-pituitary axis (Robaire et al., 1979; Nakamura et al., 2010; D'Souza et al., 2005; Goyal et al., 2001). The stimulatory and inhibitory effects are determined by the dosages of 17β-estradiol administered, route of exposure and the exposure time (Robaire et al., 1979). Three *in vivo* studies (Nakamura et al., 2010 D'Souza et al., 2005; Goyal et al., 2001) were selected from the literature to illustrate the effect of treatment with various dosages of 17β-estradiol on hormonal levels in male rats. The three studies are helpful in determining the threshold dosages that produce inhibitory and stimulatory effects via the hypothalamo-pituitary axis (Figure 4.1). The studies followed a treatment protocol that was similar to that was used in our study, including route of administration (subcutaneous), duration of treatment (10 to 24 day), animal model (adult male rats) and dosages (ranging from 5 to 1000 μg/kg/day), but they used different estrogens (17β-estradiol or diethylstilbesterol). The results of these studies indicated that estrogen at dosages between 5 and 20 μg/kg (low dose category) decreased serum LH levels (D'Souza et al., 2005; Goyal et al., 2001; Nakamura et al., 2010). This could be due to a feedback inhibitory effect on the pituitary. Estrogen at dosages between 100 and 1000 μg/kg (high dose category) did not affect serum LH levels, but was accompanied by a drastic decrease in testosterone levels (D'Souza et al., 2005; Goyal et al., 2001; Nakamura et al., 2010). Estrogens are known to suppress testosterone synthesis in a dose-dependent manner

regardless of the inhibitory or stimulatory effect on pituitary, *in vitro* and *in vivo* (Sakaue et al., 2001; Brinkmann et al., 1980; D'Souza et al., 2005). Thus, the stimulatory effect of estrogens at higher dosages on the pituitary is not directly attributed to estrogens, but could be attributed to the lower serum levels of testosterone.

In light of the above discussion, the first two EB dosages (0.004 and 0.04 μmol/kg/day, equivalent to 1.5 and 15 μg/kg/day, respectively) used in the present study could be considered as low dosages (i.e., below 20 μg/kg/day). So the suppressive effects associated with these two doses could be primarily due to feedback inhibition of the hypothalamo-pituitary axis (decreased serum levels of LH). Similarly, the remaining two dosages of EB (0.4 and 4 μmol/kg/day, equivalent to 150 and 1500 μg/kg/day, respectively) used in the present study are in the high dosage category (i.e., above 100 μg/kg), which means that these two dosages could elicit a stimulatory effect on the hypothalamo-pituitary axis (serum LH levels were not affected). Thus, the suppressive effect of EB at 0.4 and 4 μmol/kg on CYP1B1, CYP2A1 and CYP17A1 expression is not due to a change in LH levels, but it could be the direct effect of 17β-estradiol on Leydig cells.

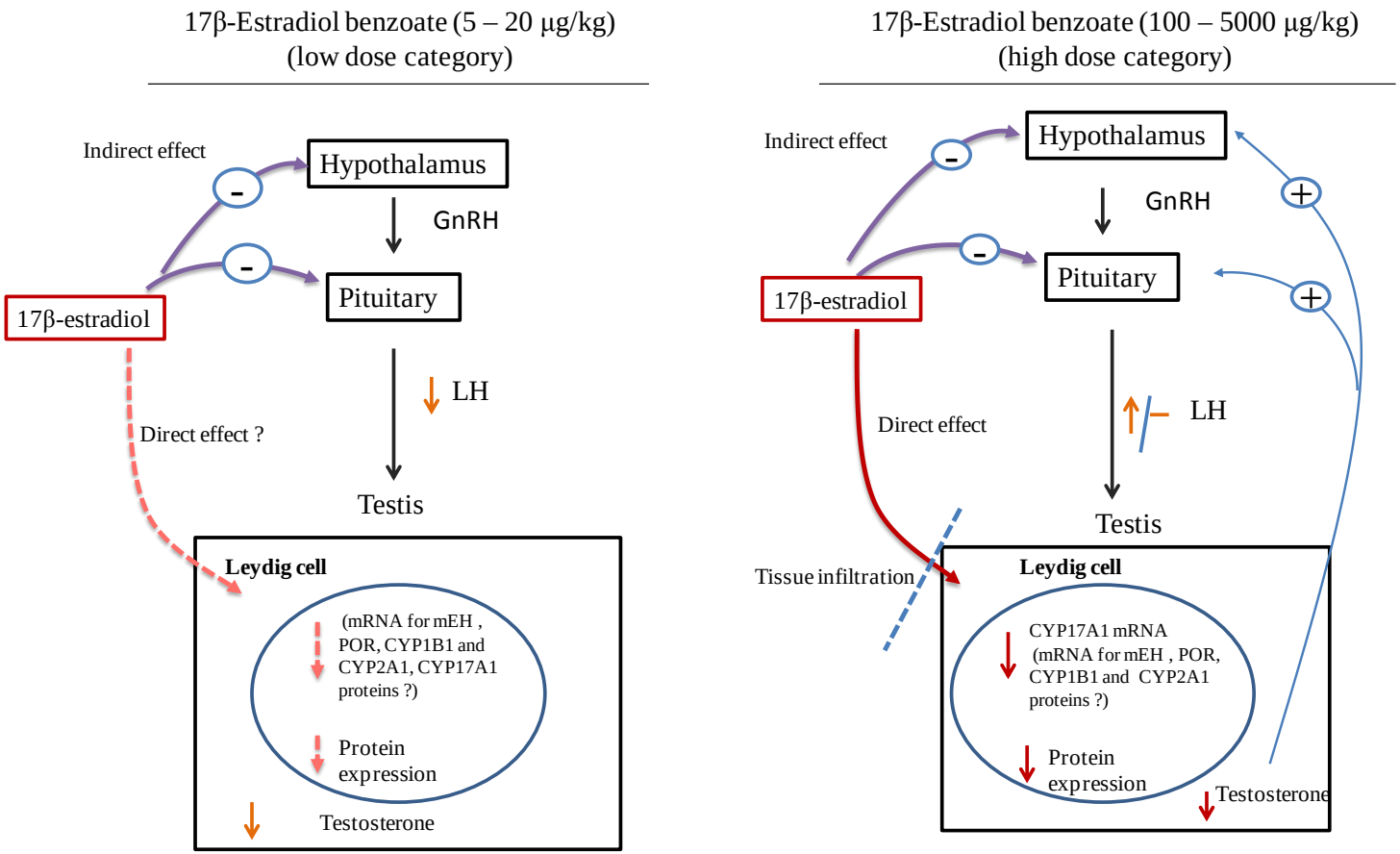

Figure 4.1 Schematic representation of possible direct and indirect effects of 17β-estradiol benzoate on the testis and on testicular expression of CYP1B1, CYP17A1, CYP2A1, mEH and POR in adult male rats (adapted from D'Souza et al., 2005; Goyal et al., 2001; Nakamura et al., 2010). Abbreviations: LH, leutinizing hormone; GnRH, gonadotropin releasing hormone.

Several studies have reported that estrogens produce anorexia either by acting on brain or by acting peripherally and affecting metabolism (Gray and Greenwood, 1982; Nunez et al., 1980; Rivera et al., 2012). In any case, estrogens ultimately suppress appetite, which leads to a decrease in daily food intake (Palmer and Grey, 1986). Malnutrition associated with a deficiency of macronutrients (carbohydrate, protein and fat), micronutrients such as vitamins (A, B and E) and minerals (zinc, magnesium and calcium) can affect protein expression of xenobiotic-metabolizing enzymes and their activities in male rat liver (Campbell and Hayes, 1974; Anthony, 1973; Mao et al 2006; Hayes and Campbell, 1974; Mgbodile et al., 1973; Xu et al., 2001). Several studies have reported that food restriction (malnutrition) or a protein-deficient diet could cause significant suppression of hepatic CYP content and its functional activity in male rats (Campbell and Hayes, 1974; Anthony, 1973; Mao et al 2006; Hayes and Campbell, 1974; Mgbodile et al., 1973; Xu et al., 2001). It has also been reported that food restriction (for 15 days), starvation (for 7 days) or a zinc-deficient diet could perturb the hypothalamo-pituitary axis and decrease serum testosterone, growth hormone and thyroid hormone levels in male rats (Oritz-Caro et al., 1984; Dickerman et al., 1968). Thus, it is possible that decreased in food intake as a consequence of treatment with 17β-estradiol produced anorexia, which partially contributed to the suppressive effect of EB on testicular expression of CYP and other xenobiotic metabolizing enzymes. However, there are no published reports on the effect of malnutrition on expression of xenobiotic-metabolizing enzymes in rat testis.

Another possible explanation is a direct effect of EB on the expression of various enzymes in the rat testis. ERα and ERβ are present in rat testis in all cell types, including Leydig, Sertoli and spermatogenic cells (Fisher et al., 1997; Saunders et al., 1998; Pelletier et al., 2000; Bois et al., 2010; Lucas et al., 2008). Because CYP and other xenobiotic-metabolizing enzymes

are primarily localized in Leydig, Sertoli and spermatogenic cells, it is possible that ERs may be involved in the regulation of these enzymes. Sakaue et al. (2002) found that 17β-estradiol could suppress the expression of CYP17A1 and CYP11A1 mRNA in rat testis, without perturbing the hypothalamo-pituitary axis. In another study, a decrease in CYP17A1 mRNA expression and catalytic activity was observed in Leydig cells collected from hypophysectomized rats that had been treated with 17β-estradiol for 7 days (Brinkmann et al., 1980). Since hypophysectomized rats are do not have a pituitary gland, this study strongly suggests the direct effect of 17β-estradiol on expression of testicular steroidogenic enzymes.

17β-Estradiol-mediated suppression of CYP1B1, CYP2A1, CYP17A1, EH and POR protein expression in rat testis is likely the result of decreased mRNA gene expression. 17β-Estradiol has been shown to decrease CYP17A1 mRNA and CYP1B1 mRNA in rat Leydig cells, *in vitro* (Brinkmann et al., 1980; Deb et al., 2011). Further, it was reported that suppression of CYP1B1 mRNA expression may be due to epigenetic modifications, such as DNA methylation (Alworth et al., 2002). However, the molecular mechanisms are yet to be discovered.

4.2.3 Clinical Implications of the Results

Usually, estrogens are not recommended for the treatment of healthy men, but they are crucial for the treatment of congenital aromatase deficiency and also used as a component in the hormonal therapy for sexual reversal in transgender and in prostate cancer patients (Rochira et al., 2000; Hermann et al., 2002; Gearhart et al., 1981). Patients with congenital aromatase deficiency lack endogenous estrogen synthesis, due to the absence of aromatase (CYP19A1), which is essential for the synthesis of estrogens from androgens. These patients are diagnosed

with abnormal bone growth with open ended epiphysis and abnormal glucose and lipid metabolism (Gearhart et al., 1981).

The EB dosages that we used in the present study are quantitatively similar (when converting to human equivalent dosages [HED]) to the 17β-estradiol doses that are used clinically in the treatment of congenital aromatase deficiency disorder. Accoding to the "guidance for industry" provided by Food and Drug Administration, the mathematical formula used to calculate HED (mg/kg) = animal dosage (mg/kg) × 0.16. Many clinical studies (Rochira et al., 2000, Hermann et al., 2002; Bilezikian et al., 1998) have used 17β-estradiol at various clinical dosages such as 0.032, 0.135 or 12.5 μg/kg/day to treat the congenital aromatase deficiency patients for a period of 2 years. These dosages are relatively similar, in terms of amount, to the dosages used in (HED, 0.24, 2.4 and 24 μg/kg/day) the present study (0.004, 0.04 and 0.4 μmol/kg/day, respectively). Although the clinical dosages of 17β-estradiol provide the minimum amount of endogenous estrogen required, it is possible that the clinical dosages may produce similar adverse effects on human testicular expression of CYP and other xenobiotic-metabolizing enzymes as we observed in the present study.

In certain disease conditions, such as obesity and obesity with myocardial infraction, serum 17β-estradiol levels has been reported to be increased by 1.6 - to 2 - fold, when compared with healthy and non-obese men (Vermeulen et al., 2002; Small et al., 1987). It has also been reported that some anti-epileptic drugs such as phenytoin and carbamazepine increase serum levels of 17β-estradiol, when compared with healthy or non-obese men (Murialdo et al., 1995). Further, elevated levels of 17β-estradiol were positively correlated with sexual dysfunction in epileptic patients (Murialdo et al., 1995).

4.3 Study 3: BPA Study

Several studies have been reported the estrogenic effects BPA on reproductive organ development and sexual function in male rats (Kato et al., 2006; Toyama and Yuasa, 2004; Akingbemi et al., 2004; Schonfelder et al., 2004). In light of the suppressive effects of 17β-estradiol benzoate on expression of various CYP and xenobiotic-metabolizing enzymes in rat testis, we were interested in determining if BPA had similar effects. The present study is the first to report the effects of BPA at three high dosages on expression of CYP1B1, CYP2A1, CYP17A1, 3β-HSD, mEH and POR in adult rat testis.

4.3.1 Effect of BPA on Body and Organ Weights

Subcutaneous injection of BPA at 400, 800 and 1600 μmol/kg reduced body weight (body weight gain) by 57-94%, when compared with saline- or propylene glycol-treated rats. Previous studies reported a suppressive effect of BPA on body weight (Takahashi and Oishi, 2003; Yamasaki et al., 2002). For instance, male rats treated with BPA at 20 and 200 mg/kg/day for 4 weeks decreased body weight gain by 20-30%, but no effect was observed when BPA was administered at 2 mg/kg (Takahashi and Oishi, 2003). The suppressive effect of BPA on body weight mediated centrally via the hypothalamus could be due to altered feeding behavior and whole-body energy homeostasis, similar to 17β-estradiol. Accordingly, a study by Batista et al. (2012) reported that administration of BPA to male mice at 100 μg/kg for 8 days decreased food intake, locomotor activity and body heat production, when compared to propylene glycol-treated animals.

BPA had no effect on relative liver weight or adrenal weight, but relative testis weight (as percent of body weight) was decreased by BPA treatment at 800 and 1600 μmol/kg. These

results suggest that testis is one of the target organs for BPA. Previous studies reported decreased relative weight of testis, epididymides and seminal vesicles in male rats that had been treated with BPA at 200 mg/kg (~ 876 μmol/kg) for 4 or 6 weeks (Nakamura et al., 2010; Takahashi and Oishi, 2003). It has been reported that BPA decreased synthesis of sex hormones, and specifically, testosterone in rat Leydig cells (Takahashi and Oishi, 2003; Tohei et al., 2001). The decrease in serum testosterone levels may cause a reduction in testis weight (Robaire et al., 1979). Overall, the present results suggest that effects of BPA on body and organ weights are similar to those of 17β-estradiol.

4.3.2 Effect of BPA on Testicular Expression of CYP1B1, CYP2A1, CYP17A1, 3β-HSD, mEH and POR in Sprague-Dawley Rats

Administration of BPA at 400, 800 and 1600 μmol/kg decreased constitutive expression of CYP1B1, CYP2A1, CYP17A1, 3β-HSD, mEH and POR in testicular microsomes prepared from adult rats. These dosages were selected on the basis of a study by Nakamura et al. (2010), in which BPA at 200 mg/kg (~ 876 μmol/kg) decreased steroidogenesis by decreasing the expression of CYP17A1 and other steroidogenic enzymes in adult male rats. In the present study, BPA decreased protein expression of CYP1B1, CYP2A1 at each of the dosages tested and maximum suppression was observed at the highest dosage of BPA (1600 μmol/kg). CYP17A1 expression was completely abolished by BPA at dosages of 800 and 1600 μmol/kg. In the case of 3β-HSD, a decrease in expression was observed at a BPA dosage of 1600 μmol/kg, but not at 400 and 800 μmol/kg. Whereas for mEH and POR, the suppressive effect of BPA increased with increasing dosages (400 and 800 μmol/kg), but no further increase in suppression was observed at the highest dosage of BPA (1600 μmol/kg).

At the lowest dosage (400 μmol/kg), protein expression of the tested enzymes, except 3β-HSD, was decreased by approximately 50%, but this dosage is (400 μmol of BPA/kg) is quantitatively 100 times greater than the highest dosage of 17β-estradiol benzoate tested (4 μmol/kg). This suggests that BPA is producing an estrogenic effect, but is less potent than 17β-estradiol, on protein expression of testicular CYP and xenobiotic-metabolizing enzymes. Several studies have reported that steroidogenic enzymes such as CYP17A1 and CYP11A1 are susceptible to BPA, but this study is the first to report that testicular CYP1B1, CYP2A1, mEH and POR are also vulnerable. In contrast, BPA had no effect on calnexin protein expression at each of the dosages tested. Calnexin is a chaperone protein located in the endoplasmic reticulum. For this reason, calnexin was used as an endoplasmic reticulum marker in the present study. It functions as a component of the protein regulatory system that allows correct folding of proteins and guides misfolded proteins to degradation (Kleizen and Braakman, 2004). Considering the lack of effect of BPA on testicular expression of calnexin, the present study suggests that the observed suppressive effect of BPA on testicular CYP and xenobiotic metabolizing enzymes was specific and was not a result of a generalized decrease in testicular protein expression.

The results demonstrated that constitutive expression of testicular CYP and xenobiotic-metabolizing enzymes exhibit differential sensitivity to BPA. CYP enzymes including CYP1B1, CYP2A1 and CYP17A1 were more sensitive to BPA and maximum suppression (by 90% or greater) was observed following treatment at a dosage of 1600 μmol/kg. mEH and POR were relatively less sensitive to BPA and maximum suppression (by 67%) was observed at 800 μmol/kg.

Possible reasons for the differential sensitivity of CYP1B1, CYP2A1, CYP17A1, mEH and POR to the effects of BPA are the cellular localization of the enzymes within testis and their

regulation by multiple hormones. CYP enzymes including CYP1B1, CYP2A1 and CYP17A1 are primarily expressed in Leydig cells, whereas mEH and POR are expressed in all cell types including Leydig, Sertoli and spermatogenic cells. Previous studies reported that BPA acts directly on Leydig cells and inhibits Leydig cell functions (Nanjappa et al., 2012; Akingbemi et al., 2004). For example, a decrease in testosterone production by 25% was observed after treatment of Leydig cells isolated from untreated rats with BPA at 0.01nM (Akingbemi et al., 2004). In the same study, analysis of steroidogenic enzyme gene expression by RT-PCR indicated that BPA reduced CYP17A1 expression, which was also suppressed by 17β-estradiol. Thus, it is possible that CYP enzymes are more vulnerable than mEH and POR because their expression is confined to Leydig cells only. Testicular expression of CYP1B1, CYP17A1 and mEH protein and mRNA is regulated mainly by LH, FSH, prolactin, 17β-estradiol and testosterone (Leung et al., 2009; Payne and Youngblood, 1995; Malaska and Payne; 1984; Lee et al., 1980; Mukhtar et al., 1978). Hormonal regulation of CYP2A1 and POR expression in testis is not known. BPA is an endocrine disrupting chemical. Several studies have reported alteration in serum hormonal levels of LH, FSH, prolactin, thyroid stimulation hormone, 17β-estradiol and testosterone after rats were treated with various dosages of BPA from 2.5 μg/kg to 200 mg/kg (Tohei et al., 2001; Akingbemi et al., 2004; Nakamura et al., 2010). It has also been reported that BPA is weak ligand for estrogen and thyroid hormone receptors and disrupts the physiological functions mediated by 17β-estradiol and thyroid hormones (Nagel et al., 1997; Moriyama et al, 2002). Taken together, the endocrine-disruptive activity of BPA and alteration in serum hormone levels may partially explain its differential effects on testicular expression of xenobiotic-metabolizing enzymes.

BPA is a xenoestrogen and exerts its estrogenic actions through binding and transactivation of estrogen receptors. In rats, estrogen receptors are expressed in the pituitary, hypothalamus, testis and other organs (Fischer et al., 1984; Azcoitia et al., 1999, Pelletier et al., 2000). Thus, BPA could act on the pituitary and hypothalamus, and perturb the hormonal regulation mediated by the hypothalamo-pituitary axis. Nakamura et al. (2010) found that administration of BPA at 200 mg/kg/ day for 4 days/week for 6 weeks decreased serum LH, testosterone levels and also decreased testicular expression of CYP17A1 and CYP11A1 in male rats. Higher dosages of BPA were used in the present study, but we presume that BPA at 800 and 1600 μmol/kg doses (equivalent doses in mg/kg units are 183 and 365, respectively) could elicit a similar inhibitory effect on the hypothalamus-pituitary axis and decrease serum LH levels. LH is essential for Leydig cell development and is also a positive regulator for the expression of CYP1B1, CYP17A1, mEH in Leydig cells (Leung et al., 2009; Malaska et al., 1984; Griffin et al., 2010; Lee et al., 1980). Thus, decreased LH levels may directly produce a suppressive effect on expression of xenobiotic metabolizing enzymes in rat testis.

Nakamura et al. (2010) also demonstrated that in male rats administrated BPA at 100 mg/kg, serum LH levels were not affected, but testicular expression of CYP17A1, CYP11A1 and other steroidogenic enzymes was decreased. Other studies (Tohej et al., 2001; Herath et al., 2004) have reported similar observations, with no change in serum LH levels when male rats were administered with BPA at 1 or 3 mg/kg for 2 or 5 weeks. Taken together, results of the studies suggest that BPA can exert its suppressive effect on enzyme expression by acting directly on Leydig cells.

Another result that provides support for the direct effects of BPA on enzyme expression in Leydig cells is the suppressive effect on 3β-HSD. Though 3β-HSD is highly expressed in

Leydig cells and involved in steroidogenesis, its expression in testis is not under the control of LH. Baker and his coworkers (2003) found that administration of human chorionic gonadotropin hormone, a known LH receptor agonist, to gonadotropin-deficient male mice resulted in increased CYP17A1 and CYP11A1 mRNA expression, but 3β-HSD mRNA expression was not affected. In the present study, BPA decreased 3β-HSD protein expression at the highest dosage tested (1600 μmol/kg). This result is in agreement with the study by Nakamura et al. (2010), in which male rats were administered BPA at 200 mg/kg/day for 4 days/week for 6 weeks, reduced testicular expression of 3β-HSD. Thus BPA might have direct effects on enzyme expression in rat testis via binding to estrogen receptors.

We cannot exclude the effect of food intake on the expression of testicular CYP and xenobiotic-metabolizing enzymes. BPA is reported to decrease daily food intake similar to 17β-estradiol. A decrease in food intake may have partially contributed to the suppressive of BPA on testicular enzyme expression (refer Section 4.2.2).

Overall, BPA suppressed protein expression of testicular CYP1B1, CYP2A1, CYP17A1, mEH and POR, similar to 17β-estradiol, but BPA is less potent in causing the suppressive effects, compared with 17β-estradiol, in rats. Suppression of these enzymes could disturb the homeostasis of xeno- and endobiotic metabolism in testis and may affect reproductive functions.

4.4 Limitations

1. In Study 2 and Study 3, we investigated the effects of EB and BPA on protein expression of testicular enzymes, but we did not determine the catalytic functions of these enzymes.
2. In this thesis we did not investigate mRNA gene expression of the testicular enzymes. Thus, it is not known whether expression of testicular enzymes was regulated at the transcriptional level by EB and BPA.
3. In Study 2 and Study 3, we did not measure serum levels of testosterone, 17β-estradiol, LH or FSH. Due to lack of this information, we were unable to conclude that whether EB or BPA had a positive or negative feedback effect on the hypothalamo-pituitary axis.
4. In Study 2 and Study 3, we did not perform immunohistochemical analysis for testicular xenobiotic-metabolizing enzymes. For this reason, we were unable to determine the histological effects of EB and BPA on testicular enzyme expression, or on Leydig cell number and volume.
5. To date, no studies are available in the literature related to the effect of BPA on testicular xenobiotic-metabolizing enzymes, thus we tried high dosages to investigate whether BPA could affect protein expression of xenobiotic-metabolizing enzymes in rat testis. The BPA dosages that were used in Study 3 were selected based on a study by Nakamura et al. 2010, in which BPA at 200 mg/kg (~ 876 μmol/kg) suppressed testicular expression of stroidogenic CYP enzymes (CYP17A1 and CYP11A1) in rat testis. However, the BPA dosages that we used in Study 3 were not environmentally relevant dosages. According to US Environmental Protection Agency guidelines

(IRIS, 1998), the lowest observed adverse effect level for BPA dosage in mice was 50 mg/kg/day, but the BPA dosages that we used were 2- to 6-fold higher. Another study reported that BPA, at approximately 2.4 μg/kg/day, is an environmentally relevant dosage that is predicted to result in serum BPA levels that are close to those observed in human serum (Nagel et al., 1997).

6. The route of administration (subcutaneous) that was used in Study 3 is not environmentally relevant, because the major routes of exposure to endocrine disrupting chemicals are oral and inhalation. We used a subcutaneous route to administer EB and we followed same protocol for BPA.

4.5 Summary

1. CYP1B1, CYP2A1, POR, and mEH were detected in testicular microsomes isolated from adult rats. By comparison, CYP1A1, CYP1A2, CYP2B1, CYP2E1, CYP2D1, CYP2D2, CYP2C6, CYP2C7, CYP2C12 CYP2C13, CYP3A1, CYP3A2, CYP4A1, CYP4A2 and CYP4A3 were not detected in the same microsomal samples. CYP17A1, 3β-HSD and calnexin were detected as reference proteins for testicular microsomes
2. Immunohistochemical results suggest that expression of CYP1B1, CYP2A1, CYP17A1 and 3β-HSD is cell type specific and is primarily confined to interstitial cells, whereas expression of POR and mEH is distributed across the testis including interstitial cells and seminiferous tubules.
3. Exogenous administration of EB suppressed protein expression of CYP1B1, CYP17A1, CYP2A1, mEH and POR, but had no effect on 3β-HSD and calnexin, in

adult rat testis at each of the dosage tested, when compared with the propylene glycol-treated rats.

4. Exogenous administration of EB decreased body weight gain, while no effect was observed on relative testis weight (as percent of body weight) across the dosages tested, when compared to control group.
5. Administration of BPA suppressed protein expression of CYP1B1, CYP17A1, CYP2A1, mEH and POR, but had no effect on calnexin, in adult rat testis at each of the dosage tested, when compared with saline- or propylene glycol-treated rats. BPA suppressed protein expression of 3β-HSD in rat testis at the highest dosage (1600 μmol/kg), but not at the lower dosages (400 and 800 μmol/kg).
6. Administration of BPA decreased body weight gain across the dosages tested, but relative testis weight (as percent of body weight) was decreased at dosages of 800 and 1600 μmol/kg, but not at the lowest dosage, when compared with vehicle or saline-treated rats.

4.6 Conclusions

1. Relatively few xenobiotic-metabolizing CYP enzymes were expressed, at protein level, in rat testis when compared with liver, which is a central organ for xenobiotic-metabolism in the body
2. The expression of xenobiotic-metabolizing CYP and other enzymes in rat testis is cell type specific
3. Estrogen and estrogen-like chemicals produce down-regulation of xenobiotic-metabolizing and steroidogenic enzymes in testis

4. Suppression of testicular xenobiotic-metabolizing enzymes expression can be used as an in vivo marker to assess estrogenic activity

4.7 Future Directions

Several studies reported that diet has a pronounced role in the regulation of xenobiotic metabolizing enzymes. Since EB and BPA were reported to suppress feeding behavior, it is possible that decreased food intake might have partially contributed to the suppressive effect observed with EB and BPA on testicular enzymes. Further studies with fasted animals may provide further insights into the extent of role of decreased food intake in the EB and BPA produced suppressive effects on testicular enzymes.

From the results of the present study, if the observed suppressive effects of EB and BPA on testicular enzymes were due to their localized action on testis, then it is important to understand the regulatory mechanisms at cellular level. Estrogen receptors may be the potential source through which EB and BPA could regulate expression of testicular enzymes. Thus, studies with estrogen receptor antagonist could help in determining the role of estrogen receptors in the regulation of xenobiotic-metabolizing enzymes in rat testis.

BPA dosages that used in the present study were not environmentally relevant. Therefore, it is unknown whether BPA at environmentally relevant dosages has suppressive effects on expression of testicular xenobiotic-metabolizing enzymes in rat testis. Although, the BPA levels are very low in the environment, but constant exposure of this chemical to humans for longer duration may have adverse effects on expression of testicular enzymes. Thus, chronic treatment of animals with environmental relevant dosages of BPA might help in determining the effects of

BPA at environmental dosages on testicular expression of xenobiotic-metabolizing enzymes in rats.

Table 4.1 Summary of Study 1, Study 2 and Study 3

Protein of interest	**Localization in testis**	**Effect of varying dosages of EB (0.004, 0.04, 0.4 and 4 µmol/kg/day) treatment on testicular expression of various enzymes when compared with propylene glycol-treated rats**	**Effect of varying dosages of BPA (400, 800 and 1600 µmol/kg/day) treatment on testicular expression of various enzymes when compared with saline- or propylene glycol-treated rats**
CYP1B1	Leydig cells	suppressed across the dosages tested	suppressed across the dosages tested
CYP2A1	Leydig cells	suppressed across the dosages tested	suppressed across the dosages tested
CYP17A1	Leydig cells	suppressed at 0.04, 0.4 and 4 µmol of EB/kg dosages, but not at the lowest dosage (0.004 µmol/kg)	suppressed across the dosages tested
mEH	Leydig, Sertoli and Spermatogenic cells	suppressed at 0.04, 0.4 and 4 µmol of EB/kg dosages, but not at the lowest dosage (0.004 µmol/kg)	suppressed across the dosages tested
POR	Leydig, Sertoli and spermatogenic cells	suppressed at 0.04, 0.4 and 4 µmol of EB/kg dosages, but not at the lowest dosage (0.004 µmol/kg)	suppressed across the dosages tested
3β-HSD (Leydig cell marker)	Leydig cells	no effect was observed across the dosages tested	no effect was observed except at the highest dosage (1600 µmol/kg)
Calnexin (Endoplasmic reticulum marker)	Not analyzed,	no effect was observed across the dosages tested	no effect was observed across the dosages tested

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http://www.fda.gov/downloads/Drugs/Guidances/UCM078932.pdf