Analysis of xenoestrogen effects in the male rat gonad

by

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Abstract

Naturally occurring estrogenic chemicals existing in food such as soybeans and synthetic xenoestrogens, termed Xenoestrogens, contaminating food and water sources have become issues of public health interest. The concern about these estrogenic compounds is particularly concerning to male individuals because of the association of estrogenic compounds with the decline in male reproductive health. This dissertation describes the adverse effects of developmental exposure to the dietary estrogenic compounds found in soybeans (genistein and daidzein) and the synthetic estrogenic compounds that commonly contaminate food and water sources (BPA, BPS, and EE2) on testicular cell function using the rat model.

The Leydig cell is the primary source of the male sex steroid testosterone which supports the male phenotype, development of secondary sexual characteristics, and maintains fertility. The Sertoli cell is the principal link between germ cells and the regulatory hormones needed for spermatogenesis. The overall aim is to analyze the effect of xenoestrogens in testicular cells under the following specific objectives:

I. Determine whether soy-based diets exposure are due to naturally occurring isoflavones and whether the effects are influenced by age at exposure
II. Investigate whether isoflavone effects in the testis are due to daidzein, genistein, or both compounds
III. Investigate the effect of synthetic estrogens, i.e., BPA, BPS, EE2, on steroid hormone secretion in the developing male rat gonad

To achieve the first objective, male rats at 21, 35, and 75 days of age were maintained either on a casein control diet, soybean meal (SBM), or control diet supplemented with daidzin and genistin (G+D) for 14 days. Feeding of SBM and G+D diets decreased testicular testosterone

(T) secretion regardless of age. Altered androgen secretion was due to decreased ($p<0.05$) Star and Hsd17β protein in the testes and was associated with increased ($p<0.05$) Lhβ and Fshβ subunit protein expression in pituitary glands.

For the second objective, male rats were fed either a casein control diet, control diet+daidzin, control diet+genistin, or control diet+genistin+daidzin (G+D). Compared to control, feeding of all isoflavone-containing diets decreased ($p<0.05$) testicular T concentrations. Interestingly, Esr1 and androgen receptor (Ar) protein and pituitary Fshβ with Lhβ subunit protein were increased ($p<0.05$) by feeding genistin and G+D diets, but not the daidzin diet. Studies of primary Leydig cell culture showed that daidzein and genistein both caused a concentration-dependent inhibition ($p<0.05$) of T secretion by Leydig cells *in vitro* with IC_{50} of 184 ηM and 36 ηM, respectively. Together, results demonstrated that altered testicular steroidogenic capacity and pituitary FSHβ and LHβ subunit expression due to soy-based diets result from specific actions by genistein and daidzein. Our data are relevant to public health due to increased consumption of soy-based products by all segments of the population and the increasing incidence of reproductive anomalies in the population. Additional studies are warranted to assess the impact of isoflavone-induced changes in testicular androgen concentrations on germ cell development, sperm production, and male fertility.

To achieve the third objective two separate experiments were conducted in which prepubertal and pubertal male rats at 21 and 35 days of age were provided test chemicals in drinking water (5 µg/L) for 14 days. At the end of the exposure period, results showed that BPS and EE2 inhibited testicular testosterone (T) concentrations in a similar manner to BPA. Our results also showed that although BPA had no effect on gonadal estradiol (E2) secretion, both BPS and EE2 increased testicular E2 concentrations. Furthermore, protein expressions of Sertoli cell-

produced factors were altered. For example, BPS and EE2 but not BPA decreased anti-Mullerian hormone (Amh) protein expression, while expression of Desert hedgehog (Dhh) protein was increased by all tested chemicals. Together, these observations not only demonstrated that BPA, BPS, and EE2 have similar inhibitory effects on gonadal steroidogenesis but have the potential to disrupt the functions of gonadal cells in the male.

These findings on the effects of dietary isoflavones (genistein and daidzein) and the synthetic estrogenic compounds (BPA, BPS, and EE2) on testicular cells reinforce the view that exposures of the population to environmental chemicals have the potential to impair reproductive health. Additional studies are warranted to understand the effects of changes in intratesticular androgen concentrations on germ cell maturation and the mechanisms by which single and chemical mixtures impact testicular cells.

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List of Abbreviations

AR (Ar)	Androgen receptor [(upper case in human, (lower case in rodents)]
ESR (Esr)	Estrogen receptor [(upper case in human), (lower case in rodents)]
E2	17β estradiol
T	Testosterone
HSD3β	3β-hydroxysteroid dehydrogenase
CYP17A	Cytochrome P450 17α -hydroxylase
CYP11A	Cytochrome P450 side cleavage enzyme
HSD17β	17β hydroxysteroid dehydrogenase
StAR	Steroidogenic acute regulatory protein
BPA	Bisphenol A
BPS	Bisphenol S
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
Gen	Genistein
Daid	Daidzein
SBM	Soybean Diet
EDCs	Endocrine-disrupting chemicals
SRY	Sex determining region Y
RIA	Radioimmunoassay
NTP	National Toxicology Program

USDA United States Department of Agriculture

PGC Primordial germ cell

FLC Fetal Leydig cell

PLC Progenitor Leydig cell

ILC Immature Leydig cell

ALC Adult Leydig cell

ARE Androgen response element

ERE Estrogen response element

AVPN Anteroventral periventricular nucleus

POA Preoptic area

ARC Arcuate Nucleus

KNDy Kisspeptin, neurokinin and dynorphin

PND Post-natal day

HPGA Hypothalamic-pituitary-gonadal axis

Chapter 1

Introduction and Literature Review

1.0.0 Background of the Problem

A continuous deterioration in male reproductive health reported over the last few decades has become a public health concern [1,2]. A decline in semen quality, increase in the incidence of testicular cancer, hypospadias, cryptorchidism, alteration in the timing of puberty, and other male reproductive disorders have all been reported [3–5]. While the etiology of these male reproductive health conditions is still unclear, epidemiological, clinical, and laboratory evidence suggest that exposure to environmental chemicals is a contributing factor [6,7].

One of the most common environmental chemicals implicated is a group of chemicals collectively termed xenoestrogens. Xenoestrogens are naturally occurring or synthetic chemicals consisting of a large and structurally diverse group of compounds that are foreign to the body but possessing estrogen-mimicking properties. Naturally occurring xenoestrogens are of the plant (phytoestrogens) and fungal (mycoestrogens) origin while synthetic xenoestrogens are manufactured and man-made. Because xenoestrogens have either structural or functional groups resembling endogenous estrogens, they are able to bind the estrogen receptors (ESRs) with various degrees of affinity and selectivity [8]. Although xenoestrogens are characterized by their ability to interact with ESRs, they can interact with other receptors and pathways that are critical to reproductive function. For example, xenoestrogens may interact with androgen receptors (AR), aryl hydrocarbon receptors, neurotransmitter receptors, enzymatic pathways involved in steroid biosynthesis, and several mechanisms controlling cell proliferation, differentiation, apoptosis, and intercellular communication [9–13]. The overall effects of xenoestrogens at these multiple receptor

types and pathways result in actions that disrupt physiologic functions involved in the control of reproduction. The link between the disruptive receptor functions, the endocrine axis, the reproductive system, and development has led to the classification of xenoestrogens as endocrine-disrupting chemicals (EDCs) [14,15]. EDCs as defined by the US Environmental Protection Agency (USEPA) "constitute 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 regulate homeostasis, reproduction, and developmental process" [15].

Exposure to natural xenoestrogens from food and inadvertent contamination by synthetic xenoestrogens from agricultural and industrial products is a public health issue[1,16,17]. The risk to the male individual is particularly concerning due to the association of a general decline in male reproductive health with xenoestrogens. A systemic review and meta-analysis on the epidemiologic evidence linking environmental chemicals to the disorders of reproduction identified 85 risk estimates of links between xenoestrogens and male reproductive disorder [6]. Several systematic reviews and meta-analyses from clinical data show that an elevated risk of male reproductive anomalies is associated with xenoestrogens exposure occurring at the early stages of development to the end of puberty [6,18]. Animal studies demonstrated that exposure to xenoestrogens caused disturbances in sex steroid biosynthesis and regulation [19]. Disturbances in androgen, the single most important sex hormone in the male responsible for the development and maintenance of reproductive functions, is of particular concern. For example, disturbances in androgen secretion and function as a result of xenoestrogen exposure in laboratory animals include genital anomalies, reduced gonadal size, feminization, demasculinization, and decreased sperm count and quality, [20,21]. Thus, the adverse consequence of xenoestrogen exposure, especially in the male individual, makes it imperative to investigate and understand the effects and mechanisms of

action of xenoestrogens. The overall aim of this study is to analyze the effect of naturally occurring xenoestrogens (soy-isoflavones) and those of three environmentally relevant synthetic xenoestrogens [bisphenol A (BPA), bisphenol S (BPS), and 17α-ethinyl estradiol (EE2)] in testicular cells of growing rats.

1.2.0 Xenoestrogens

Xenoestrogens are a common occurrence in our daily lives. Although the vast majority of xenoestrogens are synthetic chemicals used in the industrial and agricultural sectors, naturally occurring xenoestrogens such as phytoestrogens constitute a biologically significant component of many legumes consumed both by humans and livestock. Xenoestrogens of plant origin are sometimes consumed for their putative medicinal value, but nevertheless are a risk to both livestock and humans. The economic importance of synthetic xenoestrogens is due in part to their prevalence in the environment. For example, xenoestrogens have found abundant utilization in agricultural production (pesticides, preservatives), in industries (manufacturing of several products of values), and medicine (as therapeutic agents, prosthesis). Despite the wide application of xenoestrogens, their inappropriate presence in the environment constitutes a significant health hazard.

1.2.1 Naturally Occurring Xenoestrogens

Naturally occurring xenoestrogens are mainly of plant origin. Estrogenic activity of plant origin was first reported in 1926 following Allen Doisy's bioassay for estrogens in 1923 [22,23]. Subsequently, any compound of plant origin was termed phytoestrogens. Phytoestrogens are

therefore defined as plant-derived compounds with structural and/or functional similarity to ovarian and placental estrogens and their active metabolites [24]. Although of lesser importance to humans, a group of resorcylic acid lactones of fungi origin (mycoestrogens) possess estrogenic properties and are a major class of naturally occurring xenoestrogens [25]. Examples include zearalenone, zearalenol, and zearalanol. They are mycotoxins produced by fusarium and gibberella fungi and are often contaminating animal feed sources, corn silage, and hay **(Table1).**

Table 1: Classification of Naturally occurring Xenoestrogens

Major class	**Minor class**	**Subclass**	**Examples**	**Sources**
Phytoestrogen	Lignans		secoisolaricinesol, matairesinol, lariciresinol, and pinoresinol	Seed, whole grains, vegetables
	Isoflavonoids	Isoflavones	Genistein, daidzein, glycetein, formononetin, Biochanin A	Soybeans seed and sprout, grains, potatoes
		Isoflavan	Equol, vestitol, sativan	Daidzein and other isoflavones
		Coumestans	Coumestrol, 4-methoxycoumestrol	Split peas, pinto beans, lima beans, alfalfa, clover sprouts
Mycoestrogens			zearalenone, zearalenol, zearalanol	fusarium and gibberella fungi

The earliest suspicion of phytoestrogens as a compound of biological significance was in 1940 with the outbreak of infertility ("Clover disease") in sheep grazing on pasture rich in clover in Western Australia [26]. However, their significance in humans was not discovered until a few

decades ago when genistein, a phytoestrogen of dietary origin, was isolated in the urine of humans and thought to have a possible role in hormone-dependent diseases [27]. Phytoestrogens are broadly classified into two groups: lignans and isoflavonoids. Lignans are polyphenolic derivatives of phenylalanine often found as a minor component of the cell walls. Lignans are abundant in grains, nuts, berries, seeds, and cruciferous vegetables. They include secoisolaricinesol, matairesinol, lariciresinol, and pinoresinol. Lignans are metabolized by intestinal bacteria to enterolignans, such as enterodiol and enterolactone.

The isoflavonoids are the most common group of phytoestrogens and include coumestans, isoflavones, and isoflavans. Coumestrol is the best-known coumestans and is found in clover plants and soy sprouts. The isoflavones include genistein, daidzein, glycetein, formononetin, and biochanin A. The best-known isoflavan is equol, a metabolic derivative of daidzein. The presence of isoflavones in many edible legumes, such as soybeans, peanuts, as well as edible vegetables, makes human exposure almost universal. Additionally, the belief that consumption of isoflavones-rich diets is associated with a lower risk of so-called "Western" diseases such as breast cancer and cardiovascular diseases has led to the increased consumption of isoflavones from soybeans across the globe. Isoflavones are present in varieties of soy-derived food products ranging from soy milk, soy protein, soy sauce, tempeh, miso, yogurt, and many other products. A greater proportion of this dissertation is focused on the effects of soy isoflavones in the male rat gonad.

1.2.2 Isoflavones

Isoflavones are found in whole-grain products, potatoes, fruits, vegetables, and alcoholic beverages, but the highest amount is present in soybeans [28]. In humans, the most widely consumed isoflavones are those found in soybeans which exist as glycoside conjugates [i.e., genistin (50-

55%), daidzin (40-45%), and glycetin (<5%)] [29]. After the consumption of a soy-based diet, digestion and absorption of isoflavones within the gastrointestinal tract are followed by complex enzymatic conversion in the liver to metabolites that can easily be excreted. Within the small intestine, the glycoside conjugates are deconjugated mainly by the hydrolytic action of the β-glucosidase enzyme to the aglycone forms i.e., genistein and daidzein **(Fig. 1)**. In about 30% of the general population, daidzein is further metabolized in the gastrointestinal tract to equol [30]. Genistein, daidzein, and equol are all metabolically active. After ingestion, serum isoflavone levels increase in a dose-dependent fashion and peak plasma isoflavone concentration is achieved within 2 to 6 hours [31,32]. The aglycones forms of isoflavones (i.e., genistein and daidzein) transported to the liver are conjugated to glucuronic acid and or the sulfate moiety for excretion via complex enzymatic actions. The glucuronide fraction is biologically inactive and constitutes over 90% of circulating isoflavones while the sulfated form is metabolically active. Isoflavones undergo entero-hepatic circulation and may be excreted in bile, deconjugated by intestinal floral, reabsorbed, re-conjugated by the liver, and excreted in the urine and fecal matter. While the bioavailability of isoflavones is influenced by microbiota population, pH, and intestinal transit time, its absorption, distribution, metabolism, and excretion also vary among different populations, ages, and genders [33].

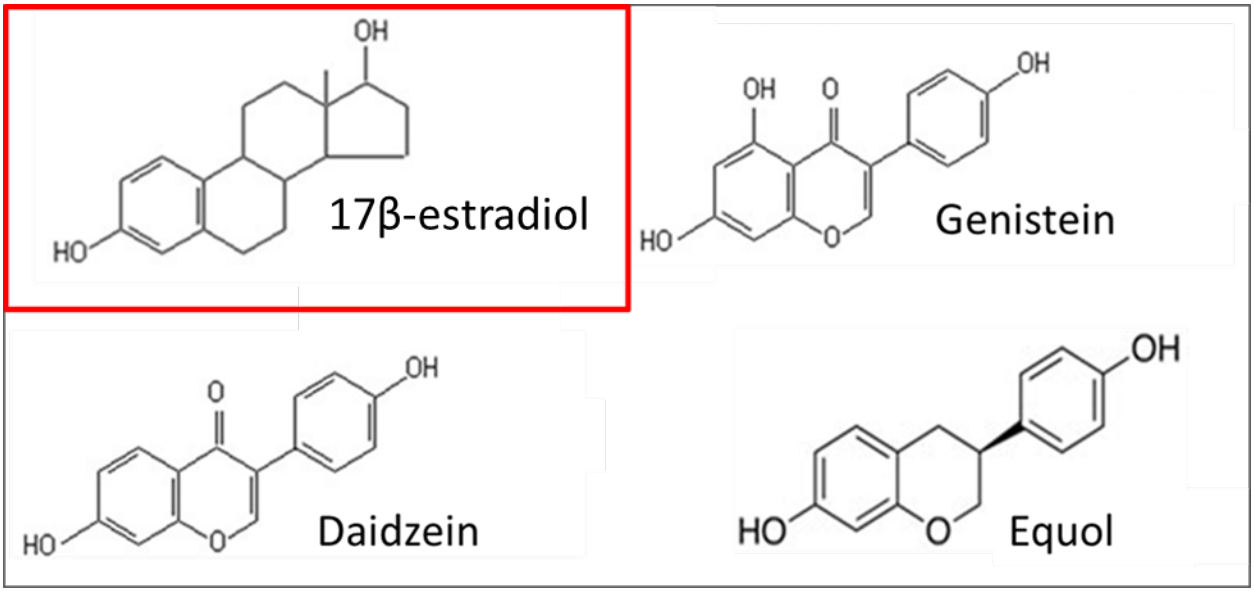

Figure 1: Structures of soy-isoflavones and estrogen: Genistein, daidzein, and equol (an isoflavan metabolically active derivative of daidzein) share the phenolic ring with the estrogens [estrone (E1), 17 β-estradiol (E2), estriol (E3), and estetrol (4)]. Hence, they are known as phytoestrogens or phytochemicals. The position of the phenolic ring determines selectivity for estrogen receptors.

1.2.3 Synthetic xenoestrogens

The list of man-made chemicals in use today is long. Among these chemicals are a group with structural and/or functional resemblance to estrogens and are capable of estrogenic activity; hence they are known as synthetic xenoestrogens (**Table 2)**. They constitute major chemicals used in the manufacturing and agricultural sectors. Exposure to synthetic xenoestrogens is mainly via contamination of food and water sources, although inhalation from the air is possible. The use of chemicals as additives in food is highly regulated by the FDA but, testing for estrogenicity is often problematic and many of these xenoestrogens were already in use before strict regulations were enforced [34]. Thus, a significant amount of synthetic chemicals with estrogenic potential are in circulation and pose a risk to both humans and livestock. Although the list of known synthetic chemicals with estrogenic potential is vast, this dissertation has devoted a section to describing

observations from the comparison of the effects of three environmentally relevant synthetic estrogenic compounds (Bisphenol A (BPA), bisphenol S (BPS), and 17α-ethinylestradiol (EE2)) on testicular development and function in growing rats.

Table 2: Common Synthetic Xenoestrogens

Class	Chemical group	Examples	Sources/Exposure	Status
Industrial	Bisphenol compounds	Bisphenol A, B, F, and S	Canned beverage, plastic food package, water bottle, tags, books	Restricted Use for BPA in infant related materials
	Phthalate esters	DEHP, DINP, DIDP, DBP	Plastics, cosmetics, fragrance, nail polish,	Still in use
	Alkylphenol and alkylphenol polyethoxylates	Nonylphenoldiethoxylate, nonylphenoxy carboxylic acid, nonylphenol,	Detergents, paints	Still in use
	Polychlorinated biphenyls	Dioxins and Furans	Hydraulic fluids, adhesives, flame retardants	Banned in the US
	Perfluorinated chemicals	PFOS and PFOA	Non-stick pots, carpeting, upholstery, floor wax,	Still in use
Agricultural	Organochlorine pesticides	DDT, DDD, DDE, dicofol, perthane, methoxychlor, chlordane, oxychlordane, heptachlor, aldrin, dieldrin hexachlorobenzene, hexachlorocyclohexanes	Herbicides, fungicides, insecticides	Mostly banned in the US in food crop production, but still
	Pentachlorophenol (PCP)	Pentachlorophenol, tetrachlorbenzol, chlordibenzofurans	Herbicides, fungicides, insecticides	Restricted use in the US
Medicaments	Modified estradiol	17α-ethinylestradiol, mestranol, quinestrol, stilbestrol	Oral tablets, creams, injectables, patches	Still in use

1.2.4 Bisphenol A (BPA)

Bisphenol A, first synthesized in 1891, has the chemical formula $(CH_3)_2C(C_6H_4OH)_2$ and shares a similar phenolic ring structure with E2 **(Fig. 2)**. At room temperature, BPA is solid with a melting point of 150-155 ^{0}C. BPA is weakly soluble in water (120-300 mg/L) but readily soluble in organic solvents [35]. It is a high production volume chemical with annual production in the US exceeding 3.8 million tons [36]. Polycarbonate plastics (PC), polyvinyl chloride plastics (PVC), epoxy resins, and lacquer are some of the products manufactured using BPA [37]. PC and PVC are components of many consumer goods and household products; hence exposure of the population is possible from daily usage. These products include infant feeding bottles, toys, medical equipment, dental sealants, and electronic devices. Epoxy resins are used in water pipes and water-resistant sealants in various products, while lacquer coating is employed as lining for food packages and beverage cans. BPA can also be found in thermal papers, luggage tags, and books. BPA exposure of the population via contamination of the environment occurs through many sources. The highest contamination of air, water, and land occurs in areas of industrial activities. BPA may be released into the air as fugitive dust emissions from closed systems during processing, handling, and transportation. In the US, atmospheric concentrations of BPA in cosmopolitan and industrial areas are estimated to be in the range of 170 to 880 pg/m^3 [38]. BPA contamination of surface water is in the low concentrations although considerable contamination has been reported in groundwater situated near waste dumps and industrial sewage [39,40]. Direct BPA exposure of humans is mainly from food products and their packages. The presence of BPA in food is linked to exposure in animals and raw plant material as well as direct leaching from canned and plastic food packages. Average daily exposure in humans is estimated to be 0.48 to 1.6 μg/kg/body weight/day [36]. Repetitive use of BPA-containing materials, increased pH, and temperature

accelerate BPA leach and exposure [41,42]. It is estimated that BPA in the range of 4 to 23 μg/tin is released from canned food during temperature pasteurization [36,42].

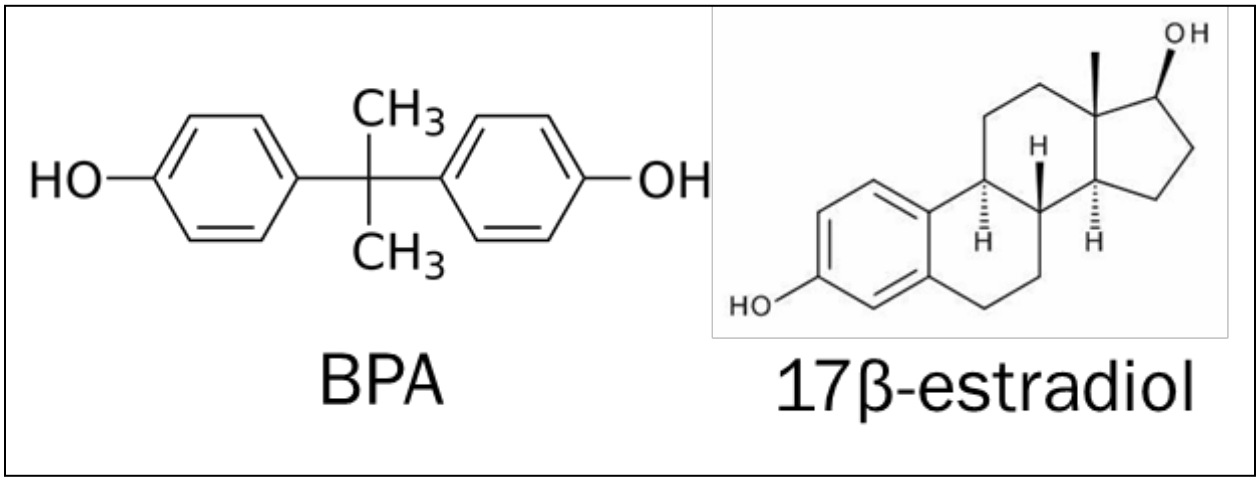

Figure 2: Structures of bisphenol A and 17β-estradiol: BPA possesses the phenolic ring structure that is present in estrogens. The hydrogen bonds of the two phenol-hydroxyl groups on BPA binds to the same pocket on ESR1 similar to the position where E2 binds. However, BPA binding is extremely weak compared to E2.

Pharmacokinetic studies in both humans and rodents show that BPA is readily absorbed after oral ingestions and is metabolized mainly in the liver [43]. Metabolism in the liver is mainly via conjugation to glucuronic acid to produce BPA-glucuronide. To a lesser degree, BPA-S and BPA-quinone are produced in the liver as products of the conjugation of BPA to sulfate and quinine moieties [44]. Conjugated BPAs were thought to be biologically inactive because they do not bind to ESRs. However, there is evidence that conjugated forms of BPA can perturb cellular responses in prolactin cells, presumably through interactions with membrane ESR-1[45]. In humans, elimination of BPA is via the urine in the form of free BPA or BPA conjugates. The elimination routes in rodents include urine as well as feces. Differences between humans and rodents are

attributed to different molecular weight cutoffs for excretion, with humans having a higher threshold than rodents [46].

Concerns about the health risks of BPA prompted the ban of BPA in infants-related food products in 2012. However, unlike Canada and European countries with stricter regulations on BPA usage, the US still permits the use of BPA in several products [47]. This is because the FDA continues to review emerging data on the safety profile of BPA. The prospect of eventual phasing out of BPA has resulted in the search and creation of several analogs of BPA as a possible safe replacement chemical for BPA.

1.2.5 Bisphenol S (BPS)

Concerns about the toxic effect of BPA led to a restriction on the use of BPA in many countries. The European Union banned the use of BPA in infant-related materials in 2011, while the Canadian government instituted a similar ban much earlier in 2008. A food additive petition by the American Chemistry Council and a subsequent citizen petition by a member of Congress paved the way to the regulation of BPA use in the United States. Thus, regulations in BPA use, increased awareness of its toxic potentials and the prospect of total phasing out of BPA made a replacement chemical a matter of urgency, which promoted the introduction of BPA analogs. These analogs include bisphenol B, F, AF, and S. BPB is used mainly in the manufacture of phenolic resins, while BPF is an alternative for BPA in polycarbonate plastics and epoxy resins. BPS was first synthesized in 1869 but was not produced on an industrial scale until the restriction on the use of BPA. BPS is by far the most common analog of BPA and a constituent of many household products including BPA-free infant-related products. Due to the high resistance to heat and sunlight, BPS serves as an excellent anticorrosive agent in epoxy resins and has also

completely replaced BPA as a dye in thermal papers, currency bills, and tags, and several households and industrial material [48,49]. Human exposure to BPS is similar to that described for BPA. It is reported that a BPS concentration of up to 11 ng/kg body weight occurs in the general population, and a concentration of up to 821 ng/kg/body weight per day was measured in occupationally exposed individuals [48]. It has also been documented to be present in 81% of human matrices and 3% of breast milk [50,51]. Despite the wide application of BPS and exposure of the population, little is known about its safety profile. However, its estrogenic potential and action have been predicted based on its similarity to BPA and E2 **(Fig. 3)**. Toxicokinetic studies showed that BPS in comparison with BPA is less biodegradable, with a greater degree of bioavailability and a longer half-life [52].

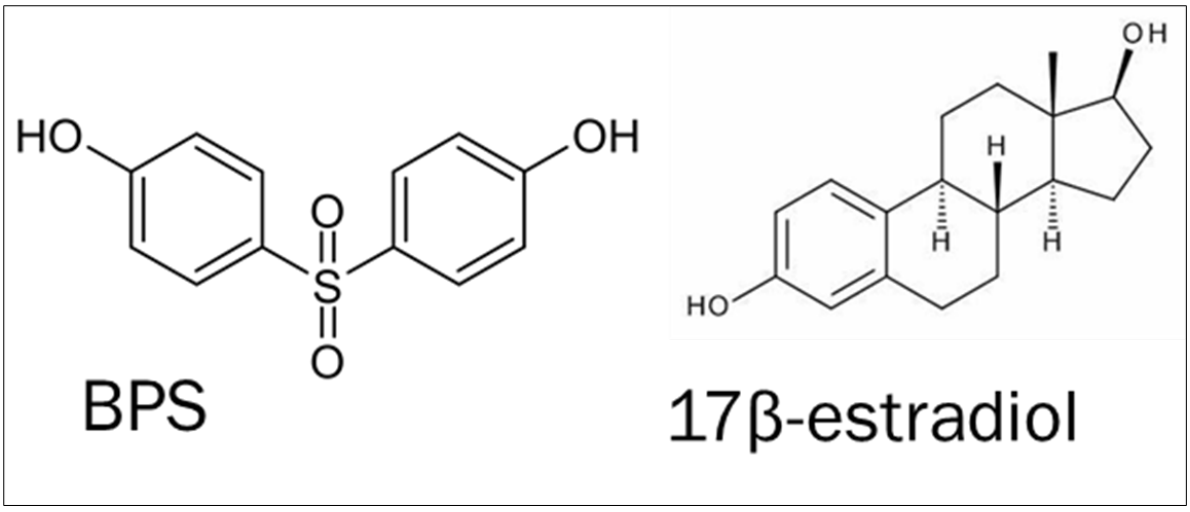

Figure 3: Structures of bisphenol S and 17β-estradiol: BPS consists of two phenolic rings that are similar to that of 17β-estradiol. BPS is proposed to be more active on ESR2 than ESR1. Activities at these receptors are weaker than E2 activity.

1.2.6 17α-ethinylestradiol (EE2)

17α-ethinylestradiol is a synthetic estrogen formulation used in female oral contraceptives and the treatment of estrogen insufficiency. It is derived from estrogen and shares a much similar structure to E2 than other synthetic xenoestrogens **(Fig. 4)**. Principally, EE2 is metabolized in humans and rodents by the liver microsomes to 2-hydroxyethinyloestradiol, a metabolically active product [53], but a substantial amount of EE2 is excreted unchanged in the urine and fecal matter [54]. EE2 has a strong lipophilic property and is relatively resistant to bacterial degradation and photodegradation. Hence, a large percentage of EE2 is not fully removed from sewage before discharge into water bodies [55]. As such unchanged EE2 contaminates water in the concentration ranging from parts per trillion in drinking water or groundwater to parts per billion in several rivers [56]. In the US, data from 139 streams across 30 states estimated EE2 concentrations to be between 5 and 273 ng/L [57]. The increased awareness of EE2 presence in the environment prompted the USEPA to add EE2 to the List-3 of the New Contaminant Candidate [58]. Similarly, the European Union has added EE2 as a substance of priority on the EU Water Framework Directive[59].

Although EE2 is mainly prescribed to female individuals, its presence as a contaminant of drinking water poses a major problem to the male individual. This is because EE2 is one of the most potent estrogenic compounds with a binding affinity to ESRs that is twice that of E2 [60,61]. Exposure to EE2 in aquatic life demonstrated the deleterious effects on reproduction in general. For example, exposures to EE2 were associated with anomalies of sexual development [62,63], altered sexual maturation [64], reduced fecundity [65], disruption of spermatogenesis [66], and reproductive tract anomalies in aquatic organisms [67]. A few available studies on the effects of EE2 on mammalian species also support the toxic effect on the reproductive axis. For example, exposure of female rats to EE2 at a concentration of 50 µg/Kg/day from conception throughout the lactation period

decreased paired testicular weight, seminal vesicle weight, and total sperm count in adult male offspring [68]. A wide range of concentrations has been used in the evaluation of the toxic effects of EE2. However, a larger percentage of the early studies are in aquatic species and female mammalian species. Thus, data on EE2 effects on the mammalian male reproductive axis as well as the mechanisms involved are limited. This is not unexpected as EE2 was largely seen as a contaminant of water bodies, and its use was generally limited to the female population. But evidence of EE2 persistence in drinking water even after treatment, increasing exposure in males, and reports linking estrogenic compounds with the decline in male reproductive health make investigations into EE2 in the male a subject of important interest. Therefore, parts of this dissertation will focus on investigating EE2 effects and mechanism of action on the gonads of developing male rats.

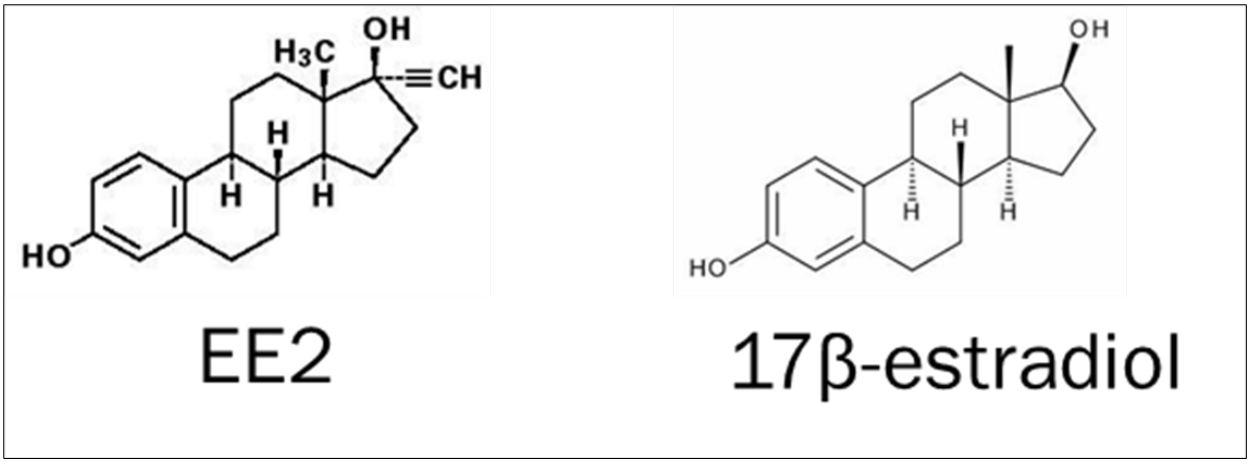

Figure 4: Structures of 17α-ethinylestradiol (EE2) and 17β-estradiol: EE2 is structurally similar in all respect to 17β-estradiol but for the unsaturated triple bond in the ethinyl group. EE2 is an agonist at all ESRs and is more potent than E2.

1.3.0 Male Reproduction and development of the testis

The male reproductive apparatus consists of the gonads and the external genitalia. The development and optimal functioning of the gonad and the accessory organs of the male reproduction are under the influence of hormones. These hormones are secreted by the gonad and the central nervous system. Locally produced hormones by the gonad early in embryonic life induce regression of potentially female structures and support the development of the male reproductive tract. Compartmentalization of the seminiferous tubules and formation of the blood-testis barrier during prepubertal development permit the steroidogenic and gametogenic activities of the adult testes to proceed normally. Hormones from the central nervous system, specifically the hypothalamus and anterior pituitary, regulate the endocrine and exocrine functions of the testes to promote normal development and function. The single most important hormone of the male reproductive axis is testosterone. It is central to male reproduction because of its importance in the prenatal period for sex determination, development of the reproductive organs during puberty, and the maintenance of reproductive physiology throughout life.

1.3.1 Development of the genital ridges and the origin of germ cells

In the mammalian early embryonic development, the first cells of the germ lineage are the primordial germ cells (PGC). They are a homogenous population of embryonic cells formed by inductive signals from embryonic and extraembryonic sources. PGCs represent the precursors of the spermatogonial stem cells in the XY embryo. In rodents, PGC precursors originate from the posterior proximal epiblast on embryonic day 6, migrating to the base of the developing allantois in the extraembryonic mesoderm [69]. In humans, two origins have been proposed for PGC. One of the putative origins is the epiblast, and the other is the extraembryonic mesoderm at the base of

the allantois [70]. Induction and migration of the PGC are under the influence of paracrine signaling factors such as BMP, WNT, β-catenin, and BLIMP [71]. Although PGC is initially located within the extraembryonic mesoderm, they subsequently migrate and colonize the gonadal ridge during the 4th and 5th week of conception in rodents and humans, respectively.

At about the embryonic day 9.5 in rodents and during the 3rd to the 4th week in humans, the proliferation of coelomic epithelium on the ventral surface of the mesonephros gives rise to paired thickenings of epithelial layers. Some of the proliferating coelomic epithelial cells undergo epithelial-to-mesenchymal transition and migrate into the dorsal inner mesenchyme region to form genital ridges. A subpopulation of coelomic epithelial cells that express *Gata4* and *Nr5a1* gives rise to the somatic lineages of the genital ridge[71]. Important regulatory genes involved in the formation of the genital ridges include *Wt1, Lhx9, Emx2, Six1, and Six4* [72]. By the end of the 4th and 5th week in rodents and humans, the genital ridges are composed of somatic cell and germ cell (gonocytes) lineages.

1.3.2 Sex determination

All the cells in the gonadal ridges have the potential to differentiate into the testicular or ovarian lineage. Sex determination in males is dependent on the expression of the *Sry* gene. This gene is located on the short arm of chromosome Y. In rodents, *Sry* is first expressed at about the embryonic day 11with peak levels of expression attained within hours (day 11.5) and a return to pre-induction levels shortly after day 12.5, whereas *Sry* is induced in humans by day 42 after conception and down-regulated by day 53 reaching baseline by day 61[73]. Thus, there is a specific window of induction of *Sry* expression during which the fate of the germ cell is determined. Failure of expression of *Sry* during this window leads to the formation of ovarian lineage cells. *Sry* serves

as the trigger for the initiation of male sex differentiation by inducing the differentiation of pre-Sertoli cells and the upregulation of the *Sox 9* gene. At the same time, *Sry* simultaneously down-regulates the expression of *Wnt4*. Upregulation of *sox 9* promotes differentiation into the testicular lineage, and the concurrent down-regulation of *Wnt4* suppresses ovarian pathway differentiation into granulosa cells. Although *Sry* and Sox9 are the most prominent genes involved in sex determination, several genes are involved in this network, including *Fgf9, NR5A1, Wt1, Fog2, and Gata4.*

1.3.3 The Sertoli cell and the formation of testis cords

Sertoli cells were described first in 1865 as "special branched cells in the seminiferous tubules of the human testicle" by Enrico Sertoli [74]. Sertoli cells are derived from Nr5a1 positive pre-Sertoli cells in the primitive gonad [75]. Pre-Sertoli cells are dispersed, non-polarized somatic cells expressing *sry* and/or *sox9,* while Sertoli cells are polarized, well organized, and expressed only Sox9. Differentiation from pre-Sertoli cells to Sertoli cell is thus characterized by the appearance of aggregates of polarized and well-organized cells that will eventually initiate testis formation. At this stage, they are termed immature Sertoli cells. The maturation of Sertoli cells continues postnatally until puberty when they stop proliferating.

Within the genital ridge, aggregation of immature Sertoli cells initiates testis cord formation. The formation of the testis results in the compartmentalization of germ cells within the solid cord of future seminiferous tubules while non-germ cells are left outside of the cords. The testis cord gradually transforms and extends into the medulla of the gonad, branching and anastomosing to form networks of cords known as rete testis. Throughout differentiation, the cord remains connected to the mesonephric cell mass until the formation of the thick fibrous capsule

known as the tunica albuginea. Gradually, the testis separates from the regressing mesonephros and is suspended by its mesentery. The formation of the cords changes the nomenclature and properties of the cell within the nascent testis. While the cords are being formed, loosely packed undifferentiated mesenchymal cells in the interstitium differentiate to become fetal Leydig cells (FLCs). PGCs that are engulfed within the cords become gonocytes (prespermatogonia or spermatogonia stem cell). The germ cells continue to differentiate and divide but are prevented from entering meiotic division by the immature Sertoli cells via the expressions of *Cyp26b1* gene [76]. The Cyp26b1 enzyme mediates the degradation of retinoic acid. Degradation of retinoic acid prevents germ cells from entering meiotic division until puberty[77]. In utero and until birth, although the gonocytes are within the testicular cords, each cord remains a solid structure with no lumen. The gonocytes briefly but rapidly proliferate, following which they become mitotically quiescent until after birth. NOTCH signaling in Sertoli cells is responsible for this mitotic quiescent period [78].

At birth, terminally differentiated Sertoli cells transform the solid cords into seminiferous tubules with lumen formation and the translocation of initially compartmentalized gonocytes to the basement membrane. Translocation of gonocytes is made possible through the formation of tight junctions by the immature Sertoli cells. Parts of these tight junctions form the blood-testis-barrier. The formation of the testicular blood vessels is influenced by signaling from endothelial growth factors (EGFs) and platelet-derived growth factors (PDGFs). EGFs and PDGFs induced the migration of vascular endothelial cells from the mesonephros to form the blood vessels and blood cells. Important genes and controlling factors involved in the aggregation and cord formation process include *Sox9, Sox8, Sox10, FGF9, and activin*s.

Sertoli cells are major effectors of the testosterone cascade that supports male germ cell development[79], but they become responsive to testosterone activation only at puberty when they begin to express androgen receptors[80]. Sertoli cells secrete many bioactive products that are important in the differentiation, regulation, and maturation of other testicular cells. Among these, the most prominent and probably one of the most critical to reproductive tract development is the anti-Mullerian hormone (Amh). Amh production starts at about the 8th week (in rodents) and the 9th week (in humans) of embryonic life, but production is maintained until puberty. As such, Amh is an important marker of testicular maturation and function [81]. Amh drives the regression of Müllerian ducts, and when its action is missing there is the persistence of Müllerian derivatives in the male urogenital tract. Recent evidence showed that Amh is involved in the control of gonocyte entry into a mitotically inactive state during fetal development and also promotes germ cell transition from gonocytes to spermatogonia [82–84]. Amh is upregulated by *NR5A1, WT1*, and *GATA4* but down-regulated by *DAX1* [85–87]. In addition to Amh, Sertoli cells secrete inhibin Bβ which has a role in testicular regulation of the pituitary gland. Other factors secreted by Sertoli cells include metal ion transport proteins (transferrin and ceruloplasmin), protease inhibitors which are important in the tissue remodeling process occurring during spermiation, and the movement of spermatocytes into the lumen of the seminiferous tubules, desert hedgehog, and glycoproteins that regulate the formation of the basement membrane between Sertoli cells and the peritubular cells. At puberty, Sertoli cells switch from the immature/proliferative phase to the mature phase, with down-regulation of Amh and up-regulation of GATA binding protein 1 (Gata1)[88].

1.3.4 The **Leydig cell**

The first description of Leydig cell was in 1850 by the German scientist Franz Leydig [89], but it was not until 1903 that Bouin and Ancel provided evidence that Leydig cells are endocrine cells important in controlling male reproduction[90]. Leydig cells constitute about 4-6% of all the cells in the testis. Leydig cells are the principal cell responsible for testosterone production in the testis. They are derived predominantly from undifferentiated mesenchymal cells, which originate from the mesonephric mesenchyme [91]. There are suggestions that Leydig cells may also originate from the neural crest cells because of their ability to express neuronal proteins. Leydig cells are found within the interstitium of the testis aggregated in clusters or in continuous strings near blood vessels[92]. Their proximity to blood vessels underly their importance as hormone-producing cells. Closely associated in the interstitium with Leydig cells are fibroblasts and blood-derived cells such as macrophages, lymphocytes, monocytes, and mast cells. The shapes and sizes of Leydig cells vary from one species to another. Leydig cells possess large and small irregular surface projections and coated pits, indicating that they are involved in pinocytic activities and intercellular trafficking of extracellular molecules via non-specific and specific interactions with the extracellular environment[93]. Senescent or damaged Leydig cells are usually phagocytosed by macrophages [94]. The earliest form of Leydig cells appearing in utero are referred to as fetal Leydig cells (FLCs), while the postnatal Leydig cells are characterized by sequential differentiation through three distinct phases of maturation. It is on these distinct phases of maturation that post-natal Leydig cells are classified into the progenitor Leydig cell (PLC), immature Leydig cell (ILC), and the adult Leydig cell (ALC) **(Fig. 5)**.

1.3.5 Fetal Leydig Cells (FLC)

Fetal Leydig cells are derived from undifferentiated mesenchymal cells originating from the ventral surface of the mesonephric mass of the genital ridges. Differentiation into Leydig cells takes place after the formation of the testis cord and the interstitium. Activation of *SF-1* and *Wt-1* genes under the influence of the *Sry* gene induce the differentiation of cells into Leydig cells. *SF-1* in fetal Leydig cells regulates the expression of steroidogenic proteins and initiates the cell to begin the process of steroidogenesis. This usually occurs at about embryonic day 14 in rats and the 8th week of gestation in humans. Fetal Leydig cells predominantly secret androstenedione and do not express androgen receptors, unlike the postnatal Leydig cells types [95]. Androstenedione produced by Fetal Leydig cells is converted to testosterone by 17β-hydroxysteroid dehydrogenase present in the peritubular cells and Sertoli cells. Testosterone from the fetal Leydig cells is critical to induce masculine differentiation of the Wolffian ducts, priming of the brain and liver to androgen sensitivity, development of the urogenital tract, differentiation of the gubernaculum ligament, and descent of the testis. Although fetal Leydig cells continue to exist after birth, they neither undergo mitosis postnatally nor transform to adult Leydig cells. Atrophy of the FLCs occurs in the first 3 weeks after birth, and the cells gradually become depleted as the testis mature postnatally.

1.3.6 Postnatal Leydig Cells

Postnatal Leydig cells are not derived from fetal Leydig cells but are independently derived from undifferentiated mesenchymal cells of the genital ridges. During embryonic development, while some of the mesenchymal cells in the interstitium transform into rounder-shaped, testosterone-producing fetal Leydig cells, others remain spindle-shaped, proliferate and transform

into Leydig stem cells. The Leydig stem cells are the precursors of postnatal Leydig cells. The Leydig stem cells proliferate neonatally within the interstitium. At about postnatal day 10 in rodents and 14 in humans, a pool of Leydig stem cells transform to progenitor Leydig cells with the appearance of 3β-hydroxysteroid (HSD-3β), cytochrome $P450_{scc}$ (Cyp11A), and $P450_{c17}$ (Cyp17A). A small pool of Leydig stem cells persists and represents the replenishing pool for the postnatal Leydig cells. Following the acquisition of steroidogenic capacity, luteinizing hormone receptors (LHR) begin to appear on the surface of PLC. Although LHR is present, PLC is not sensitive to luteinizing hormone because, structurally, the LHR on the surfaces of PLC lack an extracellular domain portion; hence they are incomplete and non-functional. PLC are spindle-shaped like the stem cells from which they are derived, and are characterized by a prominent nucleus and small cytoplasmic volume with little or no smooth endoplasmic reticulum. Although the capacity to secret androgen by the PLC is much lower compared to other stages of Leydig cells, PLC is highly proliferative. Differentiation from PLC to the ILC is completed by postnatal day 35.

The ILC that is differentiated from the PLC is morphologically distinct and has a round rather than a spindle shape appearance, numerous cytoplasmic lipid droplets, increased number of mitochondria, and a well-developed smooth endoplasmic reticulum. At this stage of maturation of the Leydig cell (ILC stage), the HSD-3β, CYP11A, and CYP17A activities are increased, and the LHR are fully formed. Hence, ILCs are stained intensely for HSD-3β and are responsive to luteinizing hormone stimulation. Although the activities of HSD-3β, CYP11A, and CYP17A are much increased, they are not maximal until full maturation. Newly formed ILCs do not contain 11β-hydroxysteroid steroid dehydrogenase 1 (HSD-11β1), an enzyme involved in the glucocorticoid suppression of androgen production [96]. However, HSD-11β1 is gradually acquired

as ILCs mature from day PND 35 to 56. Furthermore, the activity of 17β-hydroxysteroid dehydrogenase (HSD17β) is low in ILCs compared to the adult Leydig cells, and hence, the capacity for testosterone production is considerably lower than in adult Leydig cells. Activities of testosterone metabolism enzymes (i.e., 5α reductase and 3α- hydroxysteroid dehydrogenase) are exceptionally high in ILCs. Thus, 5α-reduced androgens (androstane-3,17-β-diol (3-diol) and androstenediol) instead of testosterone are the major products of ILC. Furthermore, the proliferative capacity of ILCs in comparison to PLCs is much reduced.

Adult Leydig cells (ALC) begin to appear at about PND 56. This stage of Leydig cells is characterized by increased cell size, mitochondrial number, smooth endoplasmic reticulum, and other organelles. However, there is a decline in cytoplasmic lipid droplets because at this stage the cells do not depend on intracytoplasmic lipid storage for steroidogenesis. Rather, cholesterol for steroidogenesis can be adequately synthesized de novo within the cell. ALC has a higher number of LHRs and ARs and is highly sensitive to luteinizing hormone and feedback androgen regulations. All the enzymes of the steroidogenic pathway are fully developed and functional, but the testosterone metabolizing enzymes are decreased in number and activity. Hence, more testosterone is produced, and less is metabolized in ALCs. Unlike the other Leydig cell stages, ALCs are incapable of proliferation. Overall, prepubertal and pubertal increases in Leydig cell numbers results from the differentiation of Leydig stem cells into Leydig cells between PND 14 - 28, and the mitotic division of newly formed Leydig cells from PND 28 to 56.

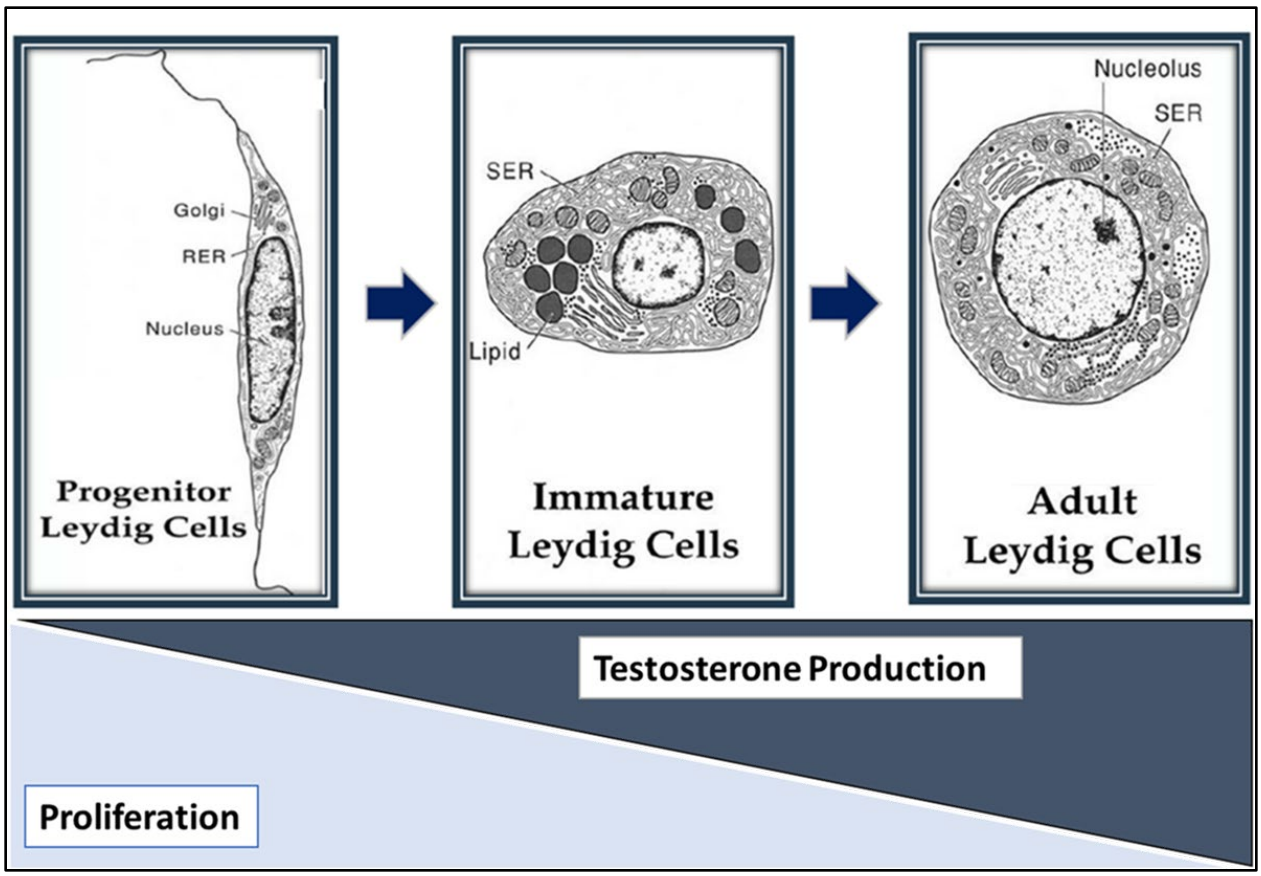

Figure 5: Stages of postnatal Leydig cell development. Progenitor Leydig cells are observed in the testis by postnatal day (PND)14- 28, immature Leydig cells by PND 35-56, and the adult Leydig cells by PND 56. As Leydig cells transition from progenitor to the adult stage, proliferative capacity reduces but testosterone secretory capacity is increased.

1.4.0 Gonadal Steroidogenesis

Testicular steroidogenesis is directly controlled by the hypothalamus and pituitary gland. Gonadotropin-releasing hormone (GnRH) from the hypothalamus binds to its membrane receptor on pituitary gonadotrophs and stimulates the biosynthesis and secretion of luteinizing hormone. The binding of luteinizing hormone to LHR on Leydig cells stimulates adenylate cyclase leading to increased intracellular cAMP, activation of protein kinase A and subsequent phosphorylation of enzymes of the steroidogenic pathway[97]. The first enzymatic step in Leydig cell steroidogenesis

is mediated by steroidogenic acute regulatory (StAR) protein **(Fig. 6)**. Phosphorylated StAR proteins mobilize cholesterol from the outer mitochondria membrane to the inner mitochondria. Cholesterol can be synthesized de novo from acetate or may be sourced from stored lipid droplets and plasma membrane. CYP11A, the side cleavage enzyme and its associated electron transport proteins located on the inner mitochondrial membrane matrix side, converts cholesterol into pregnenolone **(Fig. 6)**. The rate-limiting step in steroid biosynthesis is a subject of controversy. While the CYP11A conversion of cholesterol to pregnenolone has been suggested as the rate-limiting step in Leydig cells, newer evidence suggests that the rate-limiting activity is attributable to StAR proteins [98–100].

The subsequent enzymatic conversion of pregnenolone to testosterone is mediated by HSD-3β, CYP17A1, and HSD-17β. While HSD-3β is present in the mitochondria, it is predominantly found in the smooth endoplasmic reticulum, where it mediates the transition between Δ4 and Δ5 pathways of steroidogenesis (**Fig. 6**). Within the endoplasmic reticulum, steroidogenesis proceeds predominantly through the Δ4 pathway in rodents and the Δ5 pathway in humans. Both CYP17A1 and HSD-17β are exclusively found in the smooth endoplasmic reticulum and have specific action in both Δ4 and Δ5 pathways. Pregnenolone from the mitochondria diffuses passively to the smooth endoplasmic reticulum, where there is an abundance of HSD-3β. Conversion of pregnenolone to progesterone by HSD-3β enzymatic action drive steroidogenesis via the Δ4 pathway, while pregnenolone drives the Δ5 pathway. The first two steps in both pathways are mediated by CYP17A1 an enzyme with 17α-hydroxylase and 17, 20-lyase activities. Formation of progesterone drives Δ4 pathway. In the Δ4 pathway, CYP17A1 converts progesterone to 17α-hydroxyprogesterone in the first step, and the second step 17α-hydroxyprogesterone is converted to androstenedione. HSD-17β catalyzes the conversion of

androstenedione to testosterone to complete the Δ4 pathway. In the Δ5 pathway, CYP17A1 via its 17α-hydroxylase activity converts pregnenolone to 17α-hydroxypregnenolone, and then in a second step reaction, 17α-hydroxypregnenolone is converted to dehydroepiandrosterone (DHEA) by the 17, 20-lyase activity of CYP17A1. HSD-17β catalyzes the conversion of DHEA to androstenediol **(Fig. 6).** The final step in the Δ5 pathway is mediated by HSD-3β, which converts androstenediol to testosterone. Thus, HSD-3β has multiple actions in the steroidogenic pathway converting intermediates between the Δ4 and Δ5 pathways. For example, HSD-3β can convert 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, and also convert dehydroepiandrosterone to androstenedione. These processes can proceed in both directions depending on substrate availability. Within the Leydig cell, phosphorylation of aromatase mediates the conversion of testosterone to estradiol. Steroidogenesis in Leydig cells is precisely regulated. In addition to luteinizing hormone-mediated PKA signaling, several regulatory molecules such as kinases (MAPK, PKG, CAMKI, and AMPK) and transcription factors (NUR77, MEF2, Dhh, and GATA4) regulate steroidogenesis in Leydig cells. A short loop feedback control of the androgen pathway is mediated by the nuclear actions of locally synthesized estradiol and testosterone, which have their receptors expressed in Leydig cells.

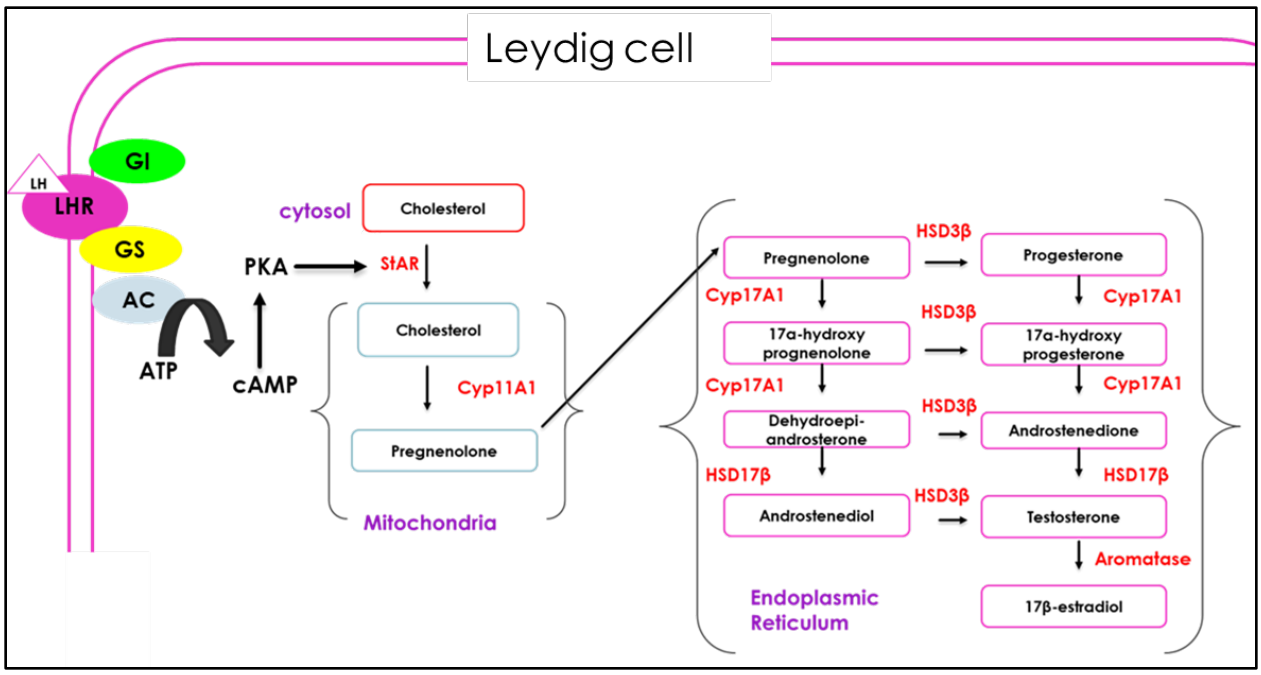

Figure 6: Leydig cell steroidogenic pathway. The Δ4 pathway predominates in rodents while the Δ5 is the preferred pathway in humans**.** 3β-hydroxysteroid (HSD-3β), cytochrome $P450_{scc}$ (Cyp11A), and $P450_{c17}$ (Cyp17A).

1.5.0 Androgen, the Androgen receptors (AR) and the Male Reproductive System

Androgens are essential for male fertility and the maintenance of reproductive health. The development of the testis, epididymis, vas deferens, seminal vesicle, prostate, and penis are essentially dependent on androgens. High levels of intratesticular testosterone, secreted by Leydig cells, are necessary for spermatogenesis. In humans, intratesticular testosterone concentrations of 25 to 125- fold (340 - 2000 nM) compared to serum levels (8.7-35 nM) are needed for optimal spermatogenesis. Outside the testis, the conversion of testosterone to dihydrotestosterone by the action of the 5α-reductase enzyme activity is needed for muscle development, bone mineralization, fat metabolism, and cognitive functions. Thus, androgens are important not just in the development and maintenance of reproductive function but also in the general physiologic function of the male

individual. Androgen functions are mediated via ARs. AR is expressed in a wide variety of tissues that are targeted by androgens. In the testis, AR is expressed in post-natal Leydig cells [101]. Sertoli cells begin to express AR after birth in a cyclic manner related to stages of the seminiferous tubule cycle [95]. Arteriole smooth muscle, vascular endothelial cells, and the peritubular cells are other cellular targets of androgen in the testis, where AR is highly expressed. Although androgens regulate germ cells, they do so by indirect action in Sertoli cells because functional ARs are not present in germ cells. In the brain, the AR has been mapped to have the highest levels of expression in the hypothalamic nuclei involved in regulating reproductive and neuroendocrine functions. The limbic regions such as the lateral septum, hippocampal, medial amygdala, anterior mammillary nuclei, median eminence, olfactory nuclei, and the brainstem have all been shown to express variable amounts of AR. In bones, AR is highly expressed in osteoblasts and hypertrophic chondrocytes, while the osteocytes and endothelial cells of blood vessels within the bone marrow exhibited moderate expression of AR. Other androgen target sites where AR is present include epididymis, prostate, adrenal gland, and the liver.

AR is a soluble protein that functions as an intracellular transcriptional factor, and it belongs to the steroid and nuclear receptor superfamily. The gene coding for AR is located on chromosome 11 (Xq11.2-q12) [102]. In a similar fashion to other steroid receptors, AR is characterized by the presence of a ligand-binding domain, a DNA-binding domain, and a transactivation domain (**Fig. 7**). The N-terminal domain is the transcriptional regulatory region of AR. The DNA- binding domain is a highly conserved region among steroid hormone receptors in general. It is composed of two zinc finger motifs. The first zinc finger confers receptor-specificity for androgen-response elements, while the second zinc finger is involved in AR dimerization and binding to the major DNA groove. The C-terminal is the ligand-binding domain and is significantly

variable among steroid hormone receptors. This ligand-binding domain folds into a ligand-binding pocket. A hinge region connects the DNA-binding domain and the ligand-binding domain.

Figure 7: Structural representation of androgen receptor. N-terminal domain (NTD), DNA-binding domain (DBD), hinge region (HR), and the Ligand-binding domain (LBD)

1.5.1 The Androgen Signaling Pathways

AR functionality is divided into two ways based on the time it takes the signaling pathways to produce a functional response. The first is through the classical pathway of activation through interaction with androgen ligands, which is deemed the slow pathway and results in switching on of genes and ultimate protein formation. It takes hours to days to observe the products of the classical pathway. A second pathway is considered the rapid pathway and is mediated via the membrane-localized AR.

1.5.2 Classical Signaling (Genomic) Pathway

The classical signaling pathway is also called the direct genomic pathway (**Fig. 8)**. The pathway is initiated with androgen binding to AR sequestered in the cytoplasm of target cells. AR in the cytoplasm is tethered to multiple chaperones, such as the heat shock proteins (hsp90, hsp70, hip, p60, p23, FKBP51) and cytoskeletal proteins. Chaperones may be either co-activators or co-repressors, depending on their function. An example of an AR co-activator is the steroid receptor coactivator family (SRC/p160). The co-activators function as enhancement of AR transactivation, regulators of histone modification, proteasomal degradation, and chromatin remodeling. The co-

repressors e.g., nuclear receptor corepressor (NCoR) act to inhibit transcription initiation of androgen-responsive genes. Testosterone or DHT acting as a ligand binds and induces a conformational change in AR. The conformational change leads to AR release from hsp, formations of ligand-receptor complex, and receptor dimerization.

The process of dimerization leads to nuclear trans-localization of ligand-receptor complex, cofactor binding, DNA binding, and transactivation. Nuclear translocation of AR is mediated by the superfamily of transport receptors known collectively as karyopherins[103]. Once in the nucleus, the ligand-receptor complex binds to specific DNA sequences called the androgen response elements (AREs) near the promoter region. Once bound, proteins regulating gene expression (i.e., co-activator or co-repressor proteins) are recruited to regulate expressions of genes that ultimately lead to a cascade of events resulting in genetic modification and protein formation. The classical pathway is characterized by the time required to produce a functional response. At least 30–45 min is required for productive transcription after testosterone stimulation, with additional time required to alter protein levels in the cell [104]. The direct genomic signaling pathway is the most important pathway for androgen regulation of spermatogenesis because a mutation in the AR gene affecting a segment of the DNA binding domain stops germ cell development at meiosis [80].

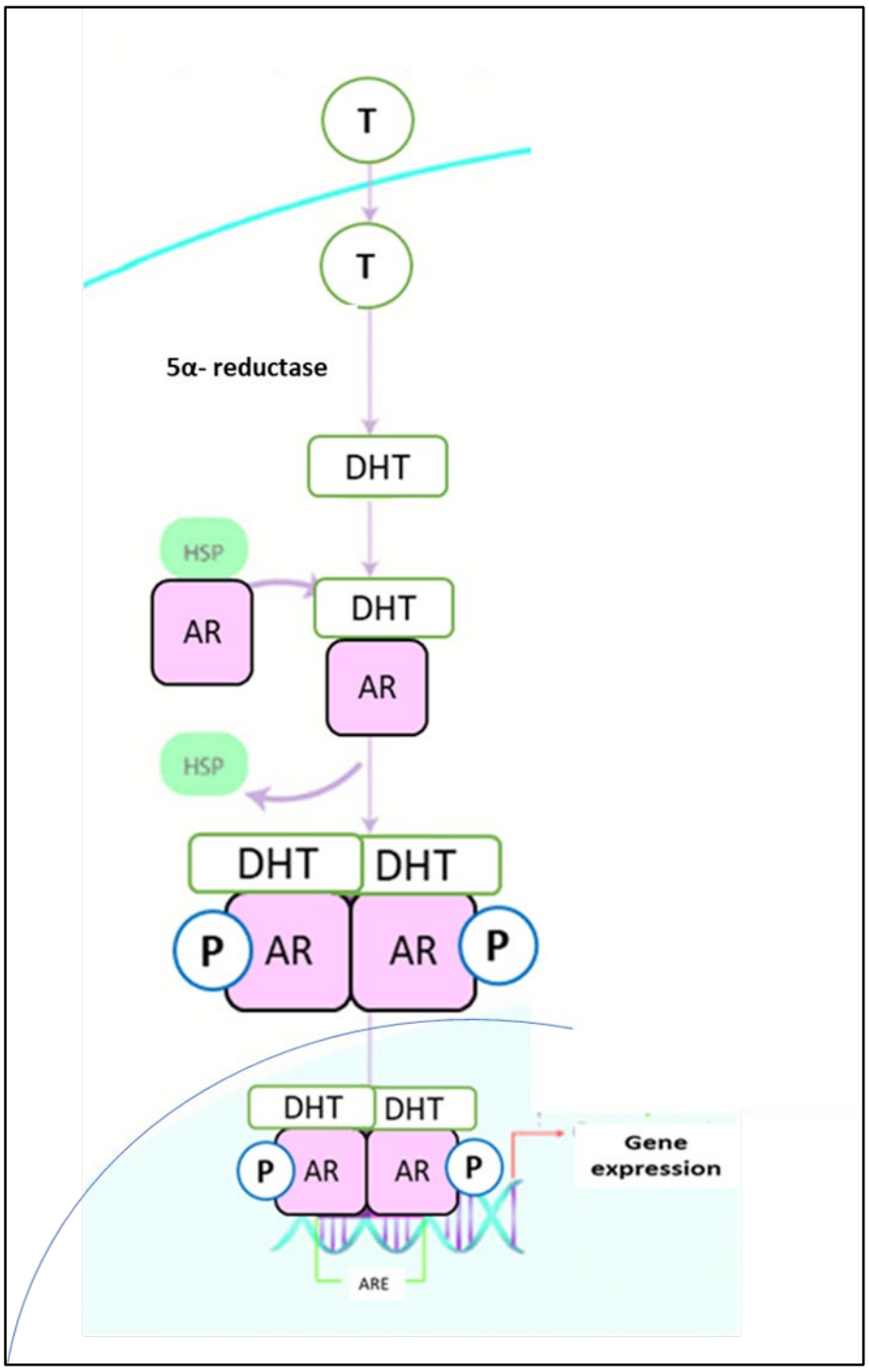

Figure 8: Classical Signaling Pathway. The pathway is initiated with androgen binding to AR sequestered in the cytoplasm of target cells with subsequent activation of genomic response through the androgen response element (ARE).

1.5.3 Non- Classical Signaling (Non-genomic) Pathway

The non-classical pathway or the indirect pathway of androgen signaling is mediated by at least two mechanisms (**Fig. 9**). The first mechanism involves androgen interaction with ARs localized to the plasma membrane. Androgen binding to ARs on the plasma membrane caused the activation of Src tyrosine kinase. Specifically, this activation is mediated by the proline-rich region of AR (amino acids 352–359) interaction with the SH3 domain of Src [105]. Activation of Src leads to the phosphorylation and activation of the epidermal growth factor receptor (EGFR). Phosphorylated EGFR activates the MAP kinase cascade along with other kinases such as RAF, MEK, and ERK. The downstream effect is the activation of the $p90^{Rsk}$ kinase and the subsequent phosphorylation of the cAMP response element-binding (CREB) transcription factor. Unlike the classical signaling pathway requiring 30-45 minutes to produce a functional response, activation of the CREB transcription factor is fast, requiring only seconds to minutes for functional response to be observed. The activation of Src and ERK kinase by non-classical signaling is critical for maintaining Sertoli-germ cell attachments during spermatogenesis [106]. The second non-classical pathway is mediated by testosterone interaction with non-AR receptors proposed to Gq sub-type of G protein-coupled receptor. This interaction causes an increased influx of $[Ca^{2+}]$ via the L-type $[Ca^{2+}]$ channel. The rapid and increased $[Ca^{2+}]$ influx caused activation of phospholipase C. Phospholipase C, in turn, hydrolyzes PIP2 in the plasma membrane to produce IP3 and diacylglycerol (DAG). IP3 and DAG caused further increase in intracellular $[Ca^{2+}]$, leading to alterations in many cellular processes.

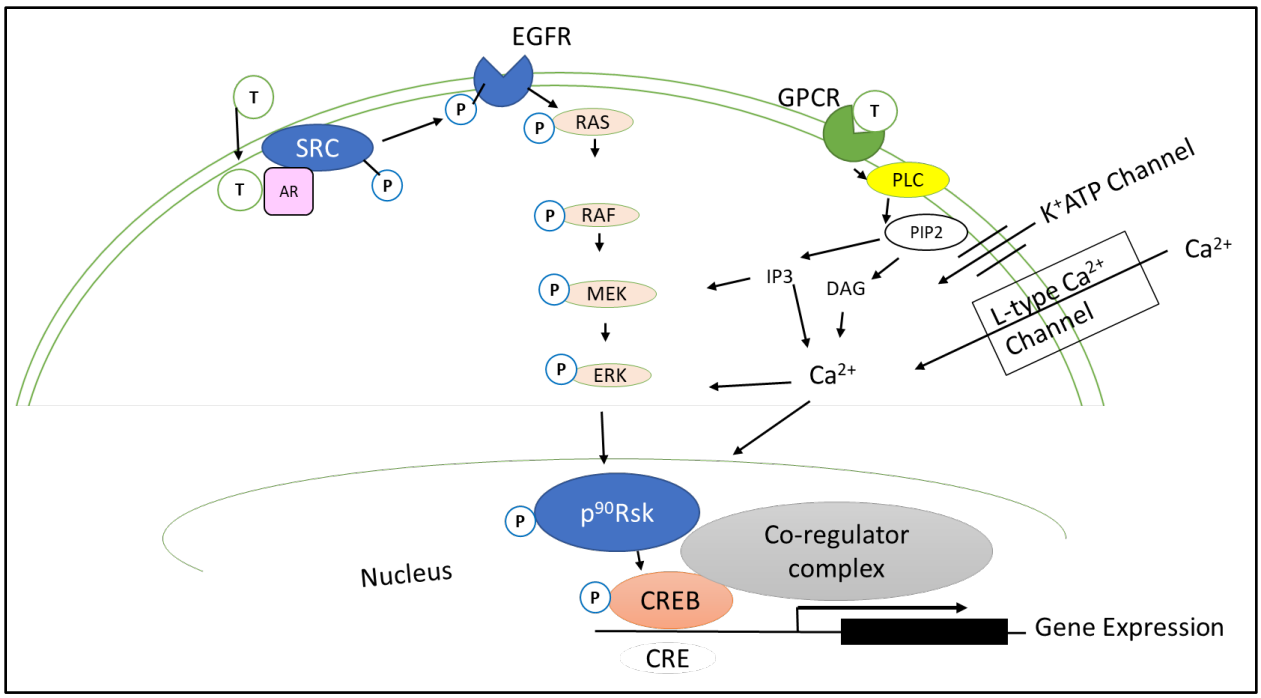

Figure 9: Non-classical Signaling Pathway. This pathway involves androgen interaction with ARs localized to the plasma membrane. Cellular response to androgen signaling through the indirect pathway is rapid, occurring within seconds to minutes.

1.6.0 Estradiol and the Male Reproductive System

Although the role of testosterone in male reproductive development and function is unquestionably the most important, estradiol contributes to the development and optimal functioning of the male reproductive system. Until the 1960s and 70s, estrogens were considered more of a female hormone, and not of much importance was attributed to E2 in the male. However, Sertoli cells, Leydig cells, and germ cells are all capable of synthesizing E2 and the role of E2 in male reproduction is linked to the presence of ESRs in the male reproductive tract. ESRs are present early in fetal development and during postnatal development and are persistent throughout life. ESRs are present in the genital ridges, Wolffian ducts, and other accessory organs of the male reproductive organ and are targets of E2. Perinatal development of distinct male neuroanatomical circuits, neuroendocrine pathways, and reproductive behavior are mediated by E2 via the

aromatase conversion of testosterone in the brain. The anteroventral periventricular nucleus (AVPN) of the hypothalamus contains multiple sexually dimorphic cell types that express ESRs. The actions of E2 on these receptors lead to masculinization of the AVPN nucleus of the hypothalamus, which is involved in the regulation of reproductive behavior. Other functions of E2 in the male include paracrine modulation of Leydig cells and Sertoli cells, regulation of seminiferous tubule luminal fluid, iron transport, feedback inhibition of the kisspeptin neurons, hypothalamic GnRH neurons, and the anterior pituitary gonadotrophs [107–110].

1.6.1 Estrogen Receptors

Three types of ESRs have been characterized. The first is ESR1(ESRα), and it is encoded by the ESR1 gene located on chromosome 6 (6q25.1). The second is termed ESR2 (ESRβ) and encoded by the ESR2 gene located on chromosome 14 (14q23-24). ESR1 and ESR2 are members of the nuclear hormone superfamily of transcription regulators. Both are composed of similar functional domains. The principal functional domains are termed A/B, C, D, and E/F and are present in both ESR1 and ESR2. A/B region is the amino-terminal (NTD) and is involved in gene transcription transactivation. The C region is the DNA binding domain (DBD) and contributes to estrogen receptor dimerization. The D domain serves as the hinge connecting the C and E domains and is capable of binding to chaperon proteins. Additionally, the D domain contains the nuclear localization signal allowing for receptor-ligand complex translocation to the nucleus. The E/F domain is the carboxyl-terminal serving as the ligand-binding domain as well as sites for coactivators and corepressors binding. Within the NTD and the DBD are two additional regulators of estrogen transcriptional activities known as activational` function domains (AF1 and AF2). The third type of ESR is a recently characterized G-coupled protein receptor known as GPR30. GPR30

is encoded by a gene located on chromosome 7 (7p22.3). Structurally, GPR30 consists of 7 transmembrane α-helical regions, 4 extracellular segments, and 4 cytosolic segments. GPR30 has a low receptor binding affinity compared to ESR1 and ESR2.

Estrogen signaling through ESRs is similar to androgen signaling (i.e., classical and non-classical signaling pathways). Estrogen may gain entry to the plasma membrane and interact with ESR1 and ESR2 to exert direct effects by binding to DNA sequences known as estrogen response elements (EREs). Estrogen may also activate intracellular signaling cascades through interaction with GPR30, and/or ESR1 and ESR2. Thus, estrogen-mediated signaling is also classified into genomic and non-genomic pathways.

1.7.0 Spermatogenesis is an Androgen-dependent Process

Spermatogenesis is the temporal event whereby germ cells in the seminiferous epithelium undergo differentiation and mature into spermatozoa. The process is characterized by mitotic proliferation of the spermatogonial pool, meiotic division of spermatocytes, and post-meiotic modification of spermatids. Once sex determination has occurred in the fetal testis, Sertoli cell sequester spermatogonia stem cells away from other cells of the testis. A rapid but brief period of proliferation of the spermatogonia stem cell occurs in utero, following which they become mitotically quiescent until after birth. NOTCH signaling in Sertoli cells is responsible for this quiescent period [78]. A significant amount of spermatogonia stem cells is lost during in utero proliferation. For example, spermatogonia stem cells that inappropriately express markers of meiotic cell division are eliminated by a process of apoptotic cell death.

In rodents, the spermatogonial stem cells re-enter the cell cycle at approximately postnatal days 1-2 and become spermatogonia by postnatal days 3-4. In humans, re-entry occurs

immediately after birth. However, differentiated spermatogonia are not recognizable until about 3-4 months after birth. Re-entry into mitotic cell division is controlled by the chromatin-modifying protein Swi-independent 3A (SIN3A) [111]. Two waves of the mitotic proliferation of the spermatogonia are initiated in succession following re-entry. In both humans and rodents, the first wave is characterized by the production of heterogeneous populations of spermatogonial (undifferentiated and differentiated type A spermatogonia). The undifferentiated type A spermatogonia are for the purpose of self-renewal. This pool of type A undifferentiated spermatogonia also supports steady-state spermatogenesis throughout life. The earliest recognizable differentiated type A spermatogonial in rodents is called type A1. It is usually observed at about PND 10. Type A1 spermatogonia mitotically divide into type A2, A3, and A4. The second wave of mitotic proliferation is not for self-renewal but represents the initiation of spermatogenesis. Here some of type A4 spermatogonia differentiate to intermediate spermatogonia, which subsequently divide once giving rise to type B spermatogonia.

In humans, the heterogeneous group of spermatogonia from the first wave of mitotic division are identified as type A_{dark} and type A_{pale} spermatogonia. The type A_{dark} represents the undifferentiated spermatogonia and serves as reserve stem cells in the testis, whereas the A_{pale} are the differentiated spermatogonia. The A_{pale} spermatogonia can divide and either give rise to new A_{pale} or further differentiate giving rise to type B spermatogonia.

Proliferation and maturation associated with the transition of type A spermatogonia involve germ cell migration from the basement membrane to the adluminal compartment of seminiferous tubules via attachment to Sertoli cells. In addition to the loos of gonocytes that occurs in-utero, a significant amount of germ cells is lost to apoptosis during neonatal differentiation of the

spermatogonia. Only about 39% of the expected numbers of germ cells survive at the completion of the two waves of mitotic cell division.

In both rodents and humans, the type B cells undergo mitotic division once giving rise to preleptotene spermatocytes. Preleptotene spermatocytes break through the blood-testis-barrier, moving from the basement membrane to the adluminal compartment of the seminiferous tubules. Here within the adluminal compartment, developing spermatocytes are in a protected micro-environment receiving nourishment from Sertoli cells. At this stage of spermatogenesis, a high level of testosterone concentration is required for the expression of junctional proteins (occludin, claudin 11, and claudin 3). These proteins are needed for the remodeling of the blood-testis-barrier following the recruitment of the preleptotene spermatocytes to the adluminal compartment.

The preleptotene spermatocytes represent the stage of premeiotic cell division during which DNA replication occurs. DNA replication signals the onsets of the first meiotic cell division during spermatogenesis. At the completion of DNA replication, the cells are tetraploid and are called primary spermatocytes. All-trans retinoic acid is critical to the initiation of this stage of spermatogenesis. Downregulation of Cyp26b1 enzyme caused reduced degradation of all-trans retinoic acid. Signal transduction from increased levels of all-trans retinoic acid induces primary spermatocytes expressing the all-trans retinoic acid receptor to commence the first meiotic cell division. Recruited primary spermatocytes undergo meiotic division during which the defining events of meiosis occur, namely alignment (pairing) and synapsis (intimate attachment) of the homologous chromosomes, and genetic recombination. The subset of recombination events called crossovers, occurs at least once on every chromosome pair, leading to an infinite number of potential chromosome constitutions in each of the potential spermatid products. At the end of the first meiotic division, secondary spermatocytes are formed, which are diploid in genetic materials.

Almost immediately after the completion of the first meiotic division, the secondary spermatocytes resume the second meiotic division, during which nuclear materials are separated and shared equally among the daughter cells. The products of the second meiotic division are haploid cells referred to as spermatids. The first meiotic division is critically dependent on androgens. Members of the transforming growth factor-beta (TGF-β) superfamily, such as the activins, inhibins, and PDGFs secreted by Sertoli cells contribute to the regulation of this phase of spermatogenesis.

Spermatids from the second meiotic cell division undergo post-meiotic modification before they are released into the lumen of the seminiferous tubule. This modification is termed spermiogenesis. The earliest modification is the gradual morphological transformation from round spermatid to elongating spermatid and biogenesis of acrosome as the cells migrate towards the lumen of the seminiferous tubules. Notable changes during spermiogenesis include the condensation and elongation of the nucleus, as well as the formation of the flagellum. During spermiogenesis, non-essential cellular organelles are packaged into cytoplasmic droplets. Spermiogenesis enhances the morphological appearance of the spermatozoa as well as its release from the Sertoli cell to the lumen of the seminiferous tubules.

Regulation of the process of spermatogenesis is not limited to locally secreted hormones and factors in the testis. Gonadotrophic hormones from the pituitary glands are involved in regulating spermatogenesis. Luteinizing hormone stimulates Leydig cells to secrete testosterone while follicle-stimulating hormone plays a role in the regulation of Sertoli cells regulation. In the absence of luteinizing hormone stimulation of Leydig cells to increase intratesticular testosterone concentration, proliferation and maturation of germ cells do not proceed normally. Gonocytes are lost, type A spermatogonia proliferation and entry of type B spermatogonial to the preleptotene stage are impacted, meiosis is halted at the pachytene stage of division and the release of

spermatids to the seminiferous tubule is disrupted. Follicle-stimulating hormone action on Sertoli cell regulates DNA synthesis in type B spermatogonia and preleptotene spermatocytes as well as preventing apoptosis in secondary spermatocytes and round spermatids. N-cadherin, an important adhesional molecule produced by the Sertoli cells is needed for normal tight junctional structure. In the absence of FSH and high levels of intratesticular testosterone, N-cadherin is not secreted by Sertoli cells leading to a poor tight junctional formation. Thus, the roles of the pituitary gland gonadotropic hormones under the influence of hypothalamic control are essential in spermatogenesis.

1.8.0 Neuroendocrine regulation of the male reproductive axis

The process of differentiation, maturation, and optimal functioning of the male reproductive system is tightly regulated by neuroendocrine activities of the hypothalamic-pituitary-gonadal (HPG) axis (**Fig. 10)**. The hypothalamic-pituitary-gonadal axis develops embryonically but, apart from the brief period of activation immediately after birth, its activities remain largely suppressed. The GnRH neurons in the hypothalamus are central to reproduction. GnRH neurons have cell bodies scattered within the hypothalamus but the axons project to the external layer of the median eminence, where GnRH is released near the hypophyseal portal vessels. Diffusion of GnRH into the anterior pituitary allows GnRH to bind to its receptor on the pituitary gland. Signal transduction initiated by the GnRH stimulates the synthesis and pulsatile release of FSH and LH by the gonadotrophs. FSH and LH in turn, act on the testis. Specifically, FSH and LH actions on the testis cooperatively promote testicular development, spermatogenesis, and steroidogenesis.

Locally synthesized testosterone and estradiol in the testis feedback to control the action of the anterior pituitary gland and the GnRH-producing neuron of the hypothalamus. A short loop of feedback control by estradiol within the testis also contributes to the regulation of the gonadal function. This short loop is mediated by the estradiol effect on Leydig cells through ESR1 activation. Both inhibin and AMH in addition to their paracrine action in the testis act on the pituitary gland to inhibit the production of FSH and LH. Recent findings have indicated that a significant subset of GnRH neurons both in rodents and humans express the AMH receptor and that AMH potently interacts with GnRH neuronal activity to directly inhibit the release of GnRH [112].

Furthermore, the control of reproduction is linked with the kisspeptin system of neurons. This is particularly important because the kisspeptin system is involved in the initiation of puberty and control of fertility through the stimulation of GnRH neurons. The Kisspeptin system consists of kisspeptin neurons and the specialized neurons secreting neurokinin B and dynorphin (KNDy neurons) [113]. The kisspeptin and KNDy neurons are in the rostral periventricular area of the third ventricle (RP3V) in rodents and are located in the preoptic area (POA) and arcuate nucleus of the hypothalamus in humans (ARC). These neurons also express receptors for gonadal steroid hormones, including those of estrogens and androgens (i.e., ESR1, AR). Thus, both testosterone and estrogen can regulate the kisspeptin system. Kisspeptin receptor is a G-coupled receptor known as GPR54 and is present on GnRH neurons. The binding of Kisspeptin to its receptor on the GnRH stimulates the release of GnRH, which in turn control pituitary gland release of FSH and LH. Thus, the kisspeptin system and the feedback control mediated by estrogen and testosterone converge on the GnRH neuron to complete the neuroendocrine regulation of reproduction.

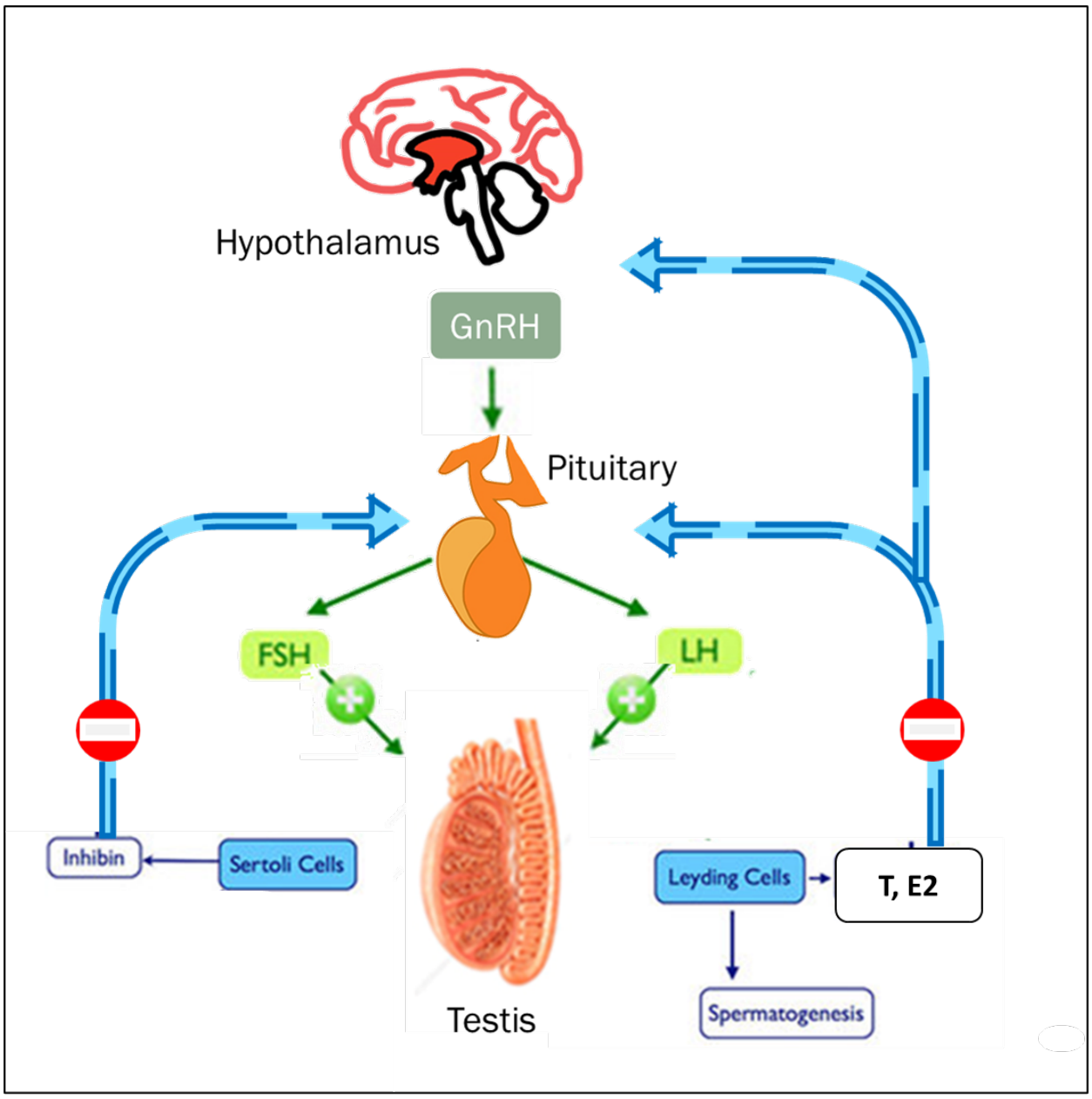

Figure 10: The hypothalamic-pituitary-gonadal axis. kisspeptin (not shown) and gonadotrophin-releasing hormone (GnRH) from the hypothalamus, LH, and FSH from the pituitary are responsible for the positive feedforward regulation of the testis. In contrast, the testis-produced hormones such as testosterone and estradiol constitute the negative feedback loop of the HPG-axis.

1.9.0 Statement of research objectives

Xenoestrogens resemble the natural estrogen in both function and structure and are capable of interacting with estrogen receptors. The affinity of xenoestrogens for ESRs are weak and are 100-1000 fold lower when compared to the activity of E2 [114]. Naturally occurring xenoestrogens such as genistein and synthetic compounds such as BPA induce tissue-specific estrogenic responses as ESRs agonists or antagonists, resulting in dysregulation of ERE-dependent transcription signaling pathways[115]. The activities of xenoestrogens are not limited to ESRs but are extended to other steroid hormone receptors such as the androgen receptor [116]. Although most xenoestrogens act via intracytoplasmic localized superfamily group of steroid hormone receptors, they are also capable of acting via localized membrane receptors [117]. Xenoestrogens are therefore capable of acting via the classical and non-classical pathways of steroid hormone signaling pathways to effect disruption in intracellular homeostasis, resulting in temporary and permanent changes affecting endocrine, reproductive, and developmental activities.

The effects of xenoestrogens on the endocrine and reproductive axis are particularly concerning in male individuals. This is because of the reports of a general decline in male reproductive health that has been linked to exposure to xenoestrogens. For example, among men with idiopathic infertility, high urinary daidzein and genistein concentrations (> 50 μg/g creatinine) were associated with low sperm counts [118]. Similarly, animal studies demonstrated that both endocrine and exocrine functions of testes are subject to regulation by soy isoflavones. Exposure of pregnant female mice to genistein at concentrations similar to human exposure levels (10 ηM) suppressed androgen secretion in male offspring [119]. Subcutaneous administration of genistein at 4 mg/kg/day to neonatal rats for 16 days stimulated germ cell development [120], and long-term genistein treatment decreased spermatogenesis in adult male rats [121]. It has also been

reported that feeding of soy-based diets to pregnant female rats from gestational day 12 to postnatal day 21 induced Leydig cell proliferation in testes early in development and suppressed steroid hormone secretion in adult male offspring [122–124].

Disruption of reproductive capacity is by no means peculiar to the naturally occurring estrogenic compounds. For example, it has been reported that perinatal exposure to BPA at environmentally relevant concentration decrease intratesticular testosterone concentration by suppressing Leydig cell testosterone secretion in adult male rats[125]. Gavage administration of BPS at 50 μg/kg/d for 28 days caused a decrease in daily sperm production and an increase in sperm DNA damage in adult rats [126]. Also, perinatal exposure of EE2 at 5 μg/kg body weight from prenatal day 7 to postnatal day 18 reduced the weight of the testis and seminal vesicle in adult male rats, while exposure at a similar duration but a greater dose of 50 ug/kg reduced sperm production [68]. Subcutaneous administration of EE2 at 1 and 10 μg/kg on alternate days in the first 6 days of life caused penile malformation, reduction in weight of testis, seminal vesicle, epididymis, epididymal fat pad, and epididymal sperm numbers in adult male rats [127]

Despite these detrimental effects of xenoestrogens on the male reproductive axis, the general population is continually exposed to xenoestrogens either in natural or synthetic forms. This is because a lot remains unknown about the mechanism underlying the effects of xenoestrogens on the male reproductive axis. Furthermore, most of the studies on the effects of xenoestrogens focused on exposures occurring continuously through gestation, lactation, and the post-weaning period, making it difficult to isolate specific effects associated with the different developmental periods (neonatal, prepubertal, pubertal, and adult stages of development). In other words, information on age-related differences in isoflavone exposure effects is limited. Additionally, the National Toxicology Board review of isoflavones noted that most studies on

dietary isoflavones had mainly focused on genistein without much consideration for the effects of daidzein [17]. Yet, daidzein constitutes greater than 40% of isoflavones in soy-isoflavones.

Moreover, a large body of evidence has shown the detrimental effects of BPA on male reproduction. But these reports are laden with many inconsistencies due in part to the different exposure paradigms and varied concentrations of BPA used in experiments. However, there is a consensus that the safety of BPA in pregnancy and infancy is not guaranteed because of the susceptibility of this period of development to toxicity. Thus, there is a restriction in the use of BPA in infant-related products, but not in prepubertal or pubertal children. This problem is further compounded by the replacement of BPA with BPS in the so-called BPA- free "safe" products. The safety profile of BPS is still not known. Additionally, EE2, as the most estrogenic emerging contaminant of drinking water has been extensively investigated in aquatic animals and female mammals with little attention paid to the males, constituting a gap of knowledge on EE2 effects in exposed males.

To address these problems (i.e., age-related effects of isoflavone exposure, the paucity of information on individual effects of daidzein and genistein, and the safety of BPA, BPS, and EE2 in the developing male gonad), we performed experiments to investigate the effects of dietary soy-isoflavones exposure in testicular cells at distinct stages of development, assess individual effects of soy-isoflavones (genistein and daidzein) on testicular cells, and compared the effects of BPA, BPS and EE2 in testes of developing rats with a central hypothesis that "xenoestrogens disrupt testicular function and development in male rats". Altogether, our objectives were to determine:

1. Whether effects associated with soy-based diets are due to isoflavones and whether the effects are influenced by age at exposure

2. Whether isoflavone effects in the testis are due to daidzein, genistein, or both compounds acting together
3. The effects of BPA, BPS, and EE2 on steroid hormone secretion in the developing male rat gonad.

Chapter 2

Effects of Exposure to Soy-isoflavones in Testis of Male Rats

2.0.0 Abstract

Soy-based foods are consumed for their health beneficial effects, implying that the population is exposed to soy isoflavones in the diet. Information on age-related differences in isoflavone exposure effects is limited. Most studies focused on isoflavone exposures occurring continuously through gestation, lactation, and the post-weaning period, making it difficult to isolate specific effects associated with different developmental periods. To address this omission, male rats at 21 (prepubertal stage), 35 (pubertal stage), and 75 (adult stage) days of age were maintained either on a casein control diet, soybean meal (SBM), or control diet supplemented with daidzin and genistin (G+D) for 14 days. Feeding of SBM and G+D diets decreased testicular testosterone (T) secretion regardless of age. Altered androgen secretion was due to decreased ($p<0.05$) Star and Hsd17β protein in the testes and were associated with increased ($p<0.05$) Lhβ and Fshβ subunit protein expression in pituitary glands. Taken together, our results demonstrated that disruptions in testicular cell function are independent of age at exposure. Further studies are required to determine the differential effects of isoflavones on testicular functions.

2.1.0 Introduction

Soy-based food products are consumed worldwide due to their putative nutritional and health beneficial effects. Raw soybeans typically contain 36.5% protein, 19.9% lipids, low cholesterol, high unsaturated fat, 9.3% dietary fiber and a small amount of isoflavones (<1%) [128]. Isoflavones (also called phytoestrogens or phytochemicals) are non-steroidal compounds, which are abundant in soybeans and other legumes [129]. Interestingly, isoflavones are similar in structure to and mimic the female hormone 17β-estradiol [130] with the capacity to interfere with the function of the endocrine axis. The most abundant isoflavones in soybeans are present as β-glycoside conjugates: 50-55% genistin, 40-45% daidzin, and less than 5% glycetin [29,131]. Genistin, daidzin, and glycetin are hydrolyzed in the gastrointestinal tract to their genistein, daidzein, and glycetein aglycones while daidzein is further broken down to equol in about a third of the population [132,133].

Importantly, individuals with lactose intolerance consume soymilk as alternatives to lactose-containing milk while vegetarians substitute soy protein for meat. Consumption of soy-based products was linked to the prevention of cancer [134–136] and other chronic diseases [137,138]. Furthermore, the American Heart Association recommends the consumption of soy-based food products for their low cholesterol content [139] while the USDA has endorsed the use of soy-based protein as an alternative to animal protein in lunch programs for preschoolers, and young and adolescent age groups [140,141]. Of particular concern are risks associated with the use of hormone-supplemented rations in livestock production [142] and the feeding of several food products containing soy products, other phytochemicals and dietary estrogens [143]. The increasing popularity of soy food products has raised public concerns regarding exposure of the population to hormonally active compounds [144]. The most vulnerable groups are developing fetuses and infants exposed *in utero* and/or during lactation and feeding of soy-based infant formulas. For example,

approximately 25% infants in the United States each year are raised on soy-based formulas [17,145] which may contain high isoflavone concentrations in the micromolar range and achieve blood concentrations of 300-600 ηM [146]. The mean daily consumption of isoflavones in four-month-old infants maintained exclusively on soy-based infant formulas was estimated at 6-9 mg⁄kg of body weight, resulting in blood isoflavone concentrations of about 980 µg⁄L which is much higher than measured in infants fed cow's milk formulas or human breastmilk (9.4 and 4.7 µg⁄L) [147,148].

Isoflavone effects were investigated in the male reproductive tract due to their high expression levels of steroid hormone receptors [149–151]. Epidemiological studies are few but a study of adult Chinese men with idiopathic infertility showed that adult men with high urinary daidzein and genistein concentrations (> 50 µg/g creatinine) had lower sperm counts in the range 38.5 – 118.4 $\times 10^6$/mL compared to individuals with lower urinary daidzein and genistein levels (< 50 µg/g creatinine) and sperm counts between 48.8 and 167.4 $\times 10^6$/mL [118]. Similarly, animal studies demonstrated that both endocrine and exocrine functions of testes are subject to regulation by soy isoflavones. Exposure of pregnant female mice to genistein at concentrations similar to human exposure levels (10 ηM) suppressed androgen secretion in male offspring [119]. Subcutaneous administration of genistein at 2.5 mg/kg/body weight/day for a period of nine days also decreased both serum and testicular androgen concentrations in adult male mice [152]. Subcutaneous administration of genistein at 4 mg/kg/day to neonatal rats for 16 days stimulated germ cell development [120] but long-term genistein treatment decreased spermatogenesis in adult male rats [121]. We observed that feeding of soy-based diets to pregnant female rats from gestational day 12 to postnatal day 21 induced Leydig cell proliferation in testes early in development and suppressed steroid hormone secretion in adult male offspring [122–124]. Results of our i*n vitro* assays showed that genistein treatment disrupted coupling of luteinizing hormone receptors (Lhr) to G proteins in

Leydig cells and diminished LH stimulation of androgen biosynthesis [153]. Isoflavones also have the capacity to impact neuroendocrine function. For example, subcutaneous administration of 250 μg of genistein at 12-hour intervals in the first 48 hours postpartum increased the number of tyrosine hydroxylase neurons in the anteroventral periventricular nucleus of the hypothalamus (AVPV) and disrupted sexual differentiation in male rats [154]. Similarly, daily administration of a high genistein dose (e.g., 1000 μg) in the first 10 days postpartum decreased Lh secretion in response to Gnrh stimulation in pubertal male rats, but a smaller dose (e.g., 100 μg) caused the opposite effect, i.e., increased Lh secretion in response to Gnrh stimulation [155]. Administration of equol to adult male rats at 100 mg/kg/bodyweight for five days upregulated expression of both Esr1 and truncated estrogen receptor product 1 (TERP1), an estrogen-induced specific Esr1 isoform that suppresses ligand-activated ESRs in the pituitary gland [156]. Overall, the inconsistencies in observations from different laboratories demonstrate that dose-response relationships of the two isoflavones are not always linear, and may be biphasic or attain a plateau for many biological endpoints [157–159].

Our laboratory is interested in the regulation of androgen secretion and gonadal function. Androgens are important for male reproductive development both *in utero* and during postnatal development [160–163]. The primary androgen testosterone (T) is produced predominantly by Leydig cells under the direct control of pituitary Lh[164] which is released in response to stimulation by Gnrh. Acting in concert with Sertoli cell-secreted factors, e.g., Anti-Müllerian hormone and inhibin Bβ, sex steroids feedback to the pituitary gland and hypothalamus and control Lh and Gnrh release [165–167]. Thus, isoflavones may act directly in testicular cells and/or perturb the entire HPG axis to regulate Gnrh and gonadotropin secretion [155,156,168].

Information on age-related differences in isoflavone exposure effects is limited [17]. Most studies focused on isoflavone exposures occurring continuously through gestation, lactation, and the post-weaning period, making it difficult to isolate specific effects associated with different developmental periods. To address this knowledge gap, we used prepubertal, pubertal, and adult exposure paradigms to assess whether dietary isoflavone effects on testicular development and function are influenced by age at exposure.

2.2.0 Materials and Methods

2.2.1 Chemicals

Genistein and daidzein were obtained from Indofine Chemical Company (Hillsborough, NJ). Trypsin inhibitor, EDTA, HEPES, BSA, bovine lipoprotein, sodium bicarbonate ($NaHCO_3$), DMEM nutrient mixture [Ham's F-12 (DMEM/F-12; 1:1 mixture without phenol red)], albumin, Percoll, etiocholan-3β-ol-17-one, and gentamicin were purchased from Sigma Chemical Company (St. Louis, MO). Dulbecco's PBS, medium 199, and 10 × Hanks' balanced salt solution were obtained from Life Technologies, Inc. (Grand Island, NY). Collagenase, dispase, and deoxyribonuclease (DNase) were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Ovine luteinizing hormone (LH) was provided by the National Hormone and Pituitary Program (NIDDK, Bethesda, MD).

2.2.2 Animal Studies

All animal and euthanasia procedures were performed in accordance with a protocol approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) based on recommendations of the panel on Euthanasia of the American Veterinary Medical Association. We performed experiments to determine whether altered sex hormone secretion by testis was due

specifically to isoflavone action and whether these effects are influenced by age at exposure. Male Long-Evans rats at different ages: [postnatal days (PND) 21 (n=45), 35 (n=36) and 75 (n=18)], were obtained from Harlan-Teklad, (Madison WI). Animals were allowed to acclimatize for three days at the College of Veterinary Medicine Division of Laboratory Animal Health Housing (DLAH) Facility. Depending on size, animals were placed in groups of 1-3 per cage (length, 0.47 m; width, 0.25 m; height, 0.22 m) (Snyder Manufacturing Company; Centennial, CO). Water was provided in glass water bottles *ad libitum*. The housing of animals in plastic cages and the use of glass bottles were designed to minimize background exposure to estrogens as may occur with resin-containing cages [17]. Animals were maintained under constant conditions of light (12L: 12D) and temperature (20–23.38C) with free access to pelleted food. There were three experimental diets: 1) casein control; 2) soybean diet (SBM); and, 3) control diet supplemented with daidzin and genistin (G+D). The SBM diet was formulated to contain 300 parts per million (ppm) genistein and 200 ppm daidzein based on the natural content of genistin and daidzin in soybeans as determined by the manufacturer (Harlan-Teklad, Madison WI). The G+D diet was formulated to contain genistein and daidzein at 300 ppm and 200 ppm. All diets (control, SBM, and G+D) were similar in the total content of protein, carbohydrates, fat, energy, and micronutrients (Harlan-Teklad, Madison WI) (**Table 3**). Animals within each age group were randomized by weight, constituted into three groups, and maintained on the appropriate diet for a period of 14 days, i.e., PND 21-35 (prepubertal exposure), PND 35-49 (pubertal exposure), and PND 75-89 (adult exposure).

Table 3: Composition of experimental diets

Contents/Diets	Control (Casein)	SBM	G+D
Daidzein	---	---	200 PPM
Genistein	---	---	300 PPM
Soybeans	---	+	---
Protein (%)	19.0	18.9	19.0
Carbohydrate (%)	49.0	47.5	49.0
Fat (%)	5.4	5.5	5.4
Total calories (Kcal/g)	3.2	3.2	3.2

Abbreviations: SBM=soybean diet, Genistein + Daidzein= G+D

2.2.3 Procedure for isolation of Leydig cells

Animals were killed by CO_2 asphyxiation after which testes were collected and digested in a dissociation buffer containing 0.25 mg/ml collagenase, 46 µg/ml dispase, and 6 µg/ml DNase for 1 h in a shaking water bath at 34°C. Seminiferous tubules from immature testis were removed by passing testicular fractions through a nylon mesh with a pore size of 0.2 µm (Spectrum Laboratories, New Brunswick NJ). The supernatant was centrifuged at 2500 rpm for 15 min at 4°C. Seminiferous tubules obtained from adult testes were removed by gravity sedimentation in dissociation buffer containing 10 mg/ml bovine serum albumin (BSA). In all cases, cell fractions were loaded onto a Percoll gradient (Sigma-Aldrich) and centrifuged at 13500 rpm for 60 min at 4°C. Leydig cells were isolated from the Percoll gradient based on density, i.e., progenitor Leydig

cells at 1.063 (21 days), immature Leydig cells (35 days) at 1.069-1.073, and adult Leydig cells (75 days) at 1.073-1.096 [169–171]. Leydig cell numbers were estimated using a hemocytometer. The purity of Leydig cell fractions was assessed by histochemical staining for 3βHSD using 0.4 mM etiocholan-3β-ol-17-one as the enzyme-substrate (Sigma-Aldrich).

2.2.4 Measurement of Steroid Hormones and Isoflavones

Serum was separated from trunk blood collected at sacrifice. Testicular explants (~100 mg) and aliquots of Leydig cells ($0.1–0.2 \times 10^6$) were incubated in microcentrifuge tubes containing DMEM/F12 culture medium buffered with 14 mm $NaHCO_3$, 15 mm HEPES, 0.1% BSA, and 0.5 mg/mL bovine lipoprotein. Incubations were conducted without (basal) and with a maximally stimulating dose of ovine LH (100 ng/mL; LH-stimulated) for 3 h at 34°C. Steroid hormone concentrations [T, 17β-estradiol (E2)] were assayed in aliquots of serum and spent media using a tritium-based RIA with an inter-assay variation of 7%–8% [172]. Hormone production was `normalized to nanogram per testicular mass (milligrams) and 10^6 Leydig cells. The concentrations of genistein and daidzein in serum were measured by Ultra performance liquid chromatography (UPLC) with mass spectrometry detection using modifications of previously reported methods [122,173,174].

2.2.5 Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis and Western Blot Analysis

We analyzed the expression of several proteins in testes (Amh, inhibin Bβ, Esr1, Ar, Star, Hsd17β) and pituitary glands (Lhβ, Fshβ, Esr1, Ar). Tissues were homogenized in T-PER lysis buffer (Pierce Biotechnology, Rockford, IL) that was freshly supplemented with a protease inhibitor cocktail (Catalog #78410; Pierce Biotechnology). Tubes were centrifuged at 3000 rpm

for 14 minutes at 4°C to remove cellular debris. Protein concentrations were determined using the Bio-Rad protein assay with BSA as standard (Bio-Rad Laboratories). Aliquots (50 μL) of whole-cell lysates were dissolved in an equal volume of Laemmli buffer containing 5% β-mercaptoethanol and were boiled for 5 min at 95°C. All samples were resolved on varying percentages of Tris-HCl acrylamide gels by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Catalog #1620147; Bio-Rad Laboratories) and subsequently incubated in blocking buffer (5% whole milk in 0.1% Tween 20 PBS) for 1 h at room temperature to reduce nonspecific binding by antibodies. Membranes were then incubated in a blocking buffer containing appropriate primary antibodies overnight at 4°C. Parameters of primary antibodies used in the present study are provided in **Table 4**. On the next day, blots were washed three times in 0.1% Tween 20 PBS to remove any unbound primary antibody before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Afterward, membranes were washed four times with 0.1% Tween 20 PBS and then scanned using a LI-COR Odyssey Infrared Scanner (Lincoln, NE). All protein measurements were normalized to β-actin.

2.2.6 Statistical Analysis

Data are presented as the mean ± SEM. Within each age group, data were analyzed by one-way ANOVA followed by Dunnett's test for multiple group comparisons, except for serum isoflavones concentration which were analyzed by independent t-test (GraphPad Prism software. San Diego. Ca). Differences of ≤0.05 were considered to be significant.

Table 4. Antibody Table

Target	Antibody sequence	Name of Antibody	Manufacturer, Catalog Number	Polyclonal or Monoclonal	Dilution Used
InhibinB-β	Belongs to the TGFβ superfamily of growth and differentiation factors. Genetic locus: INHBB (human) mapping to 2q14.2; inhbb (mouse) mapping to 1 E2.3	Inhibin β-B (B-9): sc-390959	Santa Cruz Biotechnologies, sc-390959 MW 45kDa	Mouse monoclonal IgG	1:1000
Amh (MIS)	Genetic locus: AMH (human) mapping to 19 p13.3; AMH (mouse) mapping to 10C1	MIS (C-20)	Santa Cruz Biotechnologies, Sc-6886 MW (AMH) 70/74 kDa	Mouse monoclonal IgG	1:2000
ESR1	Belongs to the nuclear hormone receptor family; NR3 subfamily; contains one nuclear receptor DNA-binding domain	Anti-ESR-*a* (33) antibody-ChIP grade	Abcam, Ab32063 MW (ESR*a*) 67 kDa	Rabbit monoclonal IgG	1:1000
AR	Belongs to the nuclear hormone receptor family; NR3 subfamily; contains one nuclear receptor DNA-binding domain	Anti-AR antibody (EP1535(2))	Abcam, Ab133273 MW (AR) 89 kDa	Rabbit monoclonal IgG	1:1000
LHβ	Genetic locus: LHB (human) mapping to 19q13.33; Lhb(mouse) mapping to 7B4	Lutropin-β(R-16)	Santa Cruz Biotechnologies, Sc-7824 MW (LHβ) 22 kDa	Goat polyclonal antibody IgG	1:1000
FSHβ	Genetic locus: FSHB (human) mapping to 11q14.1; Fshb (mouse) mapping to 2E3	FSHβ(C-19)	Santa Cruz Biotechnologies, Sc-7797 MW (FSHβ) 24 kDa	Goat polyclonal antibody IgG	1:5000
Dhh	Genetic locus: DHH (human) mapping to 12q13.12; Dhh (mouse) mapping to 15F1	Dhh (F9): sc-2711688	Santa Cruz Biotechnologies, sc-2711688 MW (Dhh) 42 kDa	Mouse monoclonal IgG	1:1000
StAR	StAR (human) mapping to 8p11.23; StAR (mouse) mapping to 8 A2	StAR (D2)	Santa Cruz Biotechnologies, Sc166821 MW (StAR) 30 kDa	Mouse monoclonal IgG	1:1000
Cyp11A1	Recombinant full-length protein corresponding to Human CYP11A1 aa 40-320 mapping to 10q24.32	Anti-Cyp11A1 antibody	Abcam, Ab175408 MW (Cyp11A1) 55 kDa	Rabbit polyclonal IgG	1:1000
17β HSD	Genetic locus: HSD17B1 (human) mapping to 17q21.2	17β-HSD (A5)	Santa Cruz Biotechnologies, Sc-376719 MW (17β-HSD) 35 kDa	Mouse monoclonal IgG	1:1000
ACTB	Epitope mapping at the C\ terminus of actin of human origin	Beta Actin antibody (GT5512)	GeneTex, GTX629630	Mouse monoclonal IgG	0.0002
Abbreviations: FSH = follicle stimulating hormone; ESR1 = estrogen receptor 1; AR = androgen receptor; ACTB= actin; MIS= Mullerian inhibiting substance; LH= luteinizing hormone; HSD= hydroxysteroid dehydrogenase; MW, molecular weight.					

2.3.0 Results

2.3.1 General Observations

No animal deaths were recorded in the course of this study. Maintenance of animals on experimental diets did not affect body weights. Although paired testicular weights were greater ($p<0.05$) in prepubertal rats fed the SBM diet compared to control (**Table 5**), the gonadosomatic index was similar ($p>0.05$) in all diet groups at this stage of development. Biochemical analysis showed that isoflavones were undetectable in serum from control animals. Total and conjugated genistein and daidzein were greater ($p<0.05$) in the SBM diet group of animals compared to the G+D group. However, daidzein, but not genistein, was greater ($p < 0.05$) in the SBM than in G+D animals. (n=3) (**Table 6**).

Table 5. Effects of diets on body weights and testicular sizes

Stages of development	Exposure groups	Average body weight (g)	Average paired testis weight (g)	Average testicular index (%)
Prepubertal (PND 21-35)	Control	181.3±4.0	2.2 ±0.03	1.2±0.02
	SBM	188.3±4.3	2.4 ±0.08[a]	1.3±0.02
	G+D	177.3±6.1	2.3 ±0.08	1.2±0.02
Pubertal (PND 35-49)	Control	225.8±4.6	2.2±0.12	1.0±0.06
	SBM	234.8±4.6	2.6±0.10	1.1±0.06
	G+D	234.0±6.8	2.5±0.08	1.1±0.03
Adult (PND 75-90)	Control	351.2±7.4	3.3±0.12	0.9±0.04
	SBM	356.7±8.8	3.3±0.05	0.9±0.02
	G+D	347.3±7.4	3.4±0.06	1.0±0.04

Abbreviations: SBM= soybean diet, G+D= genistein + daidzein
[a] $p = 0.0449$

Table 6. Serum concentrations of isoflavones in prepubertal male rats maintained on soy-based diets from 21-35 days of age

Diets/ Isoflavone	Genistein (ηg/mL)			Daidzein (ηg/mL)		
	Total	Free	Conjugated	Total	Free	Conjugated
Control	0.0	0.0	0.0	0.0	0.0	0.0
SBM	457.9±43.0*	9.3±2.0*	448.6±43.0*	249.6±16.0*	23.11±3.0*	226.5±13.5*
G+D	322.6±10.0	10.4±2.1	312.2±9.0	98.4±3.8	9.5±0.3	88.9±3.7

* $P < 0.05$ (Differences btw SBM vs G+D i.e., SBM_{total} vs $G+D_{total}$)

2.3.2 Effect of age on isoflavone regulation of steroid hormone secretion

Compared to control, serum, basal and LH-stimulated testicular T concentrations were generally decreased in rats from the SBM and G+D diet groups regardless of age at the time of exposure ($P<0.05$) (**Fig. 11**). A similar pattern of decreased basal and LH-stimulated Leydig cell T secretion ($P < 0.05$) was observed in the SBM and G+D diet groups compared to control (**Fig. 12**). Serum E2 concentrations were equivalent in all diet groups regardless of age (**Figs. 13A-C**), whereas basal testicular E2 concentrations were decreased ($P<0.05$) in adult animals fed SBM and the G+D diet compared to control (**Figs. 13D-F**). LH-stimulated testicular E2 concentrations were decreased in pubertal and adult animals fed the SBM and G+D diet; this effect was seen only in prepubertal animals maintained on the G+D diet (**Figs. 13G-I**). Maintenance of animals on SBM and G+D diets had no effect in Leydig cells from growing rats (**Figs. 14A, B**) but decreased ($P<0.05$) basal E2 secretion by adult Leydig cells (**Fig. 14C**) compared to control. LH-stimulated Leydig cell E2 production showed a similar pattern (**Figs. 14E, F**) but there was decreased E2 production in prepubertal animals from the G+D diet group (**Figs. 14D**). Altogether, it appears that feeding of the SBM and G+D diets decreased steroid hormone secretion to a greater extent in pubertal and adult animals than in prepubertal animals.

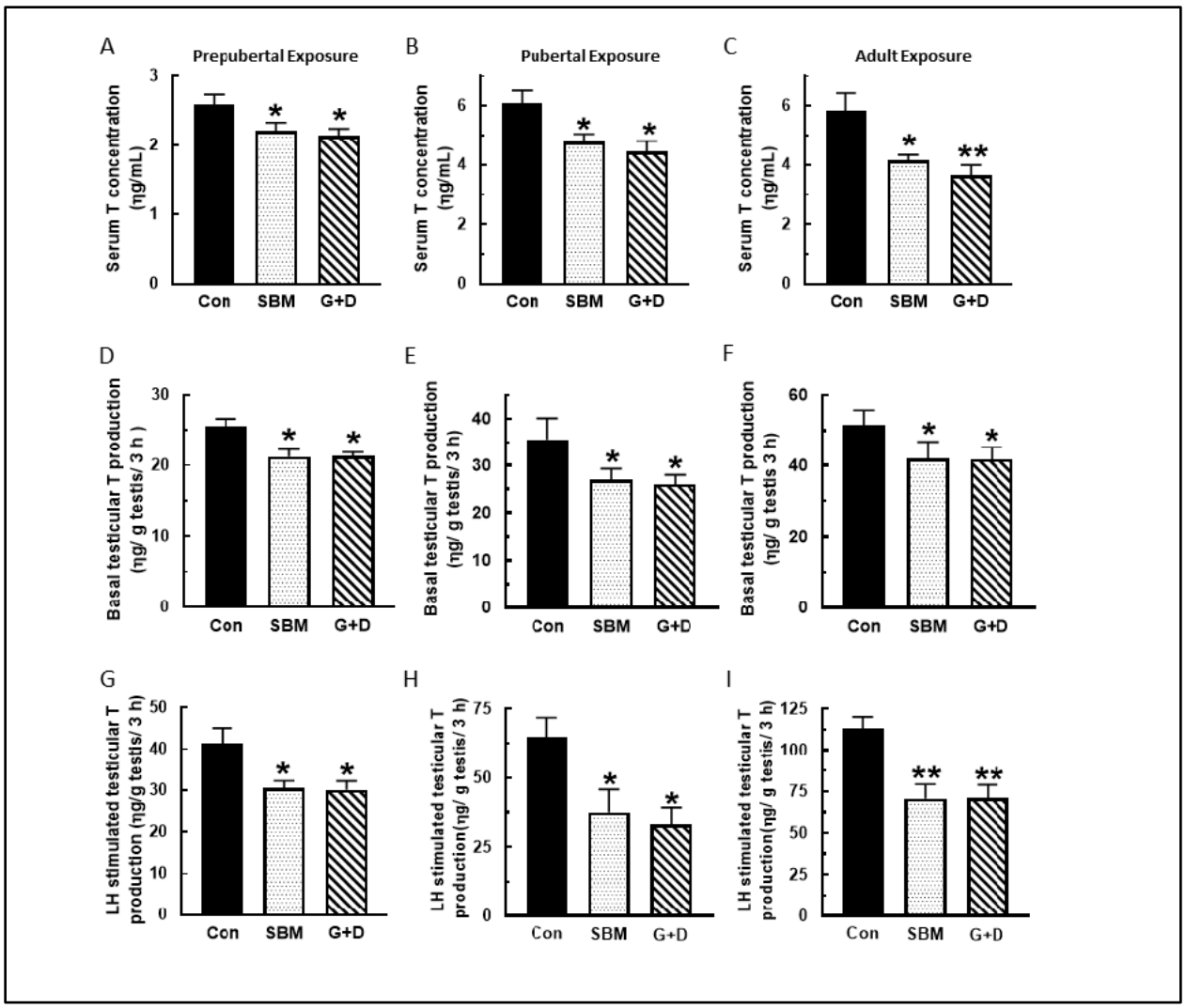

Figure 11: Effects of soy-based diets on testicular androgen secretion. Male rats at 21 (n=45), 35 (n=36) and 75 days of age (n=18) were maintained on a control diet (Con), whole soybean diet (SBM) or control diet supplemented with genistein and daidzein (G+D) for 14 days. After sacrifice, serum were separated from blood to measure testosterone (T) concentration (A, B, C). Testicular explants were obtained and incubated in DMEM/Ham's F-12 culture medium for 3 h without (D, E, F) or containing 100 ηg/ml ovine LH (NIDDK, NIH) (G, H, I). Aliquots of spent media were analyzed to measure T concentrations. Hormone concentrations were determined by RIA (*$P < 0.05$, **$P < 0.001$).

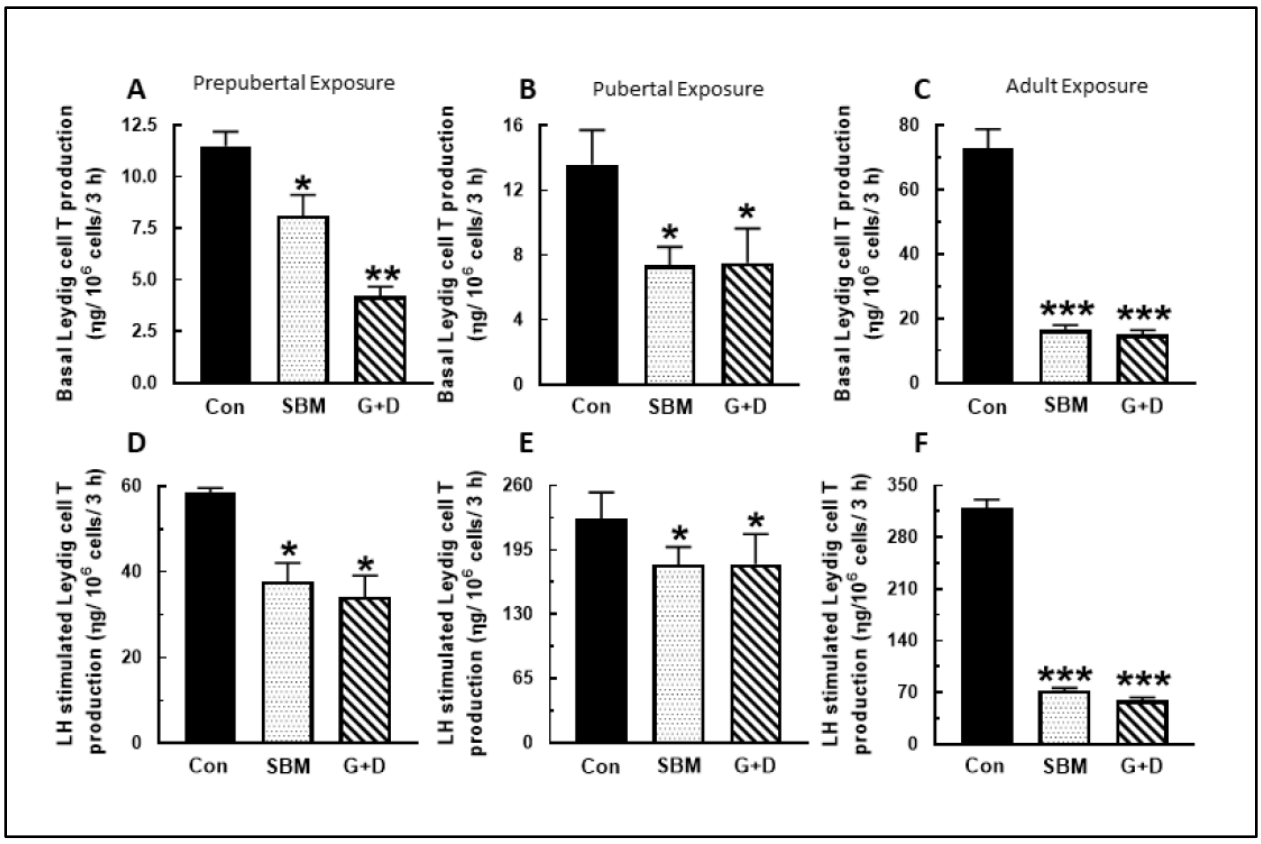

Figure 12: Effects of soy-based diets on androgen secretion by Leydig cells. Male rats at 21 (n=45), 35 (n=36), and 75 days of age (n=18) were maintained on a control diet (Con), whole soybean diet (SBM), or control diet supplemented with genistein and daidzein (G+D) for 14 days. After sacrifice, testes were pooled to isolate Leydig cells which were then incubated in DMEM/Ham's F-12 culture medium for 3 h without (A, B, C) or containing 100 ηg/ml ovine LH (NIDDK, NIH) (D, E, F). Spent media were analyzed to measure T concentrations by RIA (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

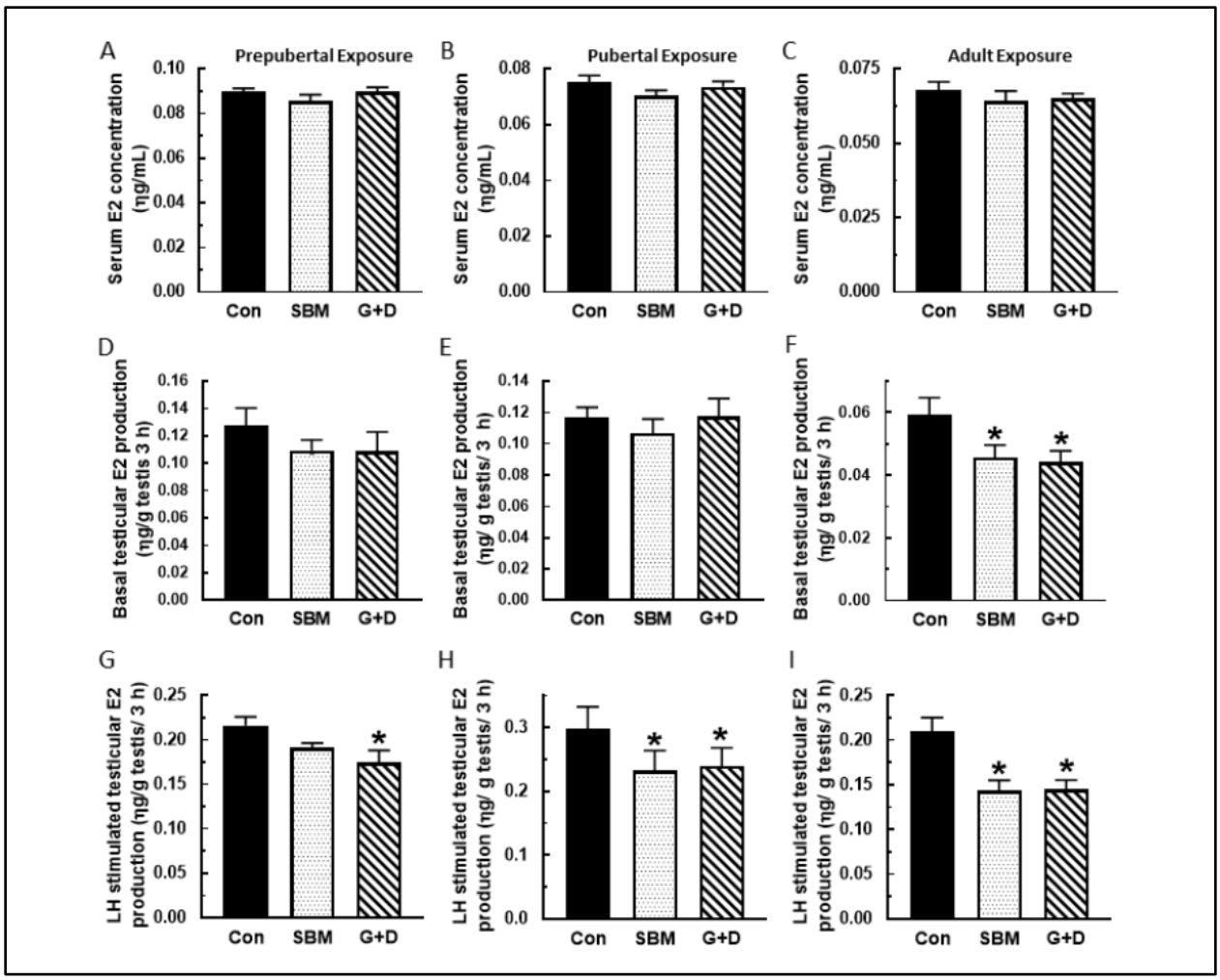

Figure 13: Effects of soy-based diets on testicular 17β-estradiol (E2) secretion. Male rats at 21 (n=45), 35 (n=36), or 75 days of age (n=18) were maintained on a control diet (Con), whole soybean diet (SBM), or control diet supplemented with genistein and daidzein (G+D) for 14 days. After sacrifice, serum were obtained to measure E2 concentration (A, B, C). Testicular explants were obtained and incubated in DMEM/Ham's F-12 culture medium for 3 h without (D, E, F) or containing 100 ηg/ml ovine LH (NIDDK, NIH) (G, H, I). Aliquots of spent media were analyzed to measure E2 concentrations by RIA (*$P < 0.05$).

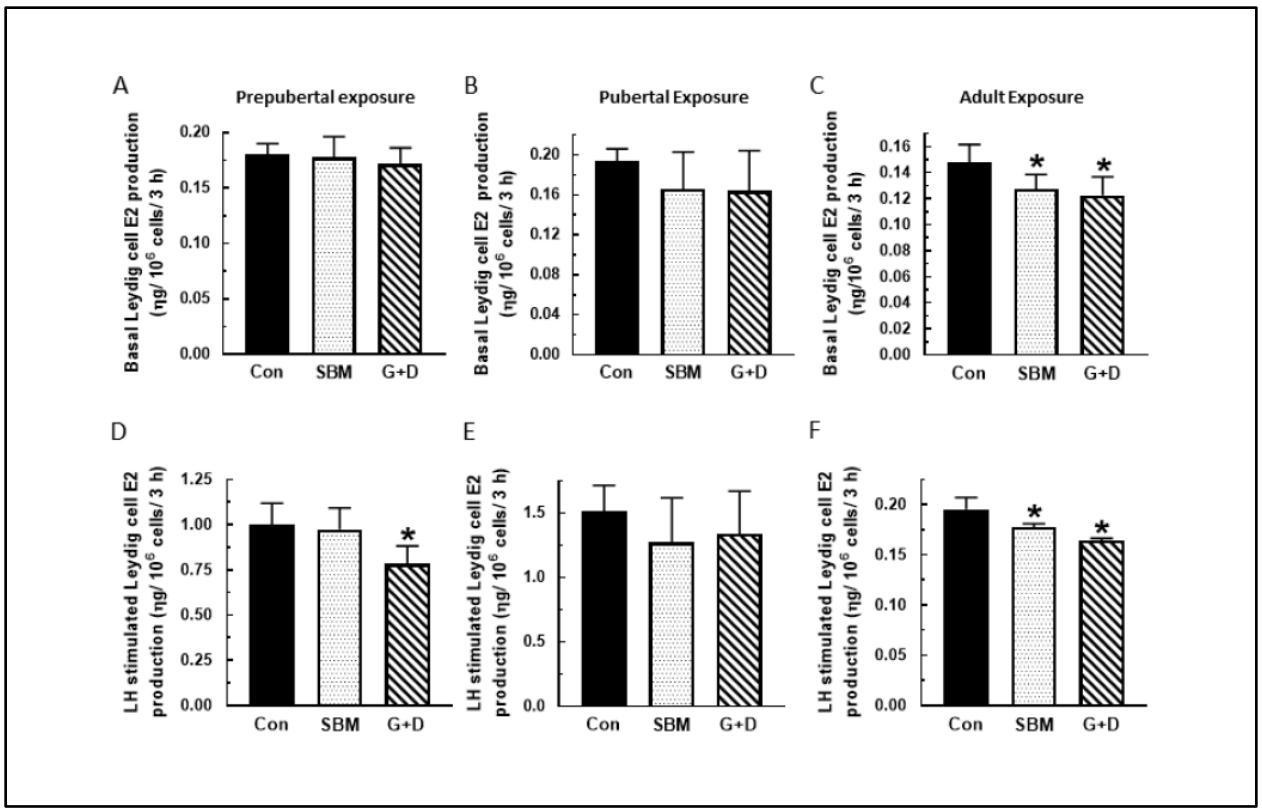

Figure 14: Effects of soy-based diets on 17β-estradiol (E2) secretion by Leydig cells. Male rats at 21 (n=45), 35 (n=36), or 75 days of age (n=18) were maintained on a control diet (Con), whole soybean (SBM), or control diet supplemented with genistein and daidzein (G+D) for 14 days. After sacrifice, testes were pooled from animals in the same group to isolate Leydig cells. Leydig cells were then incubated in DMEM/Ham's F-12 culture medium in triplicate for 3 h without (A, B, C) or containing 100 ηg/ml ovine LH (NIDDK, NIH) (D, E, F) Spent media were analyzed by RIA to measure E2 concentrations by RIA (*$P < 0.05$).

2.3.3 Isoflavone regulation of protein gene expression in the pituitary-gonadal axis

Results of western blot analysis showed that expression of Sertoli cell secreted Amh and inhibin Bβ were decreased (P<0.05) in pubertal male rats fed the SBM and G+D diet compared to control (**Figs. 15A, B**). The finding of decreased testicular Amh and inhibin Bβ protein was associated with increased (P<0.05) Lhβ and Fshβ subunit protein expression in pituitary glands of affected animals (**Figs. 15C, D**). Analysis of the steroidogenic pathway showed that Star and Hsd17β levels were decreased (P<0.05) in testes of pubertal (**Figs. 16A, B**) and adult animals (**Figs. 16C, D**) fed the SBM and G+D diet compared to control. These observations indicated that

isoflavones have the capacity to target and/or regulate specific proteins in the male neuroendocrine axis.

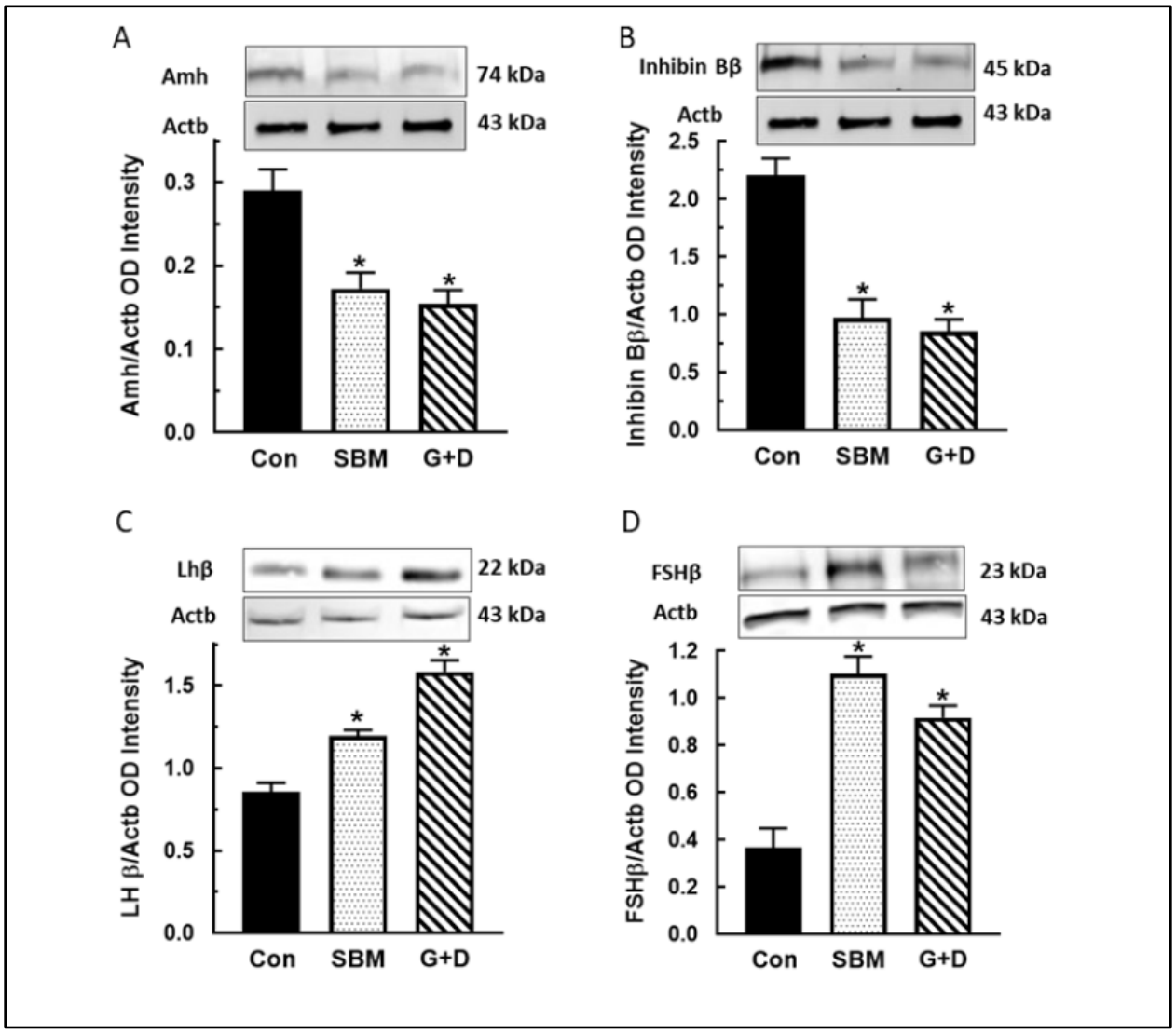

Figure 15: Effects of soy-based diets in the testis-pituitary gland axis. Pubertal male rats at 35 days of age were maintained on a control diet (Con), whole soybean (SBM) or control diet supplemented with genistein and daidzein (G+D) for 14 days. After sacrifice, tissues were processed by western blot procedures to analyze Müllerian inhibiting substance (MIS) (A) and inhibin Bβ in testes (B) and FSHβ (C) and LHβ subunit proteins in pituitary glands (D). Proteins were normalized to actin (Actb) (Inhibin Bβ = 45 kDa, Amh = 74 kDa, Fshβ = 21 kDa, Lhβ = 22 kDa, Actb = 43 kDa. *, *$P < 0.05$ vs. control)*.

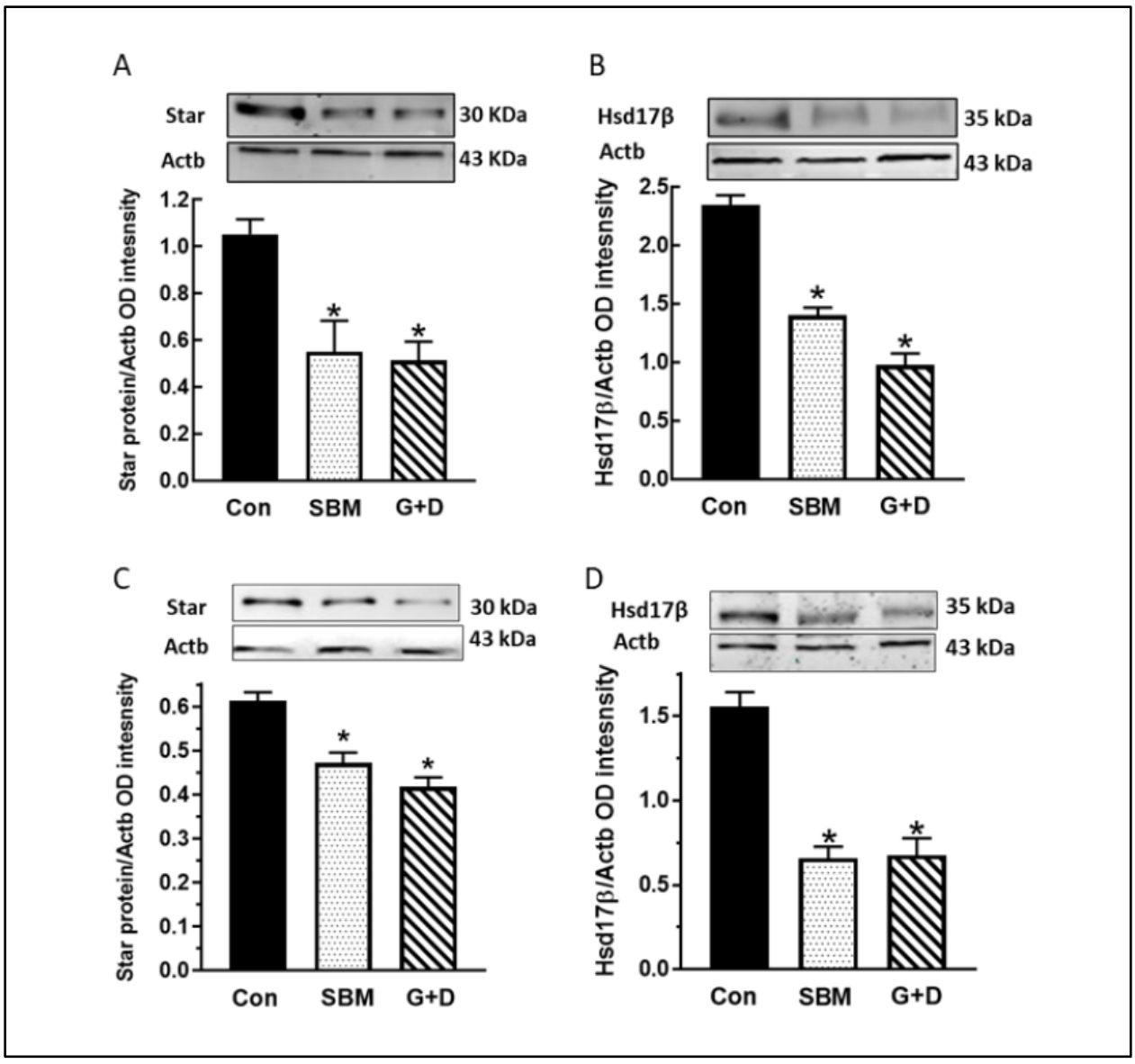

Figure 16: Effects of soy-based diets on steroidogenic proteins in the testis. Male rats at 35 (A, B) and 75 days of age (C, D) were maintained on a control diet (Con), whole soybean (SBM) or control diet supplemented with genistein and daidzein (G+D) for 14 days. After sacrifice, testes were obtained and subjected to western blotting procedures to measure steroidogenic acute regulatory protein (StAR) and the 17β -hydroxysteroid dehydrogenase enzyme (Hsd17β). Proteins were normalized to actin (Actb) (Star = 30 kDa, Hsd17β = 35 kDa, Actb = 43 kDa *, *$P < 0.05$ vs. control).*

2.4.0 Discussion

Serum isoflavone concentrations achieved in the present study are similar to those associated with consumption of soy-based diets in the population [175]. Genistein aglycone concentrations were similar in the SBM and G+D diets but daidzein aglycone measured greater in serum from animals fed the SBM but not G+D diet. The production of greater daidzein than genistein aglycones after consumption of soy-based diets has been a consistent pattern in our studies utilizing both perinatal and neonatal exposure paradigms [122,123]. There were no isoflavone-induced changes in body weights but testicular weights were increased in the SBM group, probably due to the protein content of soybean diets as was previously reported [176]. Overall, we demonstrated that rat testes at all stages of development are sensitive to hormonal activity due to soy isoflavones. We also observed that soy isoflavones in the diet impacted pituitary gonadotropin subunit protein expression although it is not clear that changes in pituitary gland gene expression were due to direct isoflavone action in pituitary gonadotrophs. It is possible that isoflavone-induced changes in sex steroid hormone secretion by the gonads disrupted negative feedback regulation of Gnrh and gonadotropin secretion.

Isoflavone inhibition of sex steroid hormone (T and E2) secretion were evident in serum, testis, and Leydig cells. We confirmed that inhibition of androgen biosynthesis was related to disruption of steroidogenic protein expression, e.g., Star and Hsd17β, which have specific roles in cholesterol mobilization and enzymatic conversion of steroid substrates into androgens in Leydig cells. A previous report also indicated that dietary isoflavones decreased expression of Hsd17β in rat testes [177]. It is perhaps not surprising that testicular E2 concentrations were affected by exposures to dietary isoflavones because T is the substrate for E2 biosynthesis [178]. Metabolism of T to E2 is catalyzed by the aromatase enzyme, and there is evidence that soy isoflavones have the

capacity to inhibit aromatase enzyme activity [177,179]. The aromatase enzyme is highly expressed in adipose tissue [180,181]. Therefore, it is possible that, unlike in the testis, isoflavone regulation of aromatase enzyme activity and E2 biosynthesis in adipose tissue is a confounding factor in E2 metabolism and measurements of serum E2 concentrations. Isoflavones are known to inhibit steroid pathways for estradiol (E2) and T biosynthesis and secretion. Although epidemiological findings are limited and inconclusive, 3β-hydroxysteroid dehydrogenase type 2 (Hsd2 β3), which catalyzes the production of sex steroid precursors, was reportedly inhibited by isoflavones along with cytochrome P450 21 hydroxylase (CYP21A2) in human subjects [182,183]. Also, ingestion of isoflavones decreased *in vivo* DHEAS and A4 levels and inhibited 17,20 lyase activity, thereby impacting the production of precursor androgens and boosting the pool of active androgens, which potentially intensifies clinical syndromes associated with androgen excess [184]. In the present study, we observed that testes from animals fed the SBM and G+D diet exhibited decreased testicular Amh and inhibin Bβ protein expression. Both proteins reflect on testicular maturation and mediate feedback regulation of pituitary Lh and Fsh secretion. Interestingly, decreased testicular Amh and inhibin Bβ protein were coupled to increased Lhβ and Fshβ gonadotropin subunit protein in the pituitary gland. This data set supports the view that dietary estrogens such as soy isoflavones have the capacity to disrupt multiple levels of the HPG axis by acting directly in testicular cells and pituitary gonadotrophs, and/or interfering with steroid hormone feedback regulation of the hypothalamus-pituitary axis [185–187]. Further studies are warranted to investigate direct isoflavone effects and the mechanisms of isoflavone modulation of steroid hormone feedback regulation of the pituitary gland and hypothalamus.

In conclusion, our results demonstrated that disruptions in testicular androgen secretion associated with consumption of soy-based diets are due to isoflavone exposure effects at all stages

of development. This clarification is important because the soy protein is known to exert biological effects and/or activate signaling pathways that regulate cellular function in many tissues [188]. Our data are relevant to public health due to increased consumption of soy-based products by all segments of the population and the increasing incidence of reproductive anomalies in the population [16]. Additional studies are warranted to assess the differential effects of daidzein and genistein in testicular cells.

Chapter 3

Differential Effects of Dietary Soy-isoflavones in Testis of Male Rats

3.0 Abstract

Dietary isoflavones in soybeans are made up of 50-55 % genistin and 40-45 % daidzin implying that these two compounds constitute the highest amounts of isoflavones to which the population is exposed. When consumed both isoflavones are hydrolyzed in the digestive tract to their aglycones genistein and daidzein. These are the metabolically active forms of soy isoflavones. Most studies on isoflavones on the male reproductive axis have focused on genistein with little attention to daidzein. This is a significant omission because both genistein and daidzein demonstrated estrogenic activities in *in vitro* models of hepG2 human hepatoma cells transfected with rat Esr1 and Esr2 [189]. To address this knowledge gap, male rats at 35 days of age were fed either a casein control diet, control diet supplemented with daidzin (Daid), control diet supplemented with genistein (Gen), or control diet supplemented with both genistin and daidzin (G+D). Compared to control, feeding of all isoflavone-containing diets decreased ($p<0.05$) testicular T concentrations. Interestingly, Esr1 and androgen receptor (Ar) protein and pituitary Fshβ with Lhβ subunit protein were increased ($p<0.05$) by feeding genistin and G+D diets, but not the daidzin diet. However, daidzein and genistein both caused a concentration-dependent inhibition ($p<0.05$) of T secretion by Leydig cells *in vitro* with IC_{50} of 184 ηM and 36 ηM, respectively. Results demonstrated that altered testicular steroidogenic capacity and pituitary FSHβ and LHβ subunit expression due to soy-based diets result from specific actions of genistein and daidzein and that genistein shows a greater inhibitory effect on testicular cell steroidogenesis. Further studies are needed to assess the effects of isoflavone regulation of intratesticular androgen concentrations on male fertility.

3.1.0 Introduction

Several biologically active chemicals have been identified in human diets of plant origin. Hormonally active chemicals of plant origin are designated phytoestrogens because they regulate estrogen biosynthesis and estrogen receptor-mediated activity to modulate physiological events in endocrine tissues. A growing body of evidence indicates that exposure to environmental levels of estrogenic agents during the period of reproductive tract development causes adverse biological effects [190,191]. In this regard, approximately 25% of infants in the United States are fed soy-based formula annually [145]. Furthermore, soy-isoflavones are increasing components of regular diets in the form of soy-based food items or commercially available nutritional supplements. Thus, the concern about the endocrine-disrupting potential of soy-isoflavones is not limited to infants but extends to older growing children whose sexual maturity is not complete until puberty. In the previous study, we demonstrated that altered testicular steroidogenic capacity and pituitary FSHβ and LHβ subunit expression due to soy-based diets result from specific actions of isoflavones and are independent of age at exposure. Although we investigated the overall effects of the dietary isoflavones, individual effects of daidzein and genistein were not evaluated. Yet the importance of the individual isoflavones (i.e., genistein and daidzein) has been emphasized by the National Toxicology Program review that noted that most studies on isoflavones on the male reproductive axis have focused on genistein [17] with little attention to daidzein which is also a major isoflavone in soybeans [192,193]. This omission is undesirable because both genistein and daidzein acted as agonists in a model of hepG2 human hepatoma cells transfected with rat Esr1 and Esr2 [189]. Moreover, genistein induced a higher rate of Esr binding to estrogen response elements (EREs) compared to daidzein [194]. However, it remains to be determined that differential binding of transcriptional factors in testicular cells influences the actions of hormonally active agents in the

male gonad. To determine the specificity of isoflavone action, in this study we performed experiments to determine whether isoflavone effects in testicular cells are due to individual action of daidzein or genistein or both compounds acting together.

3.2.0 Materials and methods

3.2.1 Chemicals: All chemicals used in this study were same as previously reported in chapter 2.

3.2.2 Animal studies

All animal and euthanasia procedures were performed in accordance with a protocol approved by the Auburn University Institutional Animal Care and Use Committee (IACUC) based on recommendations of the panel on Euthanasia of the American Veterinary Medical Association. We performed experiments to determine whether effects of soy-based diets on steroid hormone secretion by testis are due to individual actions by daidzein or genistein or both compounds acting together. Male Long-Evans rats at 35 days of age (n=48), obtained from Harlan-Teklad (Madison WI), were housed and maintained as described in chapter 2. There is general agreement that male reproductive development, which is primarily androgen-dependent, occurs in three phases: prepubertal, pubertal, and adult. Our laboratory and others have demonstrated that the 35-day old male rat signifies the pubertal and intermediate phase of development [195–197] and is the rationale for the selection of pubertal animals as a model for this study. There were four experimental diets: 1) casein control; 2) control diet plus daidzin (Dai, 200 ppm); 3) control diet plus genistin (Gen, 300 ppm); and 4) control diet plus genistin and daidzin (G+D). All diets were similar in their content of protein, carbohydrates, fat, energy, and micronutrients (Harlan-Teklad, Madison WI). (**Table 7**). Animals were randomized by weight into four groups and maintained on the appropriate diet for 14 days, i.e., PND 21-35.

Table 7: Composition of experimental diets

Contents/Diets	Control (Casein)	Daidzein (Daid)	Genistein (Gen)	G+D
Daidzein	---	200 PPM	---	200 PPM
Genistein	---	---	300 PPM	300 PPM
Protein (%)	19.0	19.0	19.0	19.0
Carbohydrate (%)	49.0	49.0	49.0	49.0
Fat (%)	5.4	5.4	5.4	5.4
Total calories (Kcal/g)	3.2	3.2	3.2	3.2

Abbreviations: Daid= daidzein, Gen= genistein, G+D= genistein + daidzein

Finally, we performed *in vitro* assays with primary Leydig cell cultures in experiments designed to eliminate isoflavone effects due to the soy or casein protein[194] and prevent isoflavone activity occurring in the hypothalamus and pituitary gland [119]. Immature Leydig cells, isolated from 35-day-old male rats not previously exposed to isoflavones, were incubated in DMEM/F-12 culture medium containing genistein or daidzein at 0, 0.01, 0.1, 1, 10 μM in the presence of LH (10 ηg/mL ovine LH) for 18 h at a temperature of 34°C. Leydig cells isolated from testes of male rats at 35 days postpartum exhibit features that are seen in prepubertal rats (e.g., proliferative capacity) and adult animals (steroid hormone secretion), and thus represent the intermediate stage of development. After treatment, aliquots of spent media were analyzed for T concentrations. We also determined the concentration of genistein and daidzein causing half-maximal inhibition (IC_{50}) of T secretion by Leydig cells.

3.2.3 Procedure for isolation of Leydig cells and measurement of hormones are as reported in chapter 2.

3.2.4 Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis and Western Blot Analysis

We analyzed the expression of several proteins in testes (Amh, inhibin Bβ, Esr1, Ar, Star, Hsd17β) and pituitary glands (Lhβ, Fshβ, Esr1, Ar). Tissues were homogenized in T-PER lysis buffer (Pierce Biotechnology, Rockford, IL) that was freshly supplemented with a protease inhibitor cocktail (Catalog #78410; Pierce Biotechnology). Tubes were centrifuged at 3000 rpm for 14 minutes at 4°C to remove cellular debris. Protein concentrations were determined using the Bio-Rad protein assay with BSA as standard (Bio-Rad Laboratories). Aliquots (50 µL) of whole-cell lysates were dissolved in an equal volume of Laemmli buffer containing 5% β-mercaptoethanol and were boiled for 5 min at 95°C. All samples were resolved on varying percentages of Tris-HCl acrylamide gels by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Catalog #1620147; Bio-Rad Laboratories) and subsequently incubated in blocking buffer (5% whole milk in 0.1% Tween 20 PBS) for 1 h at room temperature to reduce nonspecific binding by antibodies. Membranes were then incubated in a blocking buffer containing appropriate primary antibodies overnight at 4°C. Parameters of primary antibodies (Anti-ESR1 antibody, anti-AR antibody, anti-FSHβ antibody, and anti-LHβ antibody) used in the present study are listed in **Table 4**. On the next day, blots were washed three times in 0.1% Tween 20 PBS to remove any unbound primary antibody before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Afterward, membranes were washed four times with 0.1% Tween 20 PBS and then scanned using a LI-COR Odyssey Infrared Scanner (Lincoln, NE). All protein measurements were normalized to β-actin.

3.2.5 Statistical Analysis

Data are presented as the mean ± SEM. All data were analyzed by one-way ANOVA followed by Dunnett's test for multiple group comparisons (GraphPad Prism software. San Diego. Ca). Differences of ≤0.05 were considered to be significant.

3.3.0 Results

3.3.1 General Observations

No animal deaths were recorded in the course of this study. Maintenance of animals on experimental diets had no effects on body weights, paired testicular weights, and gonadosomatic index (**Table 8**).

Table 8: Body weights and testicular sizes in male rats maintained on isoflavone containing diets

Exposure groups	Average body weight (g)	Average paired testis weight (g)	Average testicular index (%)
Control	247.7±3.5	2.6±0.10	1.1±0.05
Daid	253.4±4.0	2.4±0.10	1.0±0.05
Gen	251.1±2.6	2.6±0.10	1.0±0.04
G+D	248.8±4.8	2.6±0.10	1.0±0.03

Abbreviations: Daid= daidzein, Gen= genistein, G+D= genistein + daidzein

3.3.2 Singular and combined isoflavone effects on testicular androgen secretion

Maintenance of pubertal male rats on the Dai, Gen and G+D diets decreased ($P<0.05$) serum T concentrations only in animals fed the G+D diet compared to control (**Fig. 17A**). However, basal and LH-stimulated testicular T secretion were decreased in all male rats fed isoflavone-containing diets compared to control unexposed animals (**Figs. 17B-E**).

3.3.3 Singular and combined isoflavone effects on testicular and pituitary protein gene expression

Western blot analysis showed that expression of Esr1 and Ar protein in testes was increased by feeding of the Gen and G+D diets but this effect was absent in the Dai diet group (**Figs. 18A, B**). Expression of Esr1 and Ar protein in pituitary glands was increased ($P<0.05$) by feeding of all isoflavone-containing diets compared to control (**Figs. 19A, B**). Interestingly, the patterns of pituitary FSHβ and LHβ protein expression (**Figs. 19C**, **D**) were similar to Esr1 and Ar protein (**Figs. 18A**, **B**) in testes but changes were absent in the Dai diet group.

3.3.4 Dose-dependent effects of daidzein and genistein on androgen secretion by Leydig cell

Analysis of isoflavone effects in primary Leydig cell cultures demonstrated that both daidzein and genistein caused a concentration-dependent inhibition ($P<0.05$) of T secretion by Leydig cells (**Figs. 20A, B**). The concentrations of daidzein and genistein causing half-maximal inhibition of T secretion by Leydig cells (IC_{50}) were calculated to be 184 ηM and 36.2 ηM, respectively (**Figs. 20C, D**).

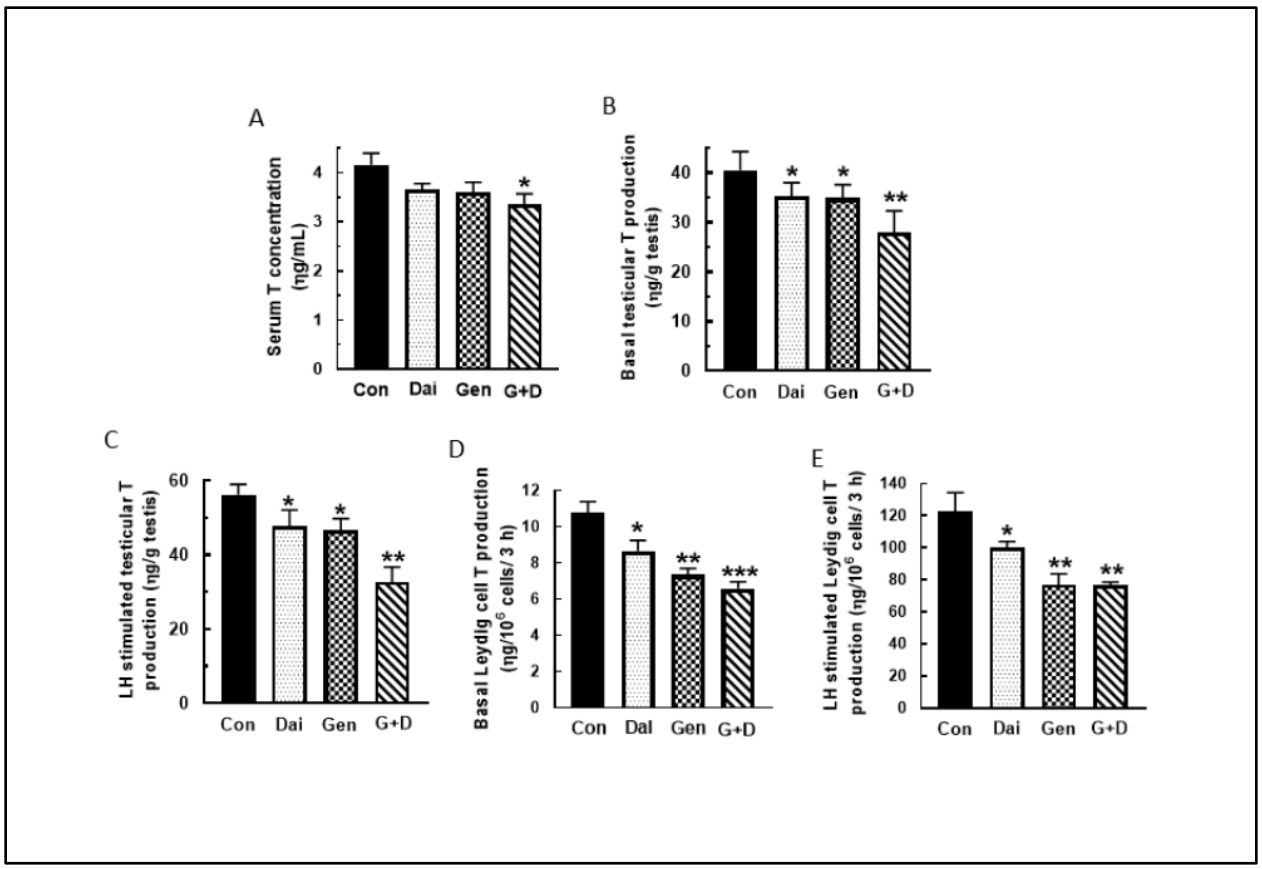

Figure 17: Differential soy isoflavone effects on testicular androgen secretion. Pubertal male rats at 35 days of age were maintained on a control diet (Con) or control diet supplemented with daidzein (Dai), genistein (Gen), or both isoflavones (G+D) for 14 days. After sacrifice, serum were obtained to measure testosterone (T) concentrations (A). Also, testicular explants and Leydig cells isolated from testes of animals in the same diet group were incubated in DMEM/Ham's F-12 culture medium in triplicate for 3 h without (B, D) or containing 100 ηg/ml ovine LH (NIDDK, NIH) (C, E). Aliquots of serum and spent media were analyzed to measure T concentrations by RIA (n = 48; *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

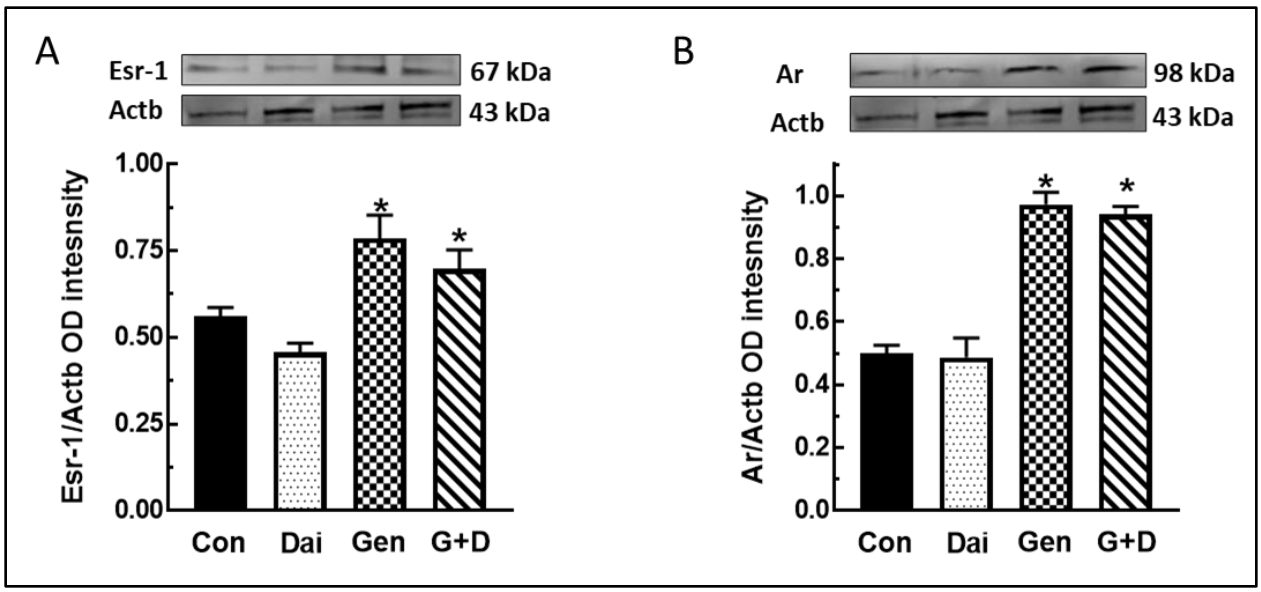

Figure 18: Differential soy isoflavone effects on protein gene expression in the testis. Male rats at 35 days of age were maintained on a control diet (Con) or control diet supplemented with daidzein (Dai), genistein (Gen), or both isoflavones (G+D) for 14 days. After sacrifice, testicular samples were obtained and processed by western blotting procedures to measure testicular estrogen receptor-1 (Esr1) (A) and androgen receptor (Ar) (B). Proteins were normalized to actin (Actb) (Esr1 = 67 kDa, Ar = 98 kDa, Actb = 43 kDa; * *P <0.05 vs. control)*.

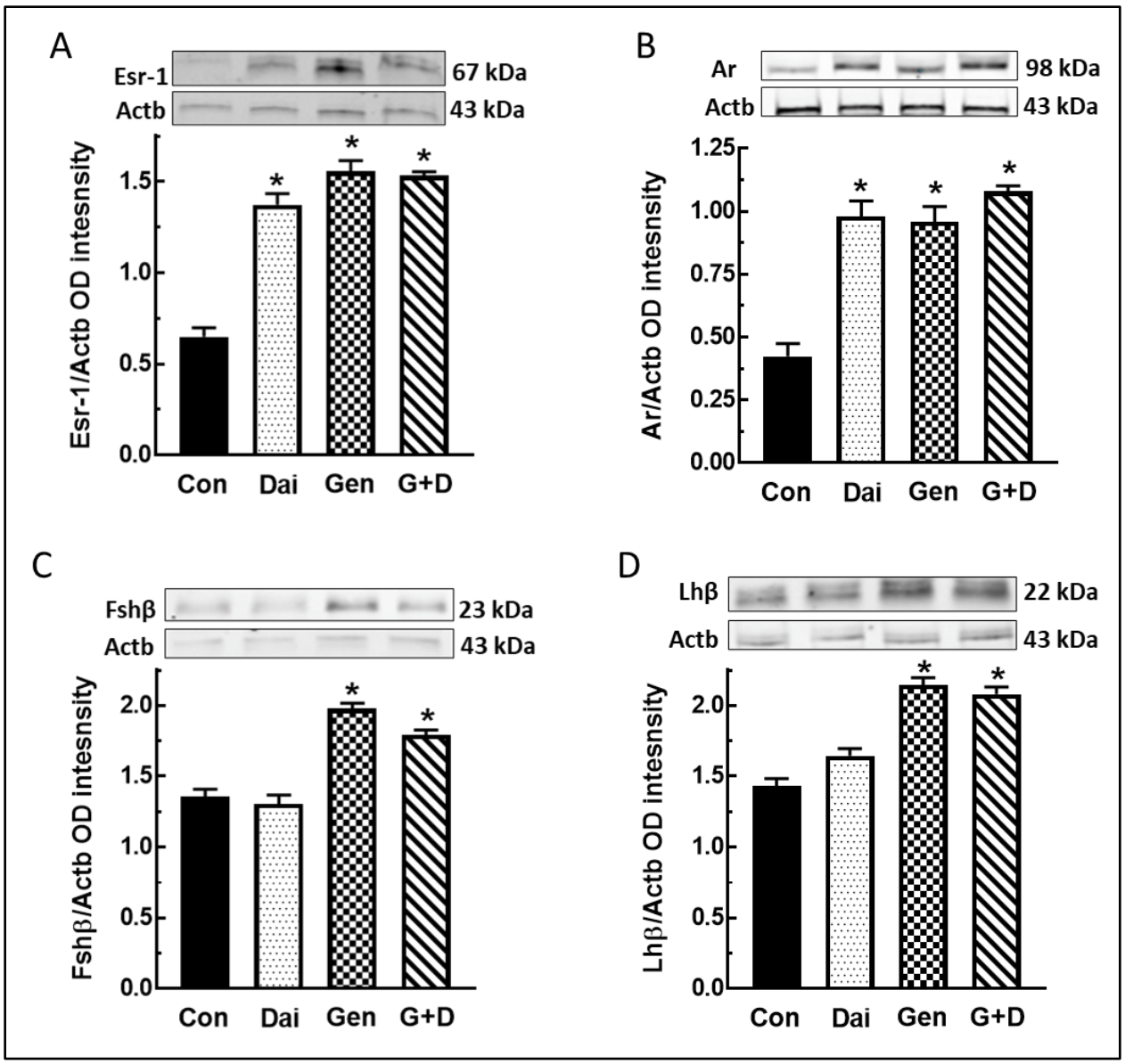

Figure 19: Differential soy isoflavone effects on gene expression in the pituitary gland. Male rats at 35 days of age were maintained on a control diet (Con) or control diet supplemented with daidzein (Dai), genistein (Gen), or both isoflavones (G+D) for 14 days. After sacrifice, pituitary gland samples were obtained and processed by western blotting procedures to measure the protein expression of estrogen receptor-1 (Esr1) (A) and androgen receptor (Ar) (B), Fshβ (C), and Lhβ subunit proteins (D). Proteins were normalized to actin (Actb) (Fshβ = 21 kDa, Lhβ = 22 kDa, Esr1 = 67 kDa, Ar = 98 kDa, Actb = 43 kDa; * *P <0.05 vs. control)*.

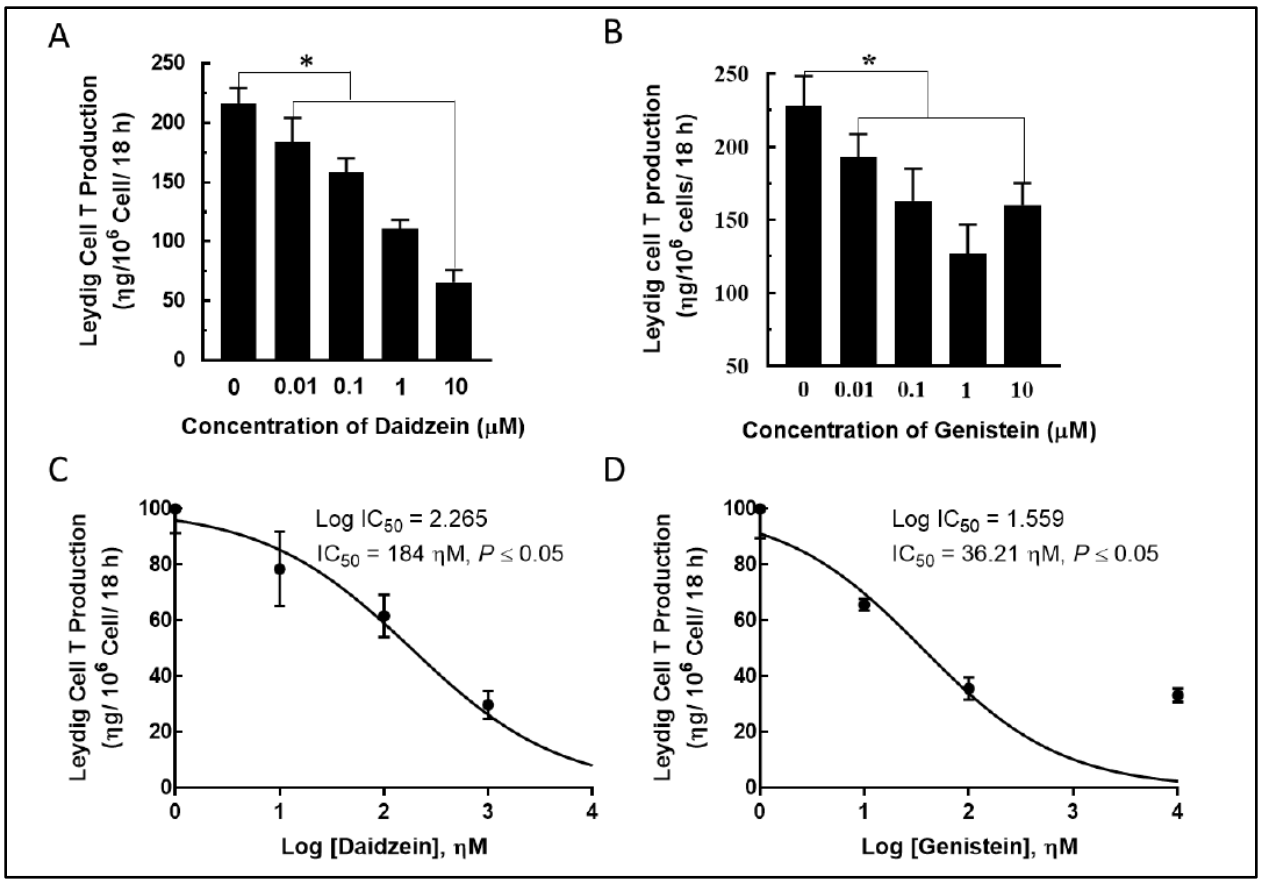

Figure 20: Effects of isoflavones on Leydig cell testosterone (T) production *in vitro*. Immature Leydig cells were isolated from 35-day-old male rats and incubated in triplicate in DMEM/Ham's F-12 culture medium containing genistein or daidzein (0, 0.1, 1, and 10 μM) and LH (10 ηg/mL; NIDDK, NIH) for 18 h. Spent media were analyzed for T concentrations by RIA (A, B). Also, the concentrations of daidzein and genistein causing half-maximal inhibition of T production (IC_{50}) were calculated (C, D). Experiments were replicated 4-6 times (*$P < 0.05$ *vs. control).*

3.4.0 Discussion

We investigated the action of individual isoflavones (genistein and daidzein) in the male reproductive axis. Serum T concentrations were unaffected but testicular and Leydig cell T secretion were decreased in animals fed diets containing daidzein and/or genistein, implying that both genistein and daidzein contribute to isoflavone suppression of testicular androgen secretion. The presence of both genistein and daidzein as major isoflavones in soybeans raises the possibility

that combined effects due to both chemicals may be more intense than those associated with single chemicals. Analysis of chemical mixture effects is outside the focus of the present study, but the effects of the genistein and daidzein combination appeared to be additive. Indeed, feeding the G+D diet in this study affected more endpoints than either Dai or Gen diet alone. On the other hand, it is possible that the combination of genistein and daidzein exerts no effects on biological parameters if both chemicals activate signaling pathways that counteract and mitigate each other's actions [198].

Moreover, our results demonstrated that genistein might be a more potent isoflavone inhibitor of Leydig cell steroidogenesis. For example, basal Leydig cell T production appear to be greater in animals from the Dai compared to the Gen diet group, although this was not statistically tested. Second, our *in vitro* studies showed that daidzein and genistein concentrations causing half-maximal inhibition of Leydig cell T secretion were 184 ηM and 36 ηM, respectively. These observations are supported by other reports of greater hormonal effects due to genistein compared to daidzein [150,189,199]. We also observed differential isoflavone effects on pituitary Fshβ and Lhβ subunit protein expression. For example, animals from the Gen diet group exhibited increased Fshβ and Lhβ subunit protein than in the Dai diet group. These observations reinforce the view that genistein might be the more hormonally active soy isoflavone in reproductive tract tissues. Furthermore, there was a lack of concordance between decreased serum T concentrations and increased pituitary Lhβ subunit protein in male rats fed the G+D diet because the latter failed to normalize Leydig cell T secretion. Although we did not measure Lh receptor expression in the present study, we observed previously that genistein diminished Lh stimulation of androgen secretion by uncoupling Lh receptors from G proteins in Leydig cells [153]. Also, we did not measure serum LH concentrations and it remains a possibility that isoflavone-induced

posttranslational modifications prevented secretion and release of pituitary Lhβ subunit protein into peripheral blood to target the gonads and stimulate androgen secretion [200]. Although increased expression of Esr1 and Ar protein after exposure to genistein was reported in other studies [201], the biological significance of increased pituitary and gonadal Ar and Esr1 protein as a factor in tissue sensitivity to isoflavone exposure remains to be determined.

In conclusion, this study showed that genistein and daidzein acting individually impact multiple levels of the endocrine and male reproductive axes. Future studies will investigate the impact of isoflavone-induced changes in testicular androgen concentrations on germ cell development, sperm production, and male fertility.

Chapter 4

Comparative effects of BPA, BPS, and EE2 on Testicular Steroidogenesis

4.0.0 Abstract

Synthetic xenoestrogens present in the environment can interfere with the endocrine axis hence they are classified as EDCs. BPS is used in the manufacture of consumer products because of its superior thermal stability and is thought to be a safe replacement chemical for its analog BPA. However, the safety profile of BPS is yet to be fully investigated. The estrogenic chemical EE2 is used in the manufacture of female oral contraceptives for women and a contaminant of water supplies. EE2's presence in water supplies implies that males are also exposed to this agent. Most studies on EE2 effects are in aquatic species and have largely ignored mammalian species. To bridge this knowledge gap, we investigated the effects of BPA, BPS, and EE2 on sex steroid secretion in the growing male rat gonad. In two separate experiments, prepubertal and pubertal male rats at 21 and 35 days of age were provided test chemicals in drinking water (5 µg/L) for 14 days. At the end of the exposure period, results showed that BPS and EE2 inhibited testicular testosterone (T) concentrations in a similar manner to BPA. Results also showed that although BPA did not affect gonadal E2 secretion, both BPS and EE2 increased testicular E2 concentrations. Furthermore, the protein expressions of Sertoli cell exclusively produced factors were altered. For example, BPS and EE2 but not BPA decreased Amh protein expression while expressions of Dhh protein were increased by all tested chemicals. Together, these observations demonstrated that BPA, BPS, and EE2 have similar inhibitory effects on gonadal steroidogenesis and showed that these compounds have the potential to disrupt testicular function. These findings reinforce the view that exposure of the population to environmental chemicals may impair reproductive health.

Additional studies are warranted to understand the mechanisms by which chemical mixtures impact testicular cells and interfere with paracrine interactions.

4.1.0 Introduction

Exposure of the population to chemicals in the environment has increased significantly in the past 100 years due to their use in the manufacture of plastic materials, pesticides, herbicides, medicaments, and other industrial products [202,203]. This is a public health concern because there is evidence that many chemicals have the capacity to interfere with the endocrine axis as several endocrine-disrupting chemicals (EDCs) [204]. Xenoestrogens are chemicals that mimic the activity of endogenous estrogens and are among the most common EDCs [205]. In the present study, we have focused on the comparative effects of three environmentally relevant xenoestrogens, namely, bisphenol A (BPA), bisphenol S (BPS), and 17α-ethinyl estradiol (EE2) in the rodent male gonad.

BPA is a high volume production chemical whose estrogenic property was first discovered in 1936[206]. About 3.4 million tons of BPA is employed each year in the production of polycarbonate plastics as electrical and household appliances, and food and water packaging products. A significant amount of BPA is present in epoxy resins that are used to coat inner surfaces of food and beverage metallic cans and as an antioxidant in polyvinyl chloride and thermal cash register paper. BPA is ubiquitous in the environment because of its widespread use in many products, contamination of water and food products. Exposure of the population by inhalation is also possible in addition to exposure in man through leaching from food and beverage containers[207]. BPA is associated with many human diseases including diabetes, cardiovascular diseases, and reproductive anomalies in both sexes[208]. Previously, we showed that perinatal BPA exposures of female rats at 2.5 and 25 μg/kg body weight increased expression of Ar, Esr1, Esr2, Wnt4, beta-Catenin, and p-Erk in testes of pubertal and adult male offspring [209]. We also demonstrated that perinatal BPA exposures disrupted seminiferous tubule development in prepubertal male rats [210]. Our work and others have led to a tightened restriction in the use of BPA

in infant-related material. Although the FDA has restricted the use of BPA in infant-related products, usage in food packages at low concentrations is still permissible for older ages. FDA is continuing to review ongoing and emerging data on BPA safety. However, the concern surrounding the safety profile of BPA and the need to withdraw it from circulation has led to the creation and embrace of several analogs of BPA as replacement chemicals in BPA-containing consumer products. Because the FDA continues to rely on emerging data to make decisions on regulations, studies on BPA continue to be relevant.

BPS is one of the many analogs of BPA and has found application as a replacement for BPA in a variety of products termed "BPA-Free" including infant feeding bottles, lacquer coating for food and beverage packaging cans, developer for thermal paper, wash fastening agent in cleaning products, electroplating solvent, and as a constituent of phenolic resin [48,211]. Despite the widespread use of BPS, its safety profile is relatively unknown due to a paucity of scientific data. However, a few of the available data on BPS shows that it has potential for toxicity. For example, daily administration of BPS by gavage at 50 µg/kg/day for 28 days decreased sperm production and increased sperm DNA damage in adult rats [51]. Also, BPS was reported to activate the GPR30 estrogen receptor, induce oxidative stress, and increase DNA fragmentation in sperm cells and HepG2 cells *in vitro* [51,212]. The high degree of structural resemblance between BPA and BPS raised similar concerns of endocrine-disrupting capacity in its usage, but there are no restrictions or regulations on its use. One objective of this study is to analyze and compare the effects of BPA and BPS in the gonad of developing male rats.

EE2 is a synthetic estrogen used in the formulation of oral contraceptives for women. It is nonpolar, non-volatile, hydrophobic, and relatively resistant to biodegradation. A substantial amount of EE2 is excreted unchanged in urine and fecal matter [54]. Although bacterial degradation

and photodegradation in sewage treatment plants are used to remove EE2, significant amounts of EE2 remain in water supplies due to its high lipophilic capacity [55,213] [56]. It has a binding affinity to estrogen receptors (ESRs) that is twice that of E2. Because of EE2 resistance to biodegradation and its high affinity for ESRs, EE2 is one of the most common and most potent estrogenic compounds contaminating drinking water [60,61]. Thus, despite EE2 been a medication that is commonly prescribed in females, its presence as a potent estrogen contaminant of treated water makes it an EDC of public health concern even for the population [214,215]. For example, exposures to EE2 were associated with anomalies of sexual development [62,63], altered sexual maturation [64], reduced fecundity[65], disruption of spermatogenesis [66], and reproductive tract anomalies in aquatic animals [67]. Although extensive studies have been reported on the toxicity of EE2 in aquatic species, there is a paucity of data on the reproductive and endocrine disruptive effects of EE2 in male mammalian species.

Given the broad spectrum of xenoestrogens with the potential to disrupt the male reproductive axis, limited data on the safety profile of those commonly encountered in our daily activity, and the increased health risks posed to developing males. We have selected three environmentally relevant xenoestrogens (i.e., BPA, BPS, and EE2) of public health concern to analyze and compare their effects on gonadal steroidogenesis in growing male rats.

4.2.0 Materials and Methods

4.2.1 Chemicals

Bisphenol A (Lot # 1065060 13706030), Bisphenol S (lot # BCBV2462), and 17α-ethinylestradiol (lot # WXBC6630V) were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin inhibitor, EDTA, HEPES, BSA, bovine lipoprotein, sodium bicarbonate ($NaHCO_3$),

DMEM nutrient mixture [Ham's F-12 (DMEM/F-12; 1:1 mixture without phenol red)], albumin, and gentamicin were purchased from Sigma Chemical Company (St. Louis, MO). Dulbecco's PBS, medium 199, and 10 × Hanks' balanced salt solution were obtained from Life Technologies, Inc. (Grand Island, NY). Ovine luteinizing hormone (LH) was provided by the National Hormone and Pituitary Program (NIDDK, Bethesda, MD).

4.2.2 Animal Studies

Male prepubertal (n=36) and pubertal (n=36) Long-Evans rats were obtained from Harlan-Teklad (Madison WI). After acclimatization for three days at the College of Veterinary Medicine Division of Laboratory Animal Health (DLAH) Facility, animals were randomly placed 1-3 per cage (length, 0.47 m; width, 0.25 m; height, 0.22 m) (Snyder Manufacturing Company; Centennial, CO). Water was provided in glass water bottles *ad libitum*. The housing of animals in plastic cages and the use of glass bottles were designed to minimize background exposure to estrogens and bisphenol compounds as may occur with resin-containing cages [216]. Due to the presence of phytoestrogens in soybeans and their capacity to interfere with the male reproductive system [217,218], animals were fed a soy-free X2020 diet (Harlan-Teklad, Madison WI) throughout the experimental period. Animals were maintained under constant conditions of light (12L: 12D) and temperature (20–23.38C) with free access to pelleted food.

Test chemical concentrations were selected based on their presence in the environment as reported in the literature [48,219–221]. Administration of test chemicals in drinking was designed to mimic the natural route of exposure and minimize activation of the pituitary-adrenal axis [222].

Prepubertal male rats at 21 days of age (*Experiment 1*) and pubertal male rats (*Experiment 2*) were randomly assigned by weight to four groups: Control, BPA, BPS, and EE2. Test chemicals were dissolved in 0.001% of DSMO in drinking water and fed to animals at 5 µg/L for 14 days. The control group was provided only with water containing 0.001% DSMO for the same period. To assess for toxicity, animal weights were obtained, and the volume of water consumed was measured on alternate days. At the end of treatment, animals were sacrificed to obtain blood and testicular tissues for hormonal and protein analyses.

4.2.3 Measurement of Steroid Hormones

Serum was separated from trunk blood collected at sacrifice. Testicular explants (~100 mg) were incubated in microcentrifuge tubes containing DMEM/F12 culture medium buffered with 14 mm $NaHCO_3$, 15 mm HEPES, 0.1% BSA, and 0.5 mg/mL bovine lipoprotein. Incubations were conducted without (basal) and with a maximally stimulating dose of ovine LH (100 ng/mL; LH-stimulated) for 3 h at 34°C. Steroid hormone concentrations (T, E2) were assayed in aliquots of serum and spent media using a tritium-based RIA with an inter-assay variation of 7%–8% [172]. Hormone production was normalized to nanograms per ml for serum and nanograms per mg for testes.

4.2.4 Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis and Western Blot Analysis

The expression of key enzymes of the steroidogenic pathway was analyzed using western blot procedures. We analyzed Cyp11A1 which catalyzes the conversion of cholesterol to pregnenolone and is the rate-limiting step in steroidogenesis while Hsd17β mediates the final step in the conversion of androstenedione to T[223]. Because Amh is a marker for Sertoli cell maturation

and Sertoli cell-secreted Dhh is critical to germ cell maturation [224,225], we also analyzed Amh and Dhh protein in western blots of testes. Briefly, tissues were homogenized in T-PER lysis buffer (Pierce Biotechnology, Rockford, IL) that was freshly supplemented with a protease inhibitor cocktail (Catalog #78410; Pierce Biotechnology). Tubes were centrifuged at 3000 *rpm* for 14 minutes at 4°C to remove cellular debris. Protein concentrations were determined using the Bio-Rad protein assay with BSA as standard (Bio-Rad Laboratories, Hercules, CA). Aliquots (50 µL) of whole-cell lysates were dissolved in an equal volume of Laemmli buffer containing 5% β-mercaptoethanol and boiled for 5 min at 95°C. All samples were resolved on varying percentages of Tris-HCl acrylamide gels by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Catalog #1620147; Bio-Rad Laboratories) and subsequently incubated in blocking buffer (5% whole milk in 0.1% Tween 20 PBS) for 1 h at room temperature to reduce nonspecific binding by antibodies. Membranes were then incubated in a blocking buffer containing appropriate primary antibodies overnight at 4°C. Parameters of primary antibodies (anti-CYP11A1 antibody, anti-HSD17β, anti-Amh antibody, anti-Dhh antibody, anti-actin antibody) used in the present study are provided in **Table 4**. On the next day, blots were washed three times in 0.1% Tween 20 PBS to remove any unbound primary antibody before incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Afterward, membranes were washed four times with 0.1% Tween 20 PBS and then scanned using a LI-COR Odyssey Infrared Scanner (Lincoln, NE). All protein measurements were normalized to β-actin.

4.2.5 Statistical Analysis

Data were analyzed by one-way ANOVA followed by Dunnett's test for multiple group comparisons, (GraphPad, San Diego. Ca). All parameters are presented as the mean ± SEM. Differences of $p \leq 0.05$ were considered significant.

4.3.0 Results

4.3.1 General Observations

No death was recorded in any animal group in this study. Exposure of animals to xenoestrogens had no effects on body weights, paired testicular weights and, gonadosomatic index (**Tables 9 and 10).**

TABLE 9: Body weights and testicular size in prepubertal rats exposed to chemicals in their drinking water

Xenoestrogen (µg/L)	Average body weight (g)	Average paired testis weight (g)	Average testicular index (%)
Cont. (0)	137.8±4.0	1.3±0.02	0.9±0.02
BPA (5)	139.7±4.0	1.3±0.03	0.9±0.02
BPS (5)	137.1±4.0	1.5±0.05	1.1±0.02
EE2 (5)	146.1±6.0	1.5±0.05	1.0±0.02

TABLE 10: Body weights and testicular size in prepubertal rats exposed to chemicals in their drinking water

Xenoestrogen (µg/L)	Average body weight (g)	Average paired testis weight (g)	Average testicular index (%)
Cont. (0)	275.7±5.0	2.9±0.06	1.0±0.02
BPA (10)	255.7±8.0	2.6±0.05	1.0±0.02
BPS (10)	288.8±3.0	2.5±0.12	0.9±0.06
EE2 (10)	258.3±7.0	2.7±0.07	1.0±0.02
BPA/EE2 (5 each)	264.5±4.0	2.7±0.05	1.0±0.02
BPS/EE2 (5 each)	275.5±4.0	2.9±0.05	1.0±0.02

4.3.2 Effect of BPA, BPS, and EE2 on serum and testicular androgen concentrations

Exposure of prepubertal and pubertal animals to BPA decreased ($P<0.05$) serum T concentrations (Figures 17A, B), whereas exposures to BPS and EE2 had no effect (**Figs. 21A, B**) compared to control animals. Basal testicular T concentrations were decreased by all test chemicals in prepubertal animals compared to control (**Fig. 22A**). More so in prepubertal animals, BPA- and EE2-treated ($P<0.0001$) animals exhibited a greater decrease in T production than BPS-treated animals compared to control (**Fig. 22A**). In pubertal animals, only BPA-exposed animals exhibited a decrease ($P<0.05$) in basal testicular T production while EE2-, and BPS-treated animals were similar compared to the control (**Fig. 22B**). Interestingly, LH-stimulated testicular T productions in both prepubertal and pubertal age groups were only decreased ($P < 0.05$) in animals treated with BPA while BPS- and EE2-treated animals showed no effect compared to control (**Figs. 22C, D**).

4.3.3 Effect of BPA, BPS, and EE2 on serum and testicular E2 concentrations

In general, testicular E2 concentrations were increased by exposure to the test chemicals. Serum E2 concentrations were not significantly affected by test chemicals in prepubertal male rats compared to control animals (**Fig. 23A**). On the other hand, basal and LH-stimulated testicular E2 productions (**Figs. 24A, C**) were increased ($P < 0.05$) after exposure to BPS, and EE2 compared to control, but this effect was absent in BPA-exposed animals ($P>0.05$). Exposure of pubertal male rats to test chemicals had no effect on serum E2 concentrations (**Fig. 23B**). However, measurement of basal (**Fig. 24B**) and LH-stimulated (**Fig. 24D**) testicular E2 production were increased ($P<0.05$) in pubertal animals exposed to EE2, but not in BPA and BPS compared to control.

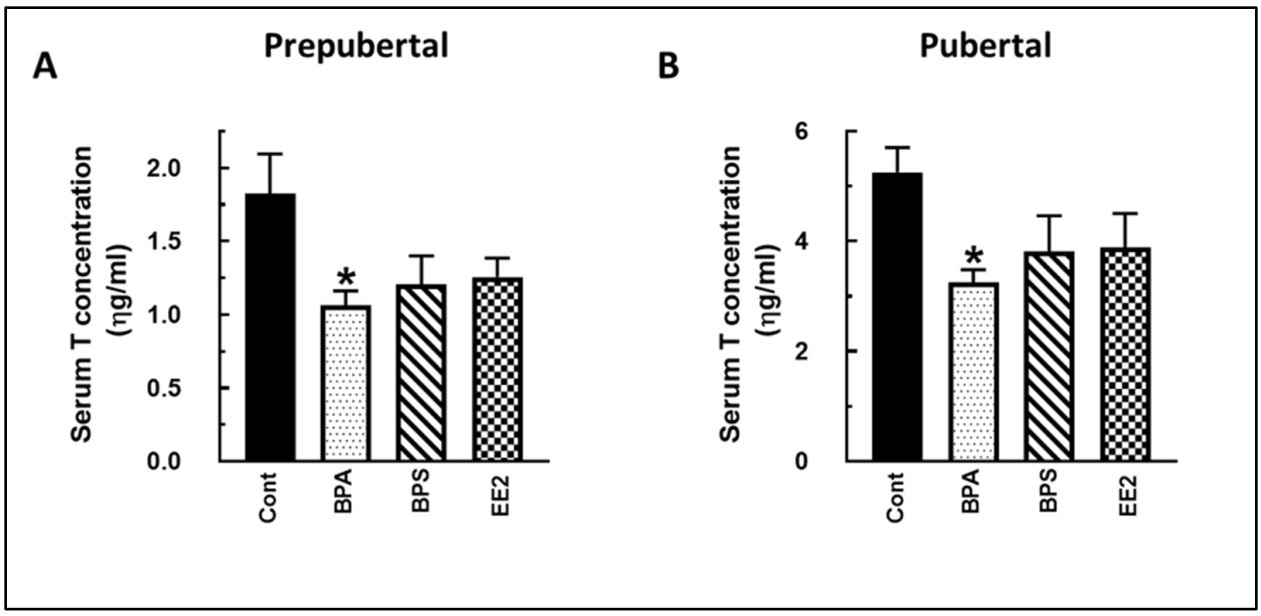

Figure 21: Effects of BPA, BPS, and EE2 on serum androgen concentrations in prepubertal and pubertal male rats. Male rats at 21 (prepubertal) and 35 (pubertal) days of age were fed drinking water containing BPA, BPS, and EE2 (5µg/L) for 14 days. At sacrifice, blood was processed to obtain serum. Aliquots of serum were analyzed to measure T concentrations by RIA (n=6; *p< 0.05).

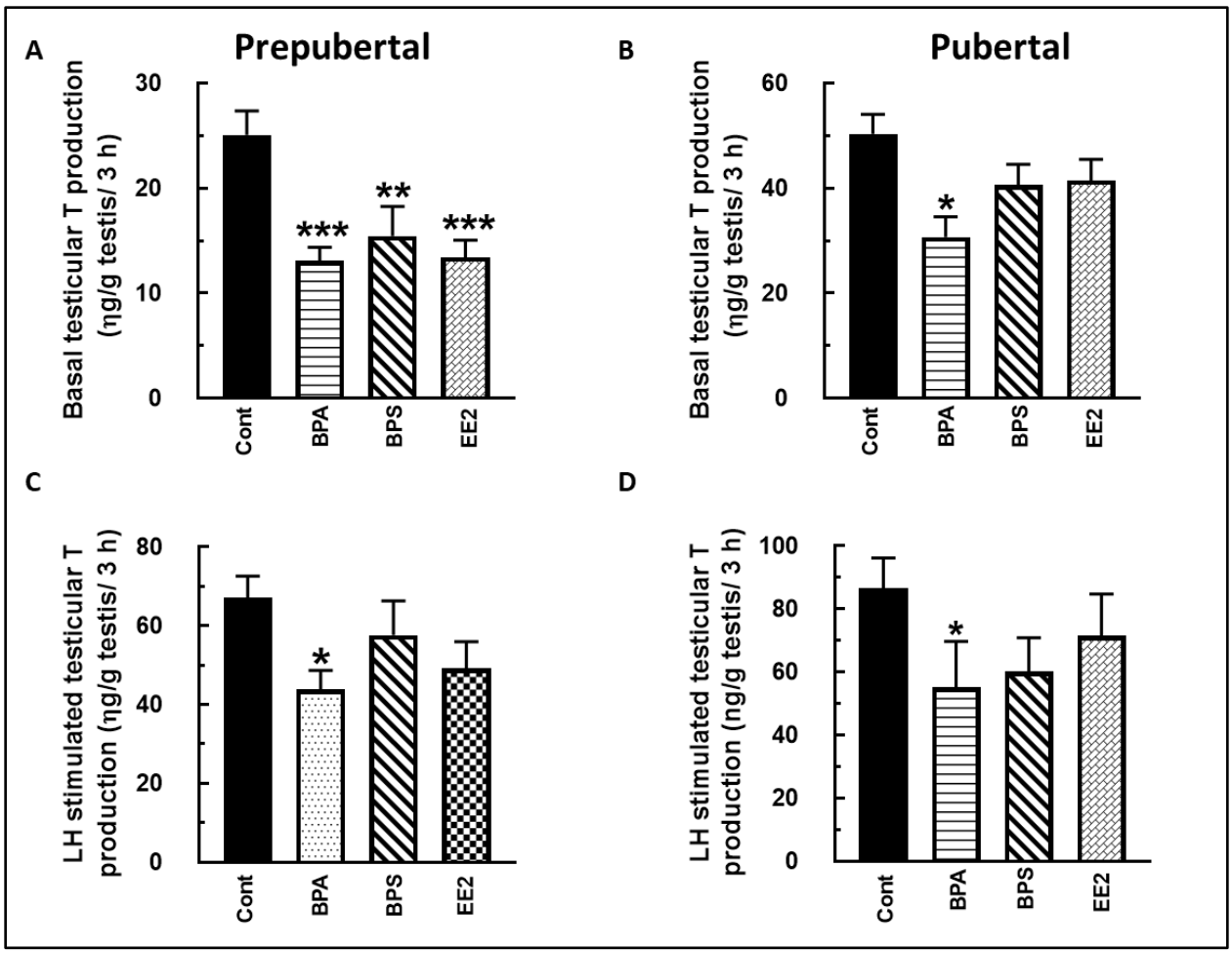

Figure 22: Effect of BPA, BPS, and EE2 on testicular androgen production in prepubertal and pubertal male rats. Male rats at 21 (prepubertal) and 35 (pubertal) days of age were fed drinking water containing BPA, BPS, EE2 (5 µg/L) for 14 days. At sacrifice, testicular explants were obtained and incubated in DMEM/Ham's F-12 culture medium in triplicate without (A, B) or with 100 ηg/ml ovine LH (NIDDK, NIH) (C, D) for 3 h. Aliquots of spent media were analyzed to measure T production by RIA (n = 6; *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

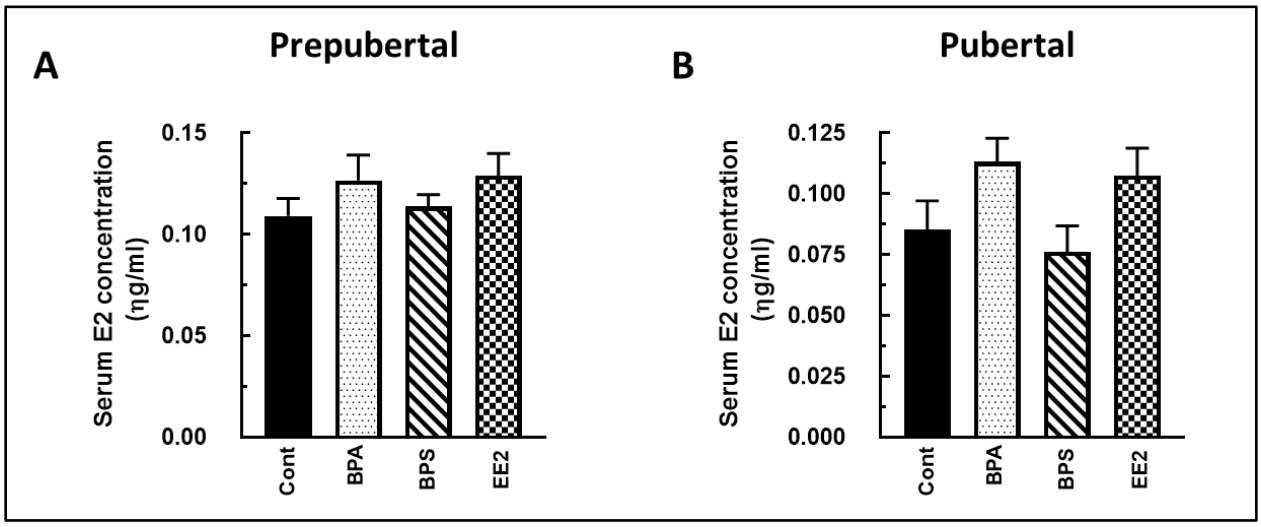

Figure 23: Effect of BPA, BPS, and EE2 on serum 17β-estradiol concentrations in prepubertal and pubertal male rats. Male rats at 21 (Prepubertal) and 35 (Pubertal) days of age were fed drinking water containing BPA, BPS, EE2 (5 µg/L) for 14 days. At sacrifice, blood was processed to obtain serum. Aliquots of serum were analyzed to measure E2 concentrations by RIA (n = 6; **P* < *0.05*).

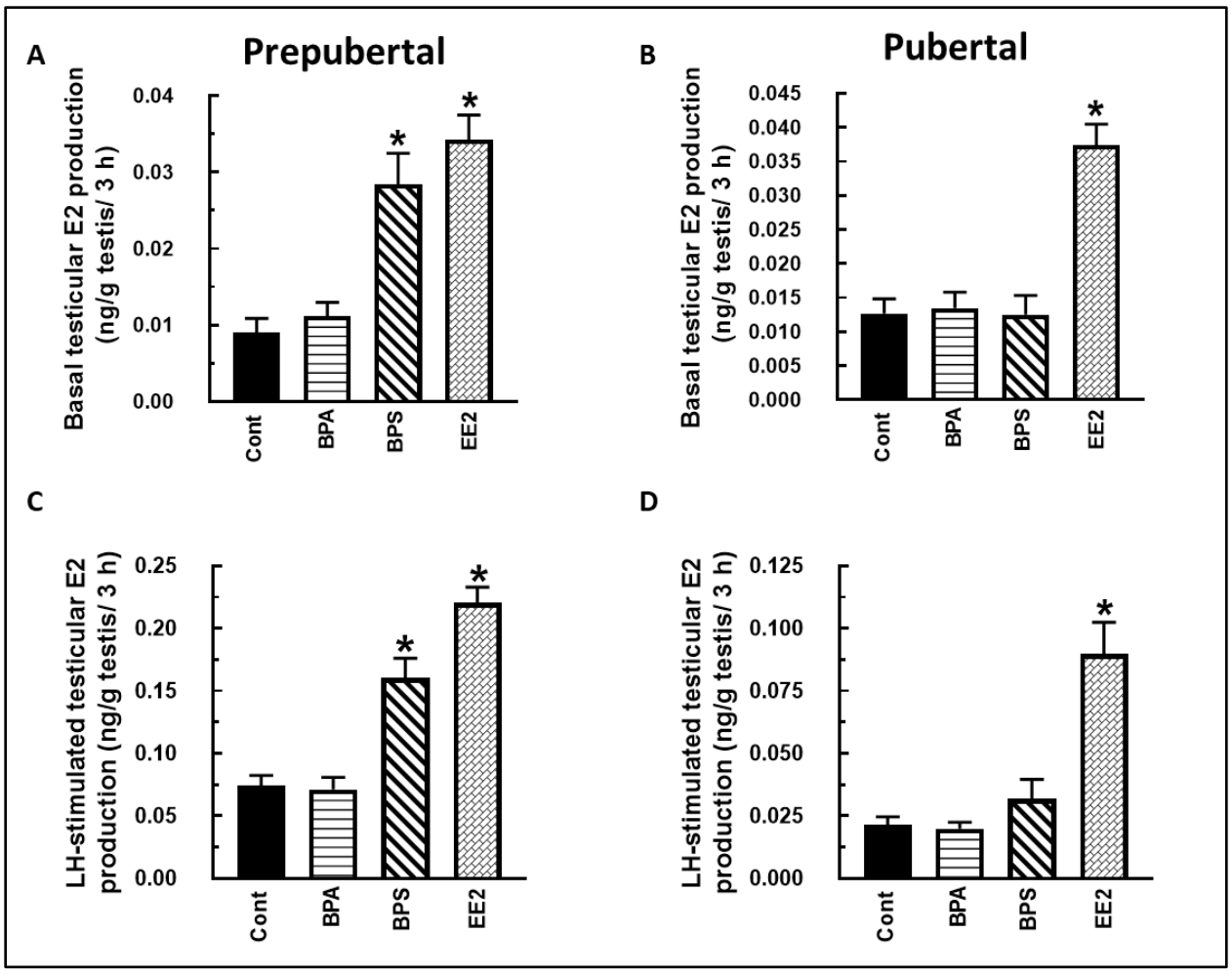

Figure 24: Effect of BPA, BPS, and EE2 on testicular androgen production in prepubertal and pubertal male rats. Male rats at 21 (prepubertal) and 35 (pubertal) days of age were fed drinking water containing BPA, BPS, EE2 (5 µg/L) for 14 days. At sacrifice, testicular explants were obtained and incubated in DMEM/Ham's F-12 culture medium in triplicate without (A, B) or with 100 ηg/ml ovine LH (NIDDK, NIH) (C, D) for 3 h. Aliquots of spent media were analyzed to measure E2 production by RIA (n = 6; $*P < 0.05$).

4.3.4 Effect of BPA, BPS, and EE2 on markers of testicular function and development

We attempted to validate observations on steroid hormone concentrations by evaluation of gonadal steroidogenic capacity. Western blot analysis showed that testicular expression of the Cyp11A1 enzyme was sensitive to the test chemicals and was decreased ($P<0.05$) in pubertal

animals exposed to all chemicals compared to control (**Fig. 25A**). However, expression of the Hsd17β enzyme was increased ($P < 0.05$) in BPA-treated animals (**Fig. 25B**) but decreased ($P < 0.05$) in EE2- and BPS-treated animals (**Fig. 25B**) compared to control. Expression of the Sertoli cell-secreted Amh was not affected by exposure to BPA but was decreased ($P<0.05$) in the BPS- and EE2- treated animals compared to control (**Fig. 26A**). On the other hand, testicular expression of the Dhh protein was increased ($P<0.05$) in all chemical-exposed animals ($P>0.05$), compared to control (**Fig. 26B**).

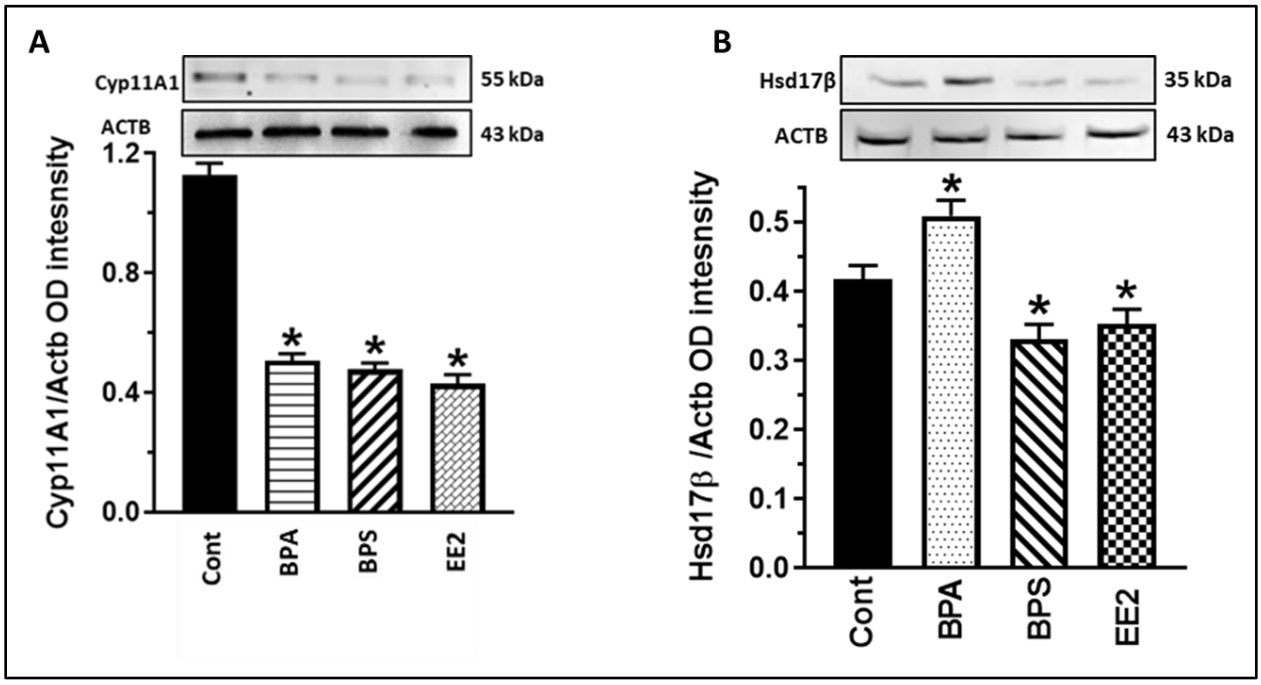

Fig. 25: Effect of BPA, BPS, and EE2 on gene protein markers of testicular steroidogenesis. Testes were obtained from pubertal male rats (PND 34-49) fed drinking water containing BPA, BPS, EE2 (5 μg/L) for 14 days and processed for western blot analysis to measure protein expression of cytochrome P45011A1 (Cyp11A1) and 17β Hydroxysteroid dehydrogenase (Hsd17β) and. Proteins were normalized to actin (ACTB). Assays were repeated at least three times. (Hsd17β = 35 kDa, Cyp11A1 = 55 kDa, ACTB = 43 kDa *, *$P < 0.05$ vs. control)*.

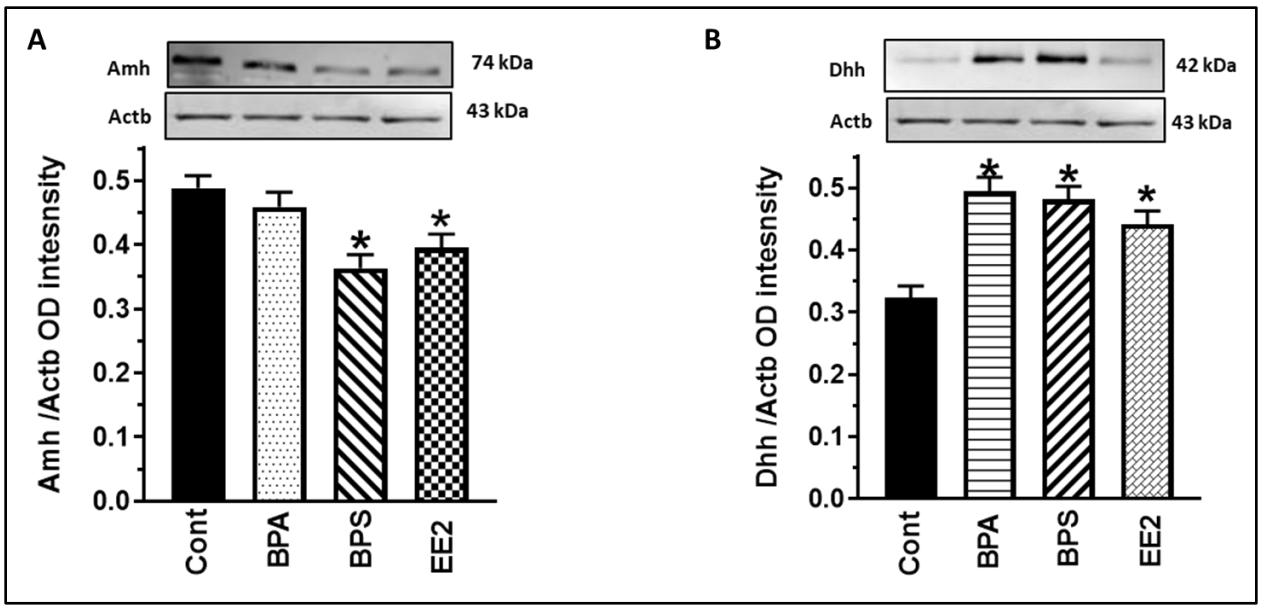

Figure 26: Effect of BPA, BPS, and EE2 on gene protein markers of testicular development and function. Testes were obtained from pubertal male rats (PND 34-49) fed drinking water containing BPA, BPS, EE2 (5 µg/L) for 14 days and processed for western blot analysis to measure Anti-Mullerian hormone (Amh) and Dhh protein expression. Proteins were normalized to actin (Actb). Assays were repeated at least three times. (Amh = 74 kDa, Dhh = 42 kDa, Actb = 43 kDa *, *$P < 0.05$ vs. control)*.

4.4.0 Discussion

The results of the present study demonstrated that developmental exposures of male rats to environmentally relevant concentrations of BPA, BPS, and EE2 altered sex steroid hormone production in the male rat gonad. Similar to the present findings, we reported previously that perinatal [19,210,226] and prepubertal [125] exposures to BPA decreased testicular T biosynthesis in male rats. The present findings also indicated that BPA may be a more potent inhibitor of testicular T biosynthesis in prepubertal than in pubertal male rats. Other studies have shown that BPS at 50 µg/kg/day for 28 days decreased intratesticular T biosynthesis in adult rats [227] [126] and may have a diminished capacity to activate the Esr compared to BPA [228] [229]. BPS may exert lesser toxicity in

the male gonad but has the capacity to regulate testicular function. Therefore, exposure of the population to this compound should remain a concern [207]. Similarly, EE2 decreased serum T and intratesticular T concentrations in prepubertal, and to a lesser degree in pubertal animals, suggesting age-dependent sensitivity of male rats to EE2. These observations are similar to reports of dose-dependent inhibition of Leydig cell T biosynthesis by EE2 [230].

The present data indicated that inhibition of androgen biosynthesis by test chemicals was associated with decreased testicular expression of Cyp11a1 similar to previous reports [231,232]. For example, we showed previously that perinatal exposures to BPA increased expression of Hsd17β in neonatal offspring [210] but a decrease in the adult testis [19,226]. Thus, it is possible that BPA action is influenced by age at exposure. Also, BPA appears to interfere with many steroidogenic enzymes because it affected Hsd17β, Cyp17a1, Hsd3β, and Cyp11a1 in the rat as was reported in the human testes [227]. Although reports on BPS effects are limited, studies in mice showed that this compound inhibited T secretion but with no effect on the Cyp11A1 enzyme [207,233,234]. In contrast, decreased Hsd17β mRNA levels were found in the testes of Zebrafish exposed to BPS [235]. In the present study, we observed that EE2 inhibition of androgen biosynthesis was related to decreased testicular Cyp11A1 and Hsd17β protein expression. Our finding related to EE2 effects on Cyp11a1 aligns with a previous report that showed that EE2 inhibited Cyp11a1 activity in Leydig cells [230]. Altogether, it is likely that testicular Cyp11a1 is a primary target for EDCs with predominant estrogenic properties such as BPA, BPS, and EE2 and it may be a marker for xenoestrogen exposure.

Moreover, we reported previously that perinatal BPA exposures did not affect E2 biosynthesis in adult male offspring [226] but E2 secretion was decreased after incubation of Leydig

cells with BPA [125]. In the present study, both BPS and EE2 increased testicular E2 secretion similar to studies in mice [233]. It is to be noted that E2 secretion by testicular cells, unlike T secretion, is not limited to a single cell type. Leydig cells, Sertoli cells [236,237], germ cells [238] may have all contributed to the observed effects. Blood E2 may also be contributed by extra-testicular sources, e.g., adipocytes [181] implying that the serum E2 levels in this study may not be a reflection only of test chemical effects on gonadal E2 secretion capacity. Because germ cells express ESRs [239], it is reasonable to expect that changes in intratesticular E2 concentrations have implications for germ cell development and sperm function. We and others reported previously that BPA [125,240], BPS, [241], and EE2 [21] regulated intratesticular T, pituitary gonadotropin release, and circulating LH concentration in male rats. The present finding of increased intratesticular E2 levels due to chemical exposures has implications for E2-mediated regulation of the hypothalamus-pituitary gland axis.

Chemical exposure effects on testicular development were evident in altered expression of Sertoli cell-produced factors. For example, testicular Amh protein was decreased by the test chemicals except for BPA. However, Dhh was increased in testes of all chemical-exposed animals. These are important observations because the Amh protein is a marker of Sertoli cell differentiation, whereas Dhh is required for the progression of germ cells through spermatogenesis and to the final stage of spermiogenesis [242]. Indeed, male mice deficient in Dhh exhibited functionally immature sperm and a decrease in the number of Leydig cells [243]. However, other studies demonstrated that Dhh overexpression leads to impaired Dhh signaling, alteration in Sertoli cell function, disturbances of spermatogenesis, and a decrease in the number of primary spermatocytes [244]. Thus, the finding of decreased Amh expression and increased Dhh expression due to BPA, BPS, and EE2 implies that the test chemicals potentially affect testicular development.

In summary, both prepubertal and pubertal stages of development are sensitive to BPA, BPS, and EE2 even at the low dose exposure paradigms used in the present study. Interestingly, the prepubertal stage of development appears to be more susceptible to the effects of all tested chemicals compared to pubertal animals. Taken together, the results showed that environmentally relevant concentrations of BPA, BPS, and EE2 disrupted steroidogenesis in the rodent male gonad. However, further studies are warranted to investigate the impact of changes in intratesticular sex steroids on spermatogenesis and the neuroendocrine axis.

Chapter 5

Summary and Conclusion

The overall aim of this dissertation was to analyze xenoestrogen effects on testicular cell development and function. We have investigated this aim under the following specific objectives.

1. Whether effects due to soy-based diet exposure can be attributed to isoflavones and whether the effects are influenced by the age at which exposure occurred.
2. Whether isoflavone effects in the testis are due to daidzein, genistein, or both compounds, and
3. Determining and comparing the effects of BPA, BPS, and EE2 on steroid hormone secretion in the developing male rat gonad.

The male rat testis is composed of numerous cells that function in coordination with the hypothalamic-pituitary axis to maintain homeostasis in support of reproductive health. Of all the cells in the testis, Leydig cells and Sertoli cells provide the best opportunity to assess postnatal development and function in the male gonad because both synthesize critical hormones and factors that support the male reproductive tract in the process of differentiation and maturation. Leydig cells in the testis are principally responsible for the biosynthesis of testosterone, the most important hormones of the male reproductive axis required for spermatogenesis and the maintenance of fertility. Measurable changes in testicular steroidogenesis as reflected in serum, testis, and Leydig cells are key to understanding the mechanisms responsible for disruptions that result from xenoestrogen exposure. On the other hand, Sertoli cells play a central role in testicular development and function by providing the structural and functional microenvironment that supports germ cell maturation. Sertoli cells serve as the channel of communication between the germ cells and pituitary gland as well as between the germ cells and all other cells types of the testis.

This dissertation has demonstrated that disruptions in the rat gonad arising from dietary soy consumption are due specifically to isoflavones and are independent of exposure time. For example, exposure to SBM or G+D diets altered sex steroid concentrations not only at the serum level but also in the testis and Leydig cells from prepubertal, pubertal and adult male rats. The decrease in androgen secretion was related in part to decreased expression of steroidogenic enzyme proteins and altered pituitary gland Fshβ and Lhβ proteins expression. Importantly, these results indicated that dietary isoflavones altered intratesticular sex steroid concentrations i.e., estradiol and testosterone, suggesting that dietary isoflavones might disrupt spermatogenesis and impact fertility. Sertoli cell expression of both anti-Mullerian hormone and inhibin Bβ proteins were decreased as a result of exposure to the SBM and G+D diets further supporting the finding of isoflavone on the male reproductive axis. Our study also demonstrated that both genistein and daidzein, acting individually or in combination, are able to exert biological effects in testicular cells and the pituitary gland. For example, expression of Ar and Esr1 protein were altered both in the testis and the pituitary gland after exposure to diets containing either genistein or daidzein or both compounds. Overall, our data set supports the view that dietary estrogens have the capacity to disrupt multiple levels of the hypothalamic-pituitary-gonadal axis by acting directly in testicular cells and pituitary gonadotrophs and/or interfering with steroid hormone feedback regulation of the hypothalamic-pituitary axis (**Fig. 26)**. Further studies are warranted to investigate direct isoflavone effects and the mechanisms of isoflavone modulation of steroid hormone feedback regulation of the pituitary gland and hypothalamus. Additional studies are also needed to assess the direct impact of changes in intratesticular androgen concentration on germ cell maturation.

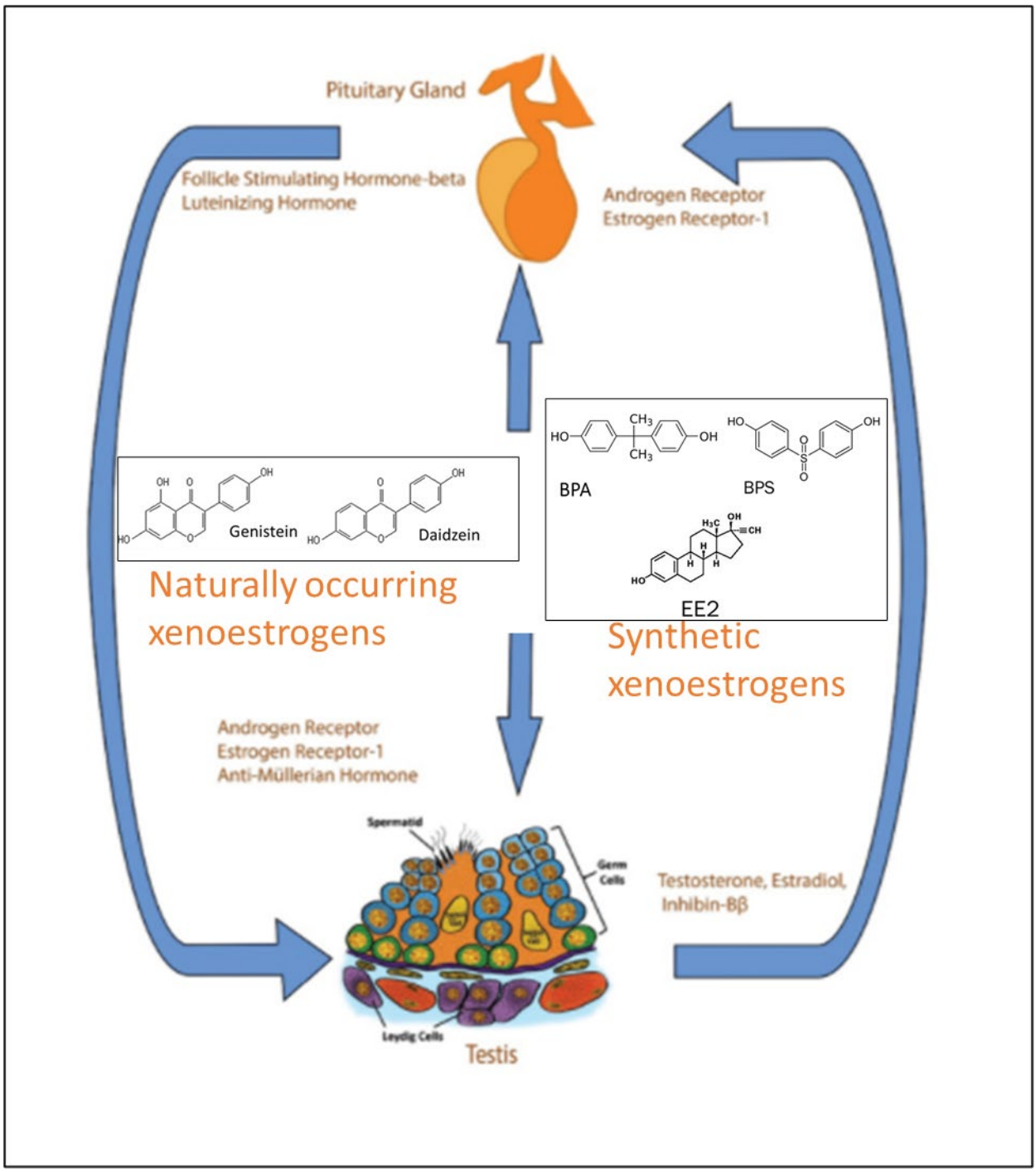

Figure 27: Xenoestrogens regulate the pituitary and the gonad in the male rats. Naturally occurring xenoestrogens (genistein and daidzein) and synthetic xenoestrogens (BPS, BPA, and EE2) exert influences on the testis and pituitary gland.

In addition to our reports on dietary isoflavone effects on testicular development and function, we analyzed and compared the effects of the synthetic estrogenic chemicals (BPA, BPS, and EE2) on the developing rat gonad. Our results demonstrated that environmentally relevant concentrations of BPA, BPS, and EE2 altered intratesticular concentrations of testosterone and estradiol in both prepubertal and pubertal male rats. The alterations in sex steroids for all three chemicals were found to be more pronounced in prepubertal than in pubertal animals, indicating that the younger age group is more vulnerable to the impacts of the tested chemicals. The mechanisms underlying altered sex steroid secretion were explained in part by decreased expression of the Cyp11A1 enzyme protein in the testis. Although BPA increased the expression of Hsd17β protein, BPS and EE2 both decreased expression of Hsd17β, suggesting that BPA and BPS, despite the similarity in structure, may act through different and perhaps multiple pathways to impact reproductive function. Furthermore, our results showed that all three chemicals increased Sertoli cell expression of the Dhh protein. The Dhh-mediated signaling in germ cells is essential for spermatogenesis. Overexpression of Dhh reduces the number of primary spermatocytes, thereby impacting the number of mature spermatozoa during spermatogenesis. Therefore, our data indicate that an environmentally relevant concentration of BPA is detrimental to the developing testes. the results also imply that BPS may not be a safe alternative to BPA. Similarly, an environmentally relevant concentration of EE2 decreased testosterone and has the potential to impact testis development and function. A challenge to understanding the relationship between xenoestrogens and reproductive health is that rather exposure to xenoestrogens tends to occur as a mixture of chemicals rather than single chemical exposure as tested in this study. Therefore, future studies will evaluate the effects of simultaneous co-exposures to these chemicals to provide information on their additive, synergistic or antagonistic actions on testicular development and functions.

Taken together, our observation on dietary soy-isoflavones and the synthetic estrogenic chemicals are relevant to public health due to:

1. Increased consumption of soy-based products in the general population

2. Inadvertent exposure to synthetic xenoestrogens by all segments of the population and

3. The increased incidence of reproductive anomalies in the population.

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