The Effects of Lactational Exposure to Soy Isoflavones on Testicular Function in the Rat

by

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Abstract

Testosterone, the male androgen responsible for maintaining the male phenotype, is produced by testicular Leydig cells. Leydig cells express estrogen receptors and are therefore regulated by estrogen. Soybeans predominantly contain the isoflavones genistin and daidzin which are hydrolyzed in the gastrointestinal tract to genistein and daidzein. Although both genistein and daidzein possess estrogenic properties, it is unclear whether the controversial effects of soy-based infant formulas on testicular function are due to the independent actions of genistein or daidzein or result from both agents acting together. In the present study, lactational exposures to both genistein and daidzein together induced Leydig cell proliferation and decreased Leydig cell T production in 22- and 35-day-old Long-Evans male rats. In addition, lactational exposure to both genistein and daidzein reduced serum testosterone concentrations in male rats 22 days of age. Decreased androgen production persisted into adulthood, during which estrogen receptor 1 protein levels were increased and expression of many steroidogenic enzymes were impaired as a consequence to lactational exposures to both genistein and daidzein. Also, lactational exposure to genistein and daidzein decreased Sertoli cell product MIS and increased MIS type II receptor expressions in 22-day-old male rats. These observations indicate that genistein and daidzein behave in a dose-additive manner, and exposures to both isoflavones during the lactational period may also interfere with Leydig-Sertoli cell interaction, with an implication for overall testis function.

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List of Abbreviations

ADIOL	5α-androstane-3α, 17β-diol
ALC	Adult Leydig cells
AR	Androgen receptor
DAID	Daidzin
DHT	Dihydrotestosterone
E2	Estradiol
ED	Endocrine disruptor
EDS	Ethane dimethanesulfonate
ERE	Estrogen response element
ESR1	Estrogen receptor 1
FSH	Follicle-stimulating Hormone
GD	Gestational Day
GEN	Genistin
17βHSD	17-beta hydroxysteroid dehydrogenase
3βHSD	3-beta hydroxysteroid dehydrogenase
ILC	Immature Leydig cells
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MIS	Mullerian-inhibiting substance

MISRII	Mullerian-inhibiting substance type II receptor
CYP17A1	Cytochrome P450 17α-hydroxylase
CYP11A1	Cytochrome P450 side-chain cleavage enzyme
PLC	Progenitor Leydig cells
PND	Postnatal day
SBM	Soybean meal
SER	Smooth endoplasmic reticulum
StAR	Steroidogenic acute regulatory protein
T	Testosterone

Chapter 1: Literature Review

Section 1: Leydig Cell Development and Function

Introduction.

The testes are the primary reproductive organs in the male because they produce testosterone, the male androgen responsible for maintaining the male phenotype, and spermatozoa, which are the male gametes. Each testis consists of two major compartments: the vascularized interstitium and the avascular seminiferous tubule. Situated within the testicular interstitium are Leydig cells, which produce testosterone. Extending from the basement membrane of the seminiferous tubule to the open lumen are Sertoli cells, which play a critical role in sperm development.

1.1: Fetal Leydig Cells.

In the rat, there are two distinct generations of Leydig cells: fetal Leydig cells and postnatal Leydig cells. Developed *in utero*, the fetal Leydig cells gain capacity to synthesize testosterone by gestational day (GD) 15.5 [1]. Subsequently, testosterone production markedly increases, with maximum steroidogenic activity attained at GD19, or just before birth [2]. The testosterone secreted by fetal Leydig cells is required for the differentiation of the male urogenital system during late gestation [2]. Fetal Leydig cells express luteinizing hormone receptor (LHR) and respond to LH stimulation [3, 4]. However, fetal Leydig cell development is independent of LH stimulation, as evidenced by analyses of LHR knock-out (LHRKO) mice, demonstrating that these mice produce similar testosterone levels as wild-types during the prenatal period [5]. Although it is unclear whether fetal Leydig cells persist in the adult testis, it has been suggested that these cells undergo postnatal apoptosis [5].

1.2: Progenitor Leydig Cells.

At postnatal day (PND) 7, spindle-shaped stem cells develop in the interstitium, mostly in the peritubular layer of the testis of the rat [6]. Proliferation of these stem cells in the testicular interstitium and their subsequent commitment to the Leydig cell lineage result in the progenitor Leydig cell (PLCs) population by PND21 [6]. The PLCs are small, spindle-shaped cells with a similar appearance to stem cells in the postnatal testis. Unlike stem cells, the PLCs are recognized as members of the Leydig cell lineage by virtue of their expression of Leydig cell markers including 3β-hydroxysteroid dehydrogenase (3βHSD), a critical steroidogenic enzyme involved in testosterone synthesis (to be discussed in detail later) [7]. The PLCs contain only small amounts of smooth endoplasmic reticulum (SER), the organelle in which several steroidogenic enzymes are localized and thus produce only small amounts of steroids, mainly androsterone [7].

1.3: Immature Leydig Cells.

By PND28, the PLCs transform from a spindle-shape to a round structure and reduce their proliferative capacity, forming immature Leydig cells (ILCs). At this time, the population of ILCs is approximately 13-14 million [8]. During the transition from PLC to ILC, the SER expands greatly and the levels of 3βHSD increases [9]. Although these transformations result in a greater capacity for steroidogenesis by the ILCs, testosterone is unexpectedly not the major steroid produced [10]. Because the ILCs possess high levels of metabolizing enzymatic activity (i.e., 3α-hydroxysteroid

dehydrogenase, 3α-HSD, and 5α-reductase), they primarily produce the androgen metabolite 5α-androstane-3α, 17β-diol (ADIOL) [8].

1.4: Adult Leydig Cells.

From day 28 to day 56, the ILC population undergoes one final round of cell division, resulting in a population of approximately 25 million adult Leydig cells (ALCs) per testis [8]. In the ALCs, there is a predominance of testosterone over ADIOL production by PND56 [8]. In fact, testosterone production is 150 times greater in 90-day-old ALCs than in PLCs at 21 days of age, and 5 times greater than in 35-day-old ILCs [7]. The ALCs have a greater abundance of SER compared to ILCs [8]. Although ALCs do not normally proliferate, they may however regenerate if the original population is eliminated. Thus, the population of ALCs is fully recovered within seven weeks of its eradication by ethane dimethanesulfonate (EDS), a cytoxin that specifically kills ALCs [11]. This regeneration involves the same cell progression that occurs during normal development of the ALCs, including the period during which ADIOL is the dominate steroid over testosterone [12].

1.5: Luteinizing Hormone Receptor Signaling Pathway.

Most of the testosterone produced by ALCs is mainly driven by LH stimulation. The LHR is a glycoprotein member of the superfamily of G protein-coupled receptors (GPCRs) [13]. The GPCR signaling pathway involves a receptor which recognizes an extracellular signal, an effector molecule which generates an intracellular signal, and a GTP-binding protein (G protein), a molecular switch which mediates signaling between

the receptor and effector molecule [13]. The G proteins are heterotrimers consisting of α, β, and γ subunits. In their inactive state, the Gα subunits bind GDP [14]. Binding of LH to the LHR activates stimulatory G proteins (G_s), which promote the release of GDP by the α subunit and binding of GTP [14]. Acting as an “on” signal, the active Gα subunit subsequently binds and activates the effector, adenylyl cyclase, which produces cyclic adenosine 3’,5’-cyclic monophosphate (cAMP) from the cellular ATP pool [14]. Subsequent hydrolysis of GTP by the GTPase domain of the Gα subunit results in termination of the hormonal signal, demoting it to an “off” position [14]. When the signal is “on”, a necessary step in the pathway of steroid hormone synthesis occurs; that is, the cAMP-dependent mobilization of cholesterol by protein transporters from cellular stores to the inner membrane of the mitochondria [15].

1.6: Transport of Cholesterol into Mitochondria.

Although details of this process remain unclear, it is possible that the cholesterol within steroidogenic cells may be stored in the form of lipoprotein and recognized by the membrane-bound scavenger receptor class B type I (SR-B1) lipoprotein receptor [16]. Also, there is the possibility that the cholesterol may be synthesized *de novo* from acetate by the 3-hydroxy-3- methyl-glutaryl-CoA (HMG-CoA) reductase enzyme [17]. In either case, cholesterol, in the form of cholesteryl esters, is subsequently stored in cytoplasmic lipid droplets [18]. Cholesterol is transported as lipid droplets in two phases in response to hormonal stimulation. The first phase involves the movement of the cholesteryl esters by cholesterol esterases and hormone-sensitive lipase towards the mitochondrial outer membrane [19, 20]. The second phase of cholesterol transfer

involves the mobilization of cholesterol from the outer mitochondrial membrane to the mitochondrial matrix. The two principal proteins that are involved in cholesterol transport across the mitochondrial membrane are the peripheral-type benzodiazepine receptor, recently renamed Translocator Protein (TSPO), and the steroidogenic acute regulatory protein (StAR) [21]. Evidence for StAR's critical role in steroidogenesis was discovered in part from studies of congenital lipoid adrenal hyperplasia, an autosomal recessive disease resulting in the near absence of adrenal and gonadal steroid synthesis [22]. Studies revealed that the manifestation of the disease was due to mutations in the StAR gene [22]. The StAR gene was cloned and the 30 kDa phosphoprotein demonstrated to be processed from a 37 kDa cytosolic precursor protein containing a mitochondrial targeting sequence [23].

TSPO is a high affinity, cholesterol binding protein, predominantly localized to the outer mitochondrial membranes of steroid-producing tissues [24]. Based on its structure, TSPO has been posited to function as a translocator of cholesterol [24]. In one study, Leydig cells were incubated with hCG, resulting in a rapid increase in TSPO ligand binding that can be inhibited by a PKA blocker, suggesting that cAMP-induced phosphorylation of TSPO may be involved in hCG-stimulated steroidogenesis [25]. Recent evidence suggests that TSPO and StAR form a complex that facilitates intramitochondrial cholesterol movement [26]. Even if this is the case, there appears to be a consensus that StAR is the transport protein primarily responsible for shuttling cholesterol into the mitochondria, thus continuing the process of testosterone production.

1.7: Testosterone Biosynthesis.

In the rat, LH stimulation promotes cAMP to transfer cholesterol to the inner mitochondrial membrane where it is metabolized into pregnenolone via the P450 cholesterol side chain cleavage enzyme (P450scc/CYP11A1) [27, 28]. Pregnenolone moves out of the mitochondria to the SER where it is converted to progesterone by 3βHSD [27, 28]. Subsequently, progesterone is converted by 17α-hydroxylase/C17-20 lyase (CYP17) to androstenedione [27. 28]. Finally, enzyme type 3 17β-hydroxysteroid dehydrogenase (17βHSD) metabolizes androstenedione into testosterone [27, 28]. Testosterone may be further metabolized into 17β-estradiol by aromatase enzyme action or into dihydrotestosterone (DHT) by 5α-reductase activity [28].

1.8.1: Sertoli Cells.

Sertoli cells are anchored to the basement membrane and surround the developing population of sperm cells [29]. Interestingly, Sertoli cells are the only somatic cells in the seminiferous epithelium [29]. Once believed to be simply a structural component of the tubule, Sertoli cells are now considered to be the cellular “nurses” that aid in spermatogenesis [29]. Each Sertoli cell may host a maximum number of developing germ cells [29]. Hence, testes with a high number of Sertoli cells are capable of producing large numbers of spermatozoa [29]. Sertoli cells are analogous to the granulosal cells of the ovarian follicle [29]. However, unlike granulosal cells, the Sertoli cell contains receptors for both follicular-stimulating hormone (FSH) and testosterone [29]. Because Sertoli cells possess receptors to different hormones, both protein- and steroid-based, they have the capability of producing a variety of

substances. Examples include androgen-binding protein, a testosterone transport protein that increases testosterone concentration in the seminiferous tubules to stimulate spermatogenesis; transferin, a blood plasma protein that deliveries iron ions to spermatocytes for proper development; and Mullerian-inhibiting substance (MIS) [29, 30].

1.8.2: Mullerian-inhibiting Substance.

Also known as anti-Mullerian hormone (AMH), MIS is a gonad-specific member of the transforming growth factor β (TGF-β) family that is synthesized early in Sertoli cell development [30]. During male sexual differentiation, MIS induces regression of the Mullerian ducts. In addition, MIS plays a critical paracrine role in the regulation of Leydig cell development and testosterone biosynthesis [30-34]. Indeed, MIS has been shown to inhibit proliferation of prepubertal progenitor Leydig cells and prevent regeneration of Leydig cells after chemical ablation by EDS [35-36]. These actions of MIS in the testis are mediated directly through the MIS type II receptor, which is abundantly expressed in Leydig cells [31]. Male transgenic mice over-expressing MIS have feminized genitalia secondary to fewer Leydig cell numbers and decreased serum testosterone concentrations [37]. Conversely, mice with targeted deletions of MIS [MIS knockout (MIS-KO)] and/or its receptor develop Mullerian structures but also manifest Leydig cell hyperplasia, focal Leydig cell tumors, and infertility [36]. Recently, MIS has been shown to induce FSH mRNA expression and enhance LH promoter activity in a pituitary cell line, indicating that changes in MIS action may also affect the hypothalamic-pituitary-gonadal axis [38]. Studies show that in adult MIS-KO mice, these is an increase in the

mRNA expression of P450c17A1, indicating that MIS may impair testosterone production by interfering with steroidogenic enzyme synthesis. [32].

In conclusion, it is clear that in the testis, testosterone production is a gradual and multi-step process that depends on endocrine as well as paracrine actions involving Leydig cells. Although these steps are critical, there are additional mechanisms that are also necessary for normal Leydig cell function. Indeed, the next section will further discuss the complex regulation of testicular function, but with a focus on estrogen receptor signaling.

Section 2: Estrogen Receptors

Introduction.

The metabolism of testosterone by the aromatase P450 enzyme results in the steroid hormone product 17β-estradiol (E2). 17β-estradiol is a key regulator of growth, differentiation, and function in a wide array of target tissues, including the male reproductive tract. The following section will focus on estrogen regulation of testicular function by way of estrogen receptor signaling.

2.1: Structure of Estrogen Receptors.

Estrogen signaling involves two estrogen receptors (ESRs), ESR1 and ESR2 (previously known as ERα and ERβ, respectively), both of which belong to the nuclear receptor family of transcription factors [39, 40]. ESR1 and ESR2 are located on chromosome 1 and 6 in the rat, respectively [40]. ESRs maintain conserved structure and possess three distinct functional domains [39, 40]. First, the N-terminal A/B domain is the most variable region, and the human ESR1 and ESR2 share less than 20% homology, suggesting that this domain may play a role in ER subtype-specific actions on target genes [40]. The A/B domain features an activation function (AF-1) that is ligand-independent and capable of promoter- and cell-specific activity [40, 41]. Second, the C-domain is the DNA-binding domain (DBD), and consists of two zinc finger motifs, which are not only responsible for DNA binding, but also for ESR dimerization, thus allowing the formation of homo- and heterodimers [40, 41]. This domain is highly conserved between ESR1 and ESR2 (95% homology), and the DBD from both

receptors bind to the same estrogen response element (ERE) [40]. Third, as part of the D/E/F ligand-binding domain (LDB), the D-domain is a flexible hinge between the DBD and the LBD [40]. The D domain, which is varies between ESR1 and ESR2 (only 30% homology), contains a nuclear localization signal that is necessary for nuclear translocation [40]. ESR1 and ESR2 share approximately 55% homology in LBD. The LBD plays an important role in ligand binding and receptor dimerization [41]. Also, the LBD contains a hormone-dependent activation function (AF-2) that works with AF-1 to recruit various co-regulatory protein complexes to the DNA-bound receptor [40]. Although the LBDs of ESR1 and ESR2 have very similar three-dimensional structures, the amino acids lining the ligand-binding pockets of ESR1 and ESR2 differ in two positions [41]. Moreover, the ligand-binding pocket of ESR1 is approximately 20% smaller than the one from ESR2, thus implying a selective affinity and pharmacology of ligands [41]. The F-domain of the LBD is less conserved between the two ESRs (less than 20% homology), and the function of this domain remains unclear [42]. One study suggested that in ESR1, the F domain plays a role in distinguishing estrogen agonists versus antagonists, possibly through interaction with cell-specific factors [42].

2.2: Estrogen Receptor Signaling Pathway.

In the absence of ligand, the receptor is sequestered in a multi-protein inhibitory complex within the nuclei of target cells [41]. The classical mechanism of ESR signaling involves estrogen binding to receptors, causing the ESR to dissociate from its chaperone proteins, and then undergoing phosphorylation and dimerization [41]. Hormone binding also induces a change in conformation within the LBD of the ESRs,

allowing coactivator proteins, such as amplified in breast cancer-1 (AIB1), nuclear-receptor coactivator-1 (NCoA-1/SRC1), p300, and CBP-associated factor (PCAF) to be recruited [41, 43]. These activated ESR-dimer complexes bind to specific estrogen receptor elements (EREs), cis-acting enhancers located within the regulatory regions of target genes [41, 43].

In humans, approximately one third of genes regulated by ESRs do not contain ERE-like sequences [41, 44]. It is possible that this mechanism of ERE-independent ESR activation is involve a tethering of the ligand-activated ESR to other transcription factors that are directly bound to DNA by their respective response elements [44]. For example, several genes are activated by 17β-estradiol through the interaction of ESRs with Fos and Jun proteins at activating protein-1 (AP-1) binding sites to induce or regulate transcriptional activity [44]. Studies suggest that genes containing GC-rich promoter sequences are regulated in an analogous manner, but include Sp-1 transcriptional factor interaction with the ESRs [43]. Other transcriptional factors such as nuclear factor kB (NF-kB) and signal transducer and activator of transcription (STAT) may also play a role in this mechanism [43]. Moreover, involvement of specific E2-ESRs-transcription factors depends on the ligand, the cell type, and the receptor subtype [41, 44]. In addition to the slow-acting, transcriptional effects of estrogen regulated by the classical nuclear ESRs, estrogen also mediates rapid effects that occur within seconds or minutes. These fast-acting effects include activation of different downstream signaling pathways, such as the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K) pathways, which regulate nuclear transcriptional events and cell proliferation [41, 45].

2.3: 17-β Estradiol and Estrogen Receptor Localization in the Testis.

During fetal development in the rodent, Sertoli cells and Leydig cells, but not spermatogonia, express aromatase. However, most cells within the adult testis express aromatase, including Leydig cells, Sertoli cells, spermatocytes, spermatids and spermatozoa [46, 47]. Indeed, aromatase expression in the postnatal rat testis is dependent on age, occurring mainly in Sertoli cells and germ cells of the prepubertal testis no more than 21 days of age (i.e., prepubertal age) and in Leydig cells past this period [48]. In contrast to rodents, aromatase activity and estrogen biosynthesis occur predominantly in adipocytes in men, and the testis produces only 10–25% of E2 in circulation [49].

Many studies indicate that in rodent testis, ESR1 is localized to Leydig cells and peritubular myoid cells, and ESR2 expression occurs in germ cells [50-52]. Beginning from 16 weeks of gestation in humans, ESR2 mRNA levels were 3 times greater than ESR1, but both were found to be present in the testis [53]. Interestingly, reports indicate that in the human testis, there are two variants of ERS2, designated ESR2.1 and ESR2.2 [54]. In fact, one study showed that ESR2.1 was more extensively expressed in Sertoli cells, germ cells and Leydig cells while ESR2.2 mRNA and protein were localized to spermatogonia in the fetal testis [55]. In the adult testis, both ESR1 and ESR2 are expressed in Leydig cells, Sertoli cells, elongating spermatids, and spermatocytes [56, 57]. In contrast, other studies show the localization of ESR1 in spermatids and mature spermatozoa, the presence of ESR2 in all germ cells, and the absence of ESR1 in Leydig cells [58, 59]. Also, there is higher ESR2.1 expression in

pachytene spermatocytes and round spermatids than in Sertoli cells and spermatogonia [58, 59]. Contrastingly, the expression of ESR2.2 is higher in Sertoli cells and spermatogonia than in spermatocytes [60]. Although the physiological importance of ESR2 isoforms in the human testis has yet to be clarified, studies indicate that ESR2.2 forms heterodimers with ESR1, thus attenuating its transcriptional activity [61]. However, ESR2.2 is unable to bind endogenous E2 or recruit cofactors via the AF-2 domain [61].

2.4: Studies Involving Transgenic Mice with Aromatase or ESR-Related Mutations.

Estrogen action is a requirement for normal testicular function, as evidenced by testicular abnormalities in men with aromatase gene mutations and in individuals lacking a functional ESR1 that resulted in undescended testis, decreased sperm production, and altered endocrine profiles [62]. Thus, development of knockout (KO) or transgenic mice with impairments to molecular pathways related to hormone production action has advanced our understanding of reproductive endocrinology. Such mutated mice may have targeted deletion of the aromatase gene (ARKO), ESR1 gene (ESR1KO), ESR2 gene (ESR2KO) or both ESR subtypes (1/2ESRKO).

ARKO mice adequately express ESR1 and ESR2 protein, but do not synthesize endogenous E2. Conversely, ESRKO mice have the capacity to produce E2 but lack either ESR1 and/or ESR2 protein [63]. Interestingly, a major problem associated with studies using ESRKO mice is the unintended abrogation of estrogen priming of extragonadal tissues during development [63]. In this respect, it is possible that the lack of endogenous E2 and/or ESR mediated activity during hypothalamic and pituitary

differentiation risks immature development of regulatory pathways in the HPT axis [63]. For example, ARKO mice have enlarged sex accessory organd, most likely due to observed elevations in serum testosterone levels and enhanced androgen action, and exhibit disruption in spermatogenesis, which is related to increased apoptosis of developing germ cells [63-65]. In contrast to the absence of E2, over expression of the aromatase gene and enhanced E2 production in mice induced cryptorchidism, spermatogenic arrest, increased Leydig cell proliferation, and decreased serum FSH and testosterone levels [63]. Disturbances in spermatogenesis were associated with decreased FSH levels while increased exposures to E2 induce Leydig cell hyperplasia [63, 66].

Unlike ESR1 regulation of testicular function, ESR2 mediates germ cell development, as documented in numerous studies. For example, inactivation of ESR1 in neonatal mice did not affect the number of Sertoli cells and spermatogonia, but ESR2 inactivation resulted in more than a 50% increase in the number of spermatogonia, the stem cells for spermatogenesis [63, 67]. However, there was no evidence of disruption in sperm production in ESR2KO mice and no indication of ESR1 expression in Sertoli cells [63]. Interestingly, spermatogenic arrest occurs in ESR1KO mice, suggesting ESR1-mediated regulation of Sertoli cell support of germ cell development [63]. Basis for this observation stems from experiments involving transplantation of germ cells from ESR1-/- donor to testes of wild-type ESR1+/+ recipient mice void of germ cells [63]. Mating of these germ cell transplant recipients with wild-type females produced offspring heterozygous for the mutation ESR1+/- but maintained the coat-color marker of the ESR1-/- donor mice [63]. These observations confirmed that ESR1 was

necessary in testicular somatic cells, but not germ cells, for normal spermatogenesis [63, 68]. Unlike ESR1KO, ESR2KO males are fertile but have a higher risk for prostate hyperplasia with senescence [63]. Moreover, male 1/2ESRKO mice are characterized by infertility, which is potentially due to ESR1 deficiency because these effects are absent in ESR2KO mice [63, 69].

In addition to deficiencies in testicular function, ARKO and ESRKO consistently display abnormal endocrine profiles. For example, serum LH concentrations were increased in adult ARKO mice [64, 65] while serum testosterone levels, though elevated at 12–14 wk of age, were similar to wild-type and mutant mice [65]. Similarly, the concentrations of serum testosterone, LH, and FSH were increased in ESRKO males in comparison to their wild-type littermates [63, 70]. It is possible that the alterations in serum gonadotropin levels result from homeostatic adjustments to estrogen feedback regulation on the hypothalamic-pituitary axis [63]. Changes in serum steroid hormone levels occur in the ESR1KO but are absent in ESR2KO mice, suggesting that testicular steroidogenesis is mainly regulated by ESR1 [63]. Furthermore, administration ICI 182,780, a pure, anti-estrogenic compound, decreased androgen biosynthesis in wild-type but not ESR1KO Leydig cells [63, 67]. The impairment in androgen biosynthesis ESR1KO Leydig cells were a consequence to changes in steroidogenic enzyme activity, as ESR1 deficiency decreased gene expression for CYP17A1 and 17βHSD, enzymes involved in testosterone production [63].

In essence, estrogen interaction with its receptors is a necessary regulator of steroidogenesis and spermatogenesis in the testis. Interestingly, estradiol is not the only substance that is capable of binding to ESRs. In this regard, there are exogenous

compounds that may activate or block ESR signaling, thereby possibly altering male reproductive function. Thus, the next section will address the subject of endocrine disruptors.

Section 3: Endocrine Disruptors

Introduction.

Endocrine disruptors (EDs) are compounds in the environment or diet that interfere with normal hormone biosynthesis, signaling, or metabolism [71]. Many EDs possess estrogenic activity and disrupt normal estrogen signaling, which is mediated by ESR1 and ESR2. ESR1 and ESR2 have both unique and overlapping physiological roles in regulating estrogen signaling which are dependent on the ligand, the availability of cofactors, and the content of the target cell [71]. EDs that interfere with ESR signaling can modify genomic and nongenomic ESR activity through direct interactions with ESRs or through modulation of metabolic enzymes that are critical for normal estrogen synthesis and metabolism [71]. Many exogenous ligands with similar affinities to endogenous estrogen, 17β-estradiol, have been shown to display selective binding affinities to ESR1 and ESR2 [72]. Both ESR subtypes have relatively large ligand binding pockets that dictate common structural characteristics of estrogenic ligands [71, 72]. In this respect, ESRs may bind numerous exogenous chemicals, and this broad specificity for ligands is what defines ESRs as promiscuous nuclear receptors [72]. Although EDs function through various mechanisms, many of them impact ESR signaling by directly binding with the ESR ligand binding pocket [71]. This section will discuss how the structural features of estrogenic compounds facilitate their interference with ESR signaling. Also, this section will highlight the general properties of EDs, such as pharmaceutical chemicals, bisphenols, and organochlorine pesticides.

3.1: Structural Features of Estrogenic Chemicals.

Crystal structures of the ligand binding domains (LBDs) of ESR1 and ESR2 identify features of the ligand binding pockets that are important for comprehending which compounds may possess estrogenic activity through direct interaction with ESR1 and/or ESR2. The ligand binding pockets of ESR1 and ESR2 are significantly larger, 450 and 390 $Å^3$, respectively, than E2, just 245 $Å^3$, thus allowing a variety of molecules access to the LBD [73, 74]. Both receptors display similarly high affinities for E2 because of the hydrophobic interactions and a network of hydrogen bonds between the hydroxyl groups on E2, a water molecule, and amino acids along the ligand binding pocket [73, 74]. Glu353 and Arg394 of ESR1 and Glu305 and Arg346 of ESR2 share hydrogen bonds with a water molecule and the hydroxyl in the A ring of E2 [74]. On the other side of the E2 molecule, the hydroxyl of the D ring shares a hydrogen bond with His524 of ESR1 that corresponds to His475 in ESR2 [74]. Ligands that bind directly to the LBD of ESRs are structurally analogous to E2 since they commonly have hydroxyl groups that undergo hydrogen bonding with the Glu, Arg, and His residues in the ligand binding pocket [74]. Although ESR1 and ESR2 have similar affinities for E2, there are many ligands that display selectivity for ESR1 and ESR2, including EDs. Differences in two amino acids within the ligand binding pocket are the main factors that define ESR subtype selectivity of many ligands [74]. Furthermore, in helix 5, Leu384 of ESR1 corresponds to Met336 of ESR2, and in loop 6-7, and Met421 of ESR1 corresponds to Ile373 of ESR2 [74]. Based on the structural similarities of known ESR selective compounds, structural features that determine ESR selectivity and binding affinity are

critical for predicting chemicals that will directly bind ESRs and subsequently interfere with ESR signaling.

3.2: Diethylstilbestrol.

One of the pioneering examples of endocrine disruption is the exposure of women to diethylstilbestrol (DES), a therapeutic medication intended for preventing miscarriages during pregnancy [75]. Prenatal exposure to DES was later discovered to correlate with vaginal cancer in daughters of mothers taking DES, and structural, functional, and cellular anomalies in the reproductive system of males exposed to DES *in utero* [75]. DES has been studied in great detail because of its significant adverse effects on humans *in utero,* thus serving as a model for predicting actions of other EDs. It appears that DES acts through both genomic and nongenomic ER signaling to induce adverse ER signaling [75]. Indeed, DES is structurally analogous to E2, and crystal structures of ESR LBD bound to DES illustrate that the hydroxyl groups of DES are located in similar positions as those of E2 [75]. Thus, DES has a higher binding affinity index for ESRs than E2 due to additional hydrophobic interactions that enhances stabilization of the ligand to the receptors [76]. Because it has a high affinity for ESRs, DES is a potent transcriptional activator by way of genomic signaling [75, 76]. Studies show that neonatal exposure to DES caused a higher incidence of uterine tumors [75]. In addition, recent evidence suggests that DES can impact nongenomic estrogen signaling as well. For example, DES treatment of MCF7 breast cancer cells results in rapid activation of PI3 kinase signaling and phosphorylation of AKT [77, 78]. Furthermore, activation of the signaling cascade leads to phosphorylation of EZH2, a

histone methyl transferase, and modification of the chromatin structure, all of which contributes to the epigenetic effects of DES [78].

3.3: Methoxychlor and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane.

Methoxychlor and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) are organochlorine pesticides that can exhibit estrogenic activity through interaction with the LBDs of both ESR1 and ESR2 [79]. DDT and methoxychlor promote uterine proliferation and cause abnormal follicle development, thereby impairing female reproductive function [80]. DDT occurs as a mixture of three isomers: *p*,*p′*-DDT, *o*,*p′*-DDT, and *o*,*o′*-DDT [81]. Interestingly, *o*,*p′*-DDT is the isomeric form responsible for the estrogenicity of DDT, for it has a high binding affinity for both ESR1 and ESR2 [80, 81]. Although DDT was banned in the 1970s, it continues to be a relevant ED of current research because of its persistence in the environment and its accumulation in adipose tissue [80]. In fact, DDT and its dechlorinated metabolite dichlorodiphenyldichloroethylene (DDE) have been detected in the adipose tissue of humans in various regions throughout the world [81].

As an alternative to DDT, methoxychlor was developed, but due to its endocrine disrupting properties, it was also banned by the United States Environmental Protection Agency in 2003 [82]. Most notably, methoxychlor stimulates uterotrophic activity and adversely affects fertility in rat models [83]. Although methoxychlor has relatively low binding affinities for ESR1 and ESR2 [79], the major metabolite of methoxychlor, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), possesses unique estrogenic properties, which likely mediates the endocrine disrupting mechanism of methoxychlor.

Interestingly, HPTE behaves as an agonist for ESR1, but an antagonist for ESR2 [84]. In HepG2 liver carcinoma cells transfected with ESR1 and a luciferase reporter associated with an estrogen responsive complement 3 promoter, HPTE greatly enhanced reporter expression relative to E2 [84]. In cells under the same conditions, except for transfection with ESR2 instead of ESR1, HPTE induced luciferase expression that was only 13% of the maximal E2 response, indicating its agonistic properties are ESR1 selective [84]. In addition, HPTE selectively antagonizes E2-mediated ESR2 activation. For example, HPTE co-treatment antagonized E2 induction of luciferase in HepG2 cells transfected with ESR2, but had no effect on E2-induced luciferase expression in cells transfected with ESR1 [84]. The selective agonistic properties of HPTE for ESR1 were also evident in similar chemicals with bishydroxyphenyl core structures, suggesting that many structurally-related compounds may regulate ESR signaling primarily through ESR1 [85]. In addition, studies show that ESR1KO mice do not respond to HPTE treatment, indicating that the effects of HPTE on gene regulation in the mouse uterus are dependent on ESR1 [86].

3.4: Bisphenol A.

Industrial chemicals are commonly used in large quantities, and such large volumes and application of these chemicals are responsible for the high risk of exposure to humans. One of the highest volume chemicals used in industry is bisphenol A (BPA), a monomer of polycarbonate plastics [86]. Although present in medical tubing and epoxy resin, exposure to BPA occurs primarily through ingestion since polycarbonate plastics are used in food and water containers [87]. Human daily intake is

estimated to be approximately 1 µg/kg [87]. A recent study conducted by the Center for Disease Control estimates that over 90% of the United States population excrete significant amounts of BPA in their urine [87]. Even with the numerous *in vitro and in vivo* experiments demonstrating the estrogenic effects of BPA, there is still controversy surrounding the potential for BPA to significantly interfere with normal endocrine function. Similar to DES, BPA has two phenolic rings, but displays much lower binding affinities and transcriptional potencies for ESR1 and ESR2 [79, 88]. Studies show that a 3-day exposure to a high dose (i.e., 100 mg/kg) of BPA can induce a uterotrophic response in immature CD-1 mice [89]. Considering the high doses of BPA necessary to elicit such responses, there continues to be uncertainty as to whether such evidence can be extrapolated to human exposure levels despite the many experimental systems in which BPA elicits estrogenic responses.

In light of the low affinity of BPA for ESRs, it is possible that the estrogenic effects of BPA are due to nongenomic ER signaling. Indeed, studies show that BPA can bind to membrane-associated ESR1 or GPR30 and initiate rapid signaling [90, 91]. Furthermore, BPA has a higher affinity for GPR30 compared to that of ESR1 and ESR2 [91]. In GH3/B6 pituitary cells, which have membrane-associated ESR1, nanomolar doses of BPA stimulated a calcium flux, indicating that rapid signaling can be triggered at environmentally relevant concentrations of BPA [90]. In micromolar ranges of BPA, nongenomic signaling is activated in MCF7 cells, as indicated by the presence of phosphorylated AKT and phosphorylated ERK proteins, and expression of temporarily-transfected reporter constructs specifically responsive to MAPK and PI3K activation [92]. Studies indicate that the ability of BPA to induce genomic ESR-regulated

transcription is dependent on the availability of cofactors [93]. In HeLa cells transfected with ESR1 or ESR2 and the co-activators TIF2 or SRC-1, BPA had greater impact on gene expression in cells expressing ESR2 and cofactor TIF-2, but similar effects in cells expressing ESR1 or ESR2 when SRC-1 was present [93]. Overall, it is clear that the complicated mechanisms through which BPA initiates signaling contribute to the controversy surrounding BPA and its role as an ED.

3.5: Phytoestrogens.

Phytoestrogens (also known as estrogen-like molecules or non-steroidal estrogens) are plant-based chemicals that have structures similar to that of E2 and display estrogenic properties, allowing them to act through ESR signaling pathways. Despite the structural similarity with E2, phytoestrogens are diphenolic yet non-steroidal compounds [93]. Phytoestrogens do not typically elicit severe, physiological abnormalities such as DES or other environmental EDs. Instead, phytoestrogens are often discussed in the context of promotion of bone and cardiovascular health, short-term treatment for menopausal symptoms, and cancer prevention [94]. However, because of their profound physiological effects mediated by ESRs, phytoestrogens are classified as EDs, especially with their widespread exposure to humans through diets containing plant material [94]. Indeed, daily phytoestrogen intake ranges from 0.15 to 3 mg per day in the United States [94, 95]. Although phytoestrogens have relatively weak affinities for ESR1 and ESR2, serum levels can reach near micromolar concentrations after a phytoestrogen-rich meal, significantly above the concentration of endogenously circulating estrogens (20-200 pg/mL) [96].

Phytoestrogenic compounds include more than 100 molecules, which are classified according to their chemical structure into 1) lignans (matairesinol, secoisolariciresinoldiglucoside), 2) coumestans (coumestrol, 4-methoxycoumestrol), and 3) stilbens (resveratrol), and 4) isoflavones (biochanin A, formonetin, genistein, daidzein) [94]. Most studies have mainly investigated the effects of isoflavones, as they are some of the most commonly ingested phytoestrogens [94].

Overall, it is clear that anthropogenic or synthetic EDs may cause detrimental physiological effects. However, naturally-occurring EDs, such as phytoestrogens, may be even more concerning since they are easily accessible within the environment. The next section will describe a specific category of phytoestrogens, namely isoflavones. There are various foods containing isoflavones. The following discussion will focus on the properties of isoflavones found in soybean products.

Section 4: Isoflavones and Soy-based Infant Formula

4.1: Isoflavones Metabolism.

In nature, isoflavones are present in more than 300 kinds of plants, but mostly found in the roots and seeds [97]. In plants, isoflavones are naturally present as sugar-bound, biologically inactive compounds called glucoconjugates [98, 99]. By action of intestinal bacteria, they are hydrolyzed to aglycones, or active forms [100]. The aglycone (i.e., unconjugated, sugar-free) forms of isoflavones may be further metabolized directly in the intestine, or are transported from the intestine to the circulatory system [100]. Isoflavones can be measured in blood within an hour of ingestion [101, 102]. In blood serum, the highest concentrations of isoflavones are reached within 2-8 hours after consumption [102]. Degradation of isoflavones occurs in the liver, where they are conjugated with glucuronic acid and to a lesser degree with sulfates [102]. Finally, most of the ingested isoflavones are eliminated from the body in urine or bile within 24 hours [103-105].

4.2: Mechanism of Isoflavones Interaction with Estrogen Receptors.

Because of their structural similarity with E2, isoflavones are able to bind to ESR1 and ESR2 [106]. Isoflavones act as agonists of ESRs, but their activity is lower than that of 17-β-estradiol [106]. In fact, most isoflavones have binding affinities that are approximately 100-500 times lower than that of 17β-estradiol [79]. However, at sufficiently high levels, the physiological effect of isoflavones may approach or exceed that of endogenous 17β-estradiol [79]. In addition, because isoflavones compete with estradiol for binding sites on ESRs, the effect of isoflavones on body homeostasis

depends also on the level of endogenous 17β-estradiol. For example, during a period of high concentrations of endogenous estrogens (e.g. women in the follicular phase of the menstrual cycle), the actions of isoflavones may be obstructed due to occupation of ESRs by 17β-estradiol, thus suppressing the full estrogenic potential of isoflavones [79, 107]. In a contrasting state of low levels of endogenous estrogens (i.e., men, women in menopause, after ovariectomy), the estrogenic activity of isoflavones may manifest because of the high availability of binding sites on ESRs [79, 107, 108]. In this regard, there is an increasing use of isoflavones as an alternative or complement to hormonal replacement therapy in postmenopausal women [109].

4.3: Isoflavone Interaction with the Metabolisms of Steroid Hormones.

Isoflavone interaction with sex steroids may occur through multiple signaling pathways. In addition, influence of isoflavones on the metabolism of sex hormones can be quite complex and may depend on several factors including species, sex, age, and hormonal status. Moreover, the dosage and duration of isoflavone administration are not always linearly related to the treatment effect, thus explaining the significant variability of research findings. For example, one study showed that rats fed with high amounts of isoflavones had reduced plasma testosterone concentrations [110], but such changes were not observed in several other studies administering lower doses of isoflavones [111-113]. Furthermore, no changes in serum 17β-estradiol levels were reported after isoflavone administration in normal rats [110-112], but increased 17β-estradiol serum levels were observed in ovariectomized rats [114].

4.4: Soy-Based Infant Formula.

Isoflavone sources include red clover, germs of alfalfa, and flaxseed, but can be produced by some types of bacteria and fungi. Soybeans are one of the richest sources of isoflavones. On average, dry soybeans contain 1.2-4.2 mg isoflavones [115]. In fact, the content of isoflavones in soybeans may vary due to many factors, including the soil in which they are grown, climate, stage of their maturity, or degree of processing [115]. In general, higher levels of processing are associated with lower concentrations of isoflavones [115]. Soy is present in a widespread of food products, including milk, ice cream, tofu, and infant formula.

Soy infant formula contains soy protein isolate and is fed to infants as a supplement to or a replacement for human milk, or as an alternative to cow milk formula. A number of studies worldwide have measured total isoflavone levels in infant formulas. In infant formulas manufactured in the United States, the range of total isoflavone levels reported in reconstituted or “ready-to-feed” formulas was 20.9-47 mg/L formula [116, 118].

The three major isoflavones found in soy infant formula are predominantly the glyconic forms: genistin, daidzin, and glycitin [119-121]. These isoflavones are biologically active in their aglycone forms (sugar-free and unconjugated): genistein, daidzein, and glycitein, respectively [120-122]. The relative abundance of the isoflavones in soy infant formula (expressed in aglycone units) is: genistein (58-66%) > daidzein (29-34%) and glycitein (5-8%) [120-122]. Studies show that 30-50% of humans metabolize daidzein into another estrogenic metabolite called equol [123]. Furthermore, based on *in vitro* studies, the relative estrogenic activity of the isoflavones is genistein > equol > daidzein > glycitein [121-123]. Thus, because of their high abundance and

estrogenic activities, genistein and daidzein are considered the most important biologically active forms of isoflavones in soy-based infant formula.

4.5: The Effects of Soy-Based Infant Formula on Male Reproductive Health.

Soy isoflavones are generating public concern due to the fact that their presence in soy-based infant formulas exposes neonates to significantly higher concentrations of exogenous estrogens than other sources of infant diet. For example, the mean isoflavone intake in breastfed or cow milk–based formula infants is 0.005–0.01 mg⁄day, while the intake of a soy-formula-fed infant in 6–47 mg⁄day [124]. In this regard, numerous studies have been conducted in order to elucidate the effects of early-life exposure to isoflavones on reproductive health.

Two studies reported the effects of feeding soy infant formula (versus standard cow milk formula) directly to infant marmosets (non-human primates) during lactational period (from PND4 or PND5 to PND35 to PND45; n=13 twin sets, plus four singletons) [30]. After treatment, the soy infant formula-fed males had significantly lower plasma testosterone levels than their cow milk formula-fed co-twins [125]. Histopathological analysis on the testes of a subset of the co-twins on PND35 to PND45 showed an increase in Leydig cell numbers per testes in the soy infant formula-fed marmosets compared to their cow milk formula–fed co-twin [125]. However, there was no significant change in testicular weight [125]. A follow up study was conducted on the remaining, sexually mature animals (80 weeks of age or older; n=7 co-twin sets) [126]. The males fed soy infant formula as infants had significantly heavier testes and an increase in the number of Leydig cells and Sertoli cells per testis as compared to cow milk formula-fed

controls [126]. However, there was no significant effect on timing of puberty, adult plasma testosterone levels, or fertility. It was suggested that the increase in testes weight was likely due to an increase in testicular cell populations [126]. Also, it is possible that the permanent change in Leydig and Sertoli cell populations may be an attempt at compensating for impaired Leydig cell function following soy infant formula exposure during lactation [126]. Furthermore, because the animals were also allowed to nurse from their mothers, the authors suggest that these studies may actually underestimate the effects of soy infant formula on human testicular development [126].

Similar to the effect described above in marmosets treated with soy infant formula during infancy, many studies reveal a generally consistent pattern of increased testicular weight in rats and mice treated with soy diet or isoflavone supplements during gestation and lactation or continuous exposure [127-130]. Exceptions include one study involving rats with a decrease in testis size [131], and two studies in rabbits resulting in no effect on testicular weight [132, 133]. One of the studies reporting an increase in testes weights (absolute and relative) was observed in rats 21 days of age with exposure to a soy-based diet supplemented with 5-1000 ppm and 50-1000 ppm isoflavones, respectively [127]. Furthermore, in 90-day-old male rats supplemented with the 50-1000 ppm isoflavones, absolute testes weights were decreased concurrently with an increase in serum testosterone levels at 1000 ppm isoflavone supplementation in comparison to controls [127]. Interestingly, decreased Leydig cell testosterone production was observed in adult male rats exposed to 1000ppm isoflavones supplementation during the perinatal period [127]. In another study, observations included an increase in absolute testes weights at PND28, but not at PND120, in male

rats continually exposed to soy-based diets containing from 36.1 to 1047 ppm isoflavones [128]. In contrast, one study documented decreased testes weights in soy-diet control males relative to soy-free diet fed males [129]. In addition, there was a decrease in spermatocyte nuclear volume per Sertoli cell on PND18 and PND25, and a decrease in Sertoli cell nuclear volume per testes at PND18 in soy-diet control males in comparison to soy-free diet males [131]. A different study observed demasculinization of the reproductive system in pups after gestational and lactational exposure to dams (0, 5, 300 ppm from gestational day 1 until postnatal day 21), including smaller testis size, fewer pups with preputial separation at postnatal day 40, lower plasma testosterone concentration (3.72 ± 0.55, 1.76 ± 0.31, and 2.23 ± 0.42 ng/ml in 0, 5, and 300 ppm, respectively), and fewer males capable of ejaculation at postnatal day 70 [134].

In closing, because numerous studies report conflict data after evaluating reproductive function in males exposed to soy isoflavones, there continues to be debate over the appropriate level of concern for this public health issue. In this regard, more research elucidating the acute and chronic effects of isoflavones exposure on testicular function is warranted.

Chapter 2: Thesis

Section 1: Background

The testis consists of two major compartments: the interstitum and the seminiferous tubules. Components of the interstitium include two generations of Leydig cells that develop consecutively between embryogenesis and puberty [1]. As the first generation, the fetal Leydig cells differentiate from the stromal cells of the testis cords on gestational day (GD) 12 in the rat [1]. On GD19, or just prior to birth, fetal Leydig cells achieve peak steroidogenic capacity [1]. In contrast to the prenatal development of the first generation, the second generation of Leydig cells appears by postnatal Day (PND) 11 [135]. These cells are known as progenitor Leydig cells and possess great proliferative capacity. In fact, the most rapid increases in Leydig cell numbers occur between PND 14 and PND 56 in the rat [6]. At approximately PND 35, progenitor Leydig cells enlarge to form immature Leydig cells [6]. At approximately PND 56, immature Leydig cells undergo one final round of cell division and transform into adult Leydig cells [8]. Although adult Leydig cells in the sexually mature testis no longer possess proliferative ability, they are fully capable of producing steroid hormones, specifically testosterone [28].

Testosterone synthesis involves transport of cholesterol by Steroidogenic Acute Regulatory (StAR) protein into the inner mitochondrial membrane where it is metabolized into pregnenolone via the P450 cholesterol side chain cleavage enzyme (P450scc/CYP11A1) [28]. Pregnenolone moves out of the mitochondria to the SER where it is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD) [28]. Subsequently, progesterone is converted by 17α-hydroxylase/C17-20 lyase

(CYP17A1) to androstenedione [28]. Finally, androstenedione is metabolized to testosterone by type 3 17β-hydroxysteroid dehydrogenase (17βHSD) [28].

Located separately from Leydig cells are Sertoli cells, which reside with the seminiferous tubule of the testis. Sertoli cells are best known for its main function of nurturing the developing sperm cells through the different stages of spermatogenesis [136]. In addition, Sertoli cells secrete a series of hormones, including Mullerian-inhibiting substance (MIS). As a member of the transforming growth factor-β family, MIS is a glycoprotein hormone responsible for inducing regression of the Mullerian ducts in the male embryo [136]. Neonatal MIS knock out (KO) mice are characterized by decreased germ cell numbers, suggesting that MIS regulates germ cell development [137]. Interestingly, MIS type II receptors (MISRII) are abundantly expressed on progenitor Leydig cells, immature Leydig cells and Sertoli cells [31]. This indicates that MIS may not only behave in an autocrine manner, but also works through a paracrine mechanism, namely regulation of Leydig cell function. Indeed, studies have demonstrated that MIS inhibits the progenitor Leydig cells proliferation, and regulates testosterone production [32, 138, 139].

Testosterone may be converted into 17β estradiol via aromatase P450, which is present in the rat Leydig cells and Sertoli cells [47]. Therefore, estrogen may influence testicular function in a paracrine or autocrine manner when bound to estrogen receptors (ESRs). In the rat testis, there are two types of ESRs: ESR1, which is predominantly expressed in Leydig cells, and ESR2, which is mainly expressed in Sertoli cells [63]. The endocrine profile of ESR1 knockout (KO) mice includes abnormal spermatogenesis, steroidogenesis, and fertility patterns [63]. Conversely, ESR2KO mice

retain normal characteristics of male reproduction, implying that ESR1 is a major regulator of testicular function [63].

In addition to localization within the testis, pituitary gonadotropes and hypothalamic nuclei mainly express ESR1 and ESR2, respectively, thus indicating a role for estrogen in the hypothalamus-pituitary-gonadal (HPG) axis, a major component of the endocrine system. The hypothalamus secretes GnRH, which binds to its receptors located on the anterior pituitary. Then, the anterior pituitary gland secretes follicle-stimulating hormone (FSH) and luteinizing hormone (LH), the primary tropic hormones that regulate testicular function. In the testis, FSH binding sites are exclusively expressed in Sertoli cells, and LH receptors (LHR) are solely located in Leydig cells. LH binding to LHR on Leydig cells results in testosterone secretion, which exerts a negative feedback onto both the hypothalamus and anterior pituitary and subsequently regulating GnRH and LH release, respectively.

It is clear that the male reproductive tract is closely regulated by the endocrine system, and thus justifies the increasing concern about exogenous compounds that may potentially disrupt this hormonal homeostasis. These compounds are known as endocrine disruptors, chemicals in the environment (i.e., food, air, water) that interfere with normal endocrine function. Endocrine disruptors include anthropogenic substances such as bisphenol A (BPA), a plasticizer, and dichlorodiphenyltrichloroethane (DDT), the controversial and subsequently discontinued ingredient of pesticides. There are also naturally-occurring endocrine disruptors such as phytoestrogens, a diverse group of plant compounds that are structurally and functionally homologous to that of 17β-estradiol. Increasing number of studies indicate that prolonged environmental exposure

to phytoestrogens may cause severe abnormalities in the male reproductive system, especially during fetal development.

A highly-consumed source of phytoestrogens by man is soybeans. Soybeans predominantly contain a mixture of the isoflavones genistin and daidzin, which are hydrolyzed in the gastrointestinal tract to genistein and daidzein, respectively [140]. Soy isoflavones are generating public concern due to the fact that their presence in soy-based infant formulas exposes neonates to significantly higher concentrations of exogenous than other sources of infant diet. For example, the mean isoflavone intake in breastfed or cow milk–based formula infants is 0.005–0.01 mg⁄day, while the intake of a soy-formula-fed infant in 6–47 mg⁄day [124]. Furthermore, previous studies demonstrated that perinatal exposure of male rats to high isoflavone concentrations significantly alters testosterone production that persists into adulthood [141]. Therefore, further investigation of the effects of early-life exposure to isoflavones on reproductive health is warranted.

In order to address the issue of neonatal exposure to soy-based diets, the National Toxicology Program (NTP) commissioned a panel of experts to evaluate soy infant formula in 2010. After reviewing studies which, most of the time, focus on adverse effects of human exposure to genistein only, the NTP released a brief concluding that there was minimal concern for isoflavone exposure through soy infant formula. The panel also identified data gaps and research needs in the current literature in laboratory animals that limited its utility for reaching conclusions for infants fed soy infant formula. According to the panel, one major experimental design component lacking in the current research of soy infant formula was isoflavone exposure during the lactational period,

which is the most accurate period for comparison of human exposure to soy-based formula. Many of the animal studies considered in the NTP evaluation of soy infant formula included isoflavone exposure during the continuous period of gestation, lactation, and post-weaning, making it difficult to distinguish the effects that might have occurred as a result of exposure during lactation alone. Therefore, data from young animals exposed directly to soy infant formula or isoflavones during the period of lactation only would provide a better approximation of human exposure of infants fed soy infant formula. Another problem with current studies investigating soy infant formula was the disregard for possible mixture effects. Although humans are exposed to mixtures of various chemicals present in the environment, research evaluating the effects of substances on living systems is frequently conducted using one compound at a time. For example, laboratory animal studies that involve administration of genistein only do not address the potential for interaction between other estrogenic isoflavones present in soy infant formula (i.e., daidzein). Indeed, numerous compounds may exhibit primarily independent actions, and many complex interactions may occur if two chemicals act at different but related targets. Therefore, in addition to continual research differentiating the individual actions of soy isoflavones, more studies considering the additive behavior of genistein and daidzein in a living system are necessary.

There are three objectives to the present study. First, we will examine the importance of lactational period as a critical window of exposure to isoflavones. Second, we will determine whether the isoflavone-induced changes in testicular function are due to dose-additive behavior of genistein and daidzein or singular action by each compound. And third, we will investigate the ability of isoflavones indirectly regulating

Leydig cells via Sertoli cell product MIS, and thereby perturb the paracrine relationship between Leydig and Sertoli cells.

Section 2: Experimental Protocol

All animal procedures were performed using a protocol approved by the Institutional Animal Care and Use Committee of Auburn University and in compliance with the Guide for the Care and Use of Laboratory Animals.

2.1: Housing of Animals.

Time-bred Long-Evans dams (n =9-11 per group), weighing approximately 250 g, were given three days of acclimation time in the housing facility of the Department of Laboratory Animal Health, College of Veterinary Medicine, Auburn University. Each dam resided in a standard plastic cage (length, 0.47 m; width, 0.25 m; height, 0.22 m) containing wood chip bedding (Lab Products, Inc.) and glass water bottles. Polypropylene cages and glass bottles were used to eliminate background exposure to estrogens, which may occur when using polycarbonate cages [17]. Animals lived under constant conditions of light (12L:12D) and temperature (20-23°C), with free access to pelleted food.

2.2: Diet Formulation and Experimental Design.

In the present study, diets containing casein (control), genistin, daidzin, or whole soybeans, were used and formulated to be identical in terms of energy, micronutrients, cholesterol, calcium, and phosphorus (Table 1, 2). The isoflavone concentrations in experimental diets were 516 ppm genistin (GEN), 484 ppm daidzin DAID), and soybean meal (containing 516 ppm genistin with 484 ppm daidzin (SOY) (1000 ppm total isoflavone) based on the assayed content of genistin and daidzin, and calculation of the

equivalent aglycone as specified by the manufacturer, Harlan-Teklad (Table 1). Pregnant dams were maintained on a soy-free, casein-based diet from GD 12 to PND 1, and then fed experimental diets from PND 2 to PND 22. All male rats were reared with their natural littermates and not cross-fostered within groups. Because of the small population of Leydig cells in the prepubertal rat testis, each isolation procedure required at least 22 rats from each group. Therefore, male rats were selected randomly from members of each litter per group for analysis of Leydig cell function and other parameters from PND 22. At weaning (i.e., PND 22), all male offspring were fed the soy-free, casein-based control diet until they were sacrificed at 22-, 35-, and 96-days of age to collect serum and testicular samples.

2.3: Leydig Cells Isolation Procedure.

CO_2 asphyxiation was performed on all Long-Evans rats for sacrifice, followed by castration. To begin Leydig cell isolation, the capsule surrounding each testis was surgically removed. Whole testicular tissue was digested in dissociation buffer (DB) containing 0.25mg/mL collagenase and 46µg/mL dispase for one hour in a shaking bath, with manual shaking every twenty minutes. Seminiferous tubules were removed by draining the mixture of DB and testicular fractions through nylon mesh (pore size, 0.2 µm; Spectrum Laboratories, Inc.). The filtered DB containing the remaining testicular fractions were centrifuged at 2500 revolutions per minute (rpm) for 15 min at 4ºC. Cell fraction filtration was omitted from Leydig cell isolation procedure involving rats 96 days of age, when seminiferous tubules are sedimented by gravity in sedimentation buffer containing 10 mg/ml of bovine serum albumin. Cell fractions were added to Percoll

solution, and then centrifuged at 13500 rpm for 60 min to isolate gradient of Leydig cells. Leydig cell numbers were estimated using a hemocytometer, followed by purity assessment via histochemical staining for 3BHSD using 0.4 mM etiocholan-3β-ol-17-one enzyme substrate (catalog no. E-5251, lot no. 11K4058; Sigma).

2.4: Investigation of Leydig Cell Proliferation.

After in vivo exposure to isoflavones, progenitor and immature Leydig cell proliferative activity were assessed by [^{3}H] thymidine incorporation assays. Separate aliquots of 22- and 35-day-old Leydig cells were separately incubated in triplicate in culture medium containing 10 ng/ml of LH and 1 µCi/ ml of [^{3}H] thymidine for labeling (specific activity: 80 Ci/mmol; lot no. 3106516; DuPont-NEN Life Science Products). After labeling for three hours, progenitor and immature Leydig cells were washed in Dulbecco phosphate-buffered saline (PBS) containing ethylenediaminetetra-acetic acid (EDTA; catalog no. E-5134, lot no. 074K0004; Sigma) and were divided into aliquots of 0.5 X 10^6 cells and lysed in microcentrifuge tubes containing 500µL of hyamine hydroxide (catalog no. 802387, lot no. 8493J; MP Biomedicals) followed by scintillation counting. To assess whether Leydig cell proliferation was associated with cell-cycle progression and mitosis, cyclin D3 expression was measured from whole-cell lysates.

2.5: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis **(***SDS-PAGE) and Western Blot Analysis.*

Leydig cells were homogenized in lysis buffer containing a protease inhibitor cocktail, including 80 µM aprotinin, 5mM bestatin, 1.5 mME-64, 0.5MEDTA, 2mM

leupeptin, and 1 mM peptatin (catalog no. 78410; Pierce Biotechnology, Inc.). To remove cellular debris, homogenized Leydig cells were centrifuged at 12,000 rpm for 10 min at 4ºC. Protein concentration was measured using the BioRad protein assay (Bio-Rad), with bovine serum albumin as standard. Protein aliquots (5-15 µg) diluted with Laemmli sample buffer (catalog no. 1610737; Bio-Rad) and 2-mercaptoethanol (catalog no. M7154; Sigma,Inc.) working solution were loaded onto 10% Tris-HCl mini acrylamide gels for SDS-PAGE and electrotransferred to nitrocellulose membranes (catalog no. 1620096; Bio-Rad) for 70-90 minutes. Membranes were blocked with 5% blotto (non-fat, dried milk in 0.1% PBS Tween 20) and subsequently incubated overnight at 4ºC with primary antibodies (Table 3). Blots were washed in 0.1% PBS Tween 20 three times (five minutes per wash) to remove unbound antibodies. Membranes were incubated with the respective horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 90 minutes at room temperature. Then, membranes were incubated with chemiluminescent developing reagent (catalog no. E2400; Denville) for 2 minutes. Membranes were subsequently exposed to x-ray film (catalog no. E-3012; Denville) to visualize the presence of the appropriate proteins. Protein expression was quantified using the Epson 4490 Perfection scanning software (Epson-America). Analysis of immunoblots involved measuring protein levels as the optical density of the bands using Doc-It LS software (Ultra-Violet Products Ltd.). Proteins were normalized to B-Actin.

2.6: Investigation of Steroid Hormone Production.

To measure testosterone production, testicular explants (0.05-0.10 g from PND 22 male rats, 0.1-0.15 g from PND 35 male rats, 0.15-0.30 g from PND 96 male rats) and aliquots of Leydig cells (0.5 to $1X10^6$) were incubated in microcentrifuge tubes. The culture medium consisted of DMEM/F-12 buffered with 14 mm NaHCO3 and 15 mm HEPES (Sigma Chemical Co., St. Louis, MO), and containing 0.1% BSA (MP Biomedicals LLC, Aurora, OH) and 0.5 mg/mL bovine lipoprotein (Sigma Chemical Co., St. Louis, MO). Incubations were conducted with a maximally stimulating dose of 100 ng/ml ovine LH [(National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)] at 34 C for 3 h. The concentration of T was assayed in duplicate in testicular explant samples and aliquots of spent media by tritium-based radioimmunoassay (RIA). To measure serum levels of testosterone and estradiol, serum was separated from trunk blood collected at the time that male rats were sacrificed at d 22, 35, and 96 postpartum. Serum testosterone and estradiol levels were measured by RIA using tritium-labeled testosterone and estradiol (both from PerkinElmer Life and Analytical Sciences, Boston, MA), respectively.

2.7: Statistical Analysis.

Data are presented as the mean ± SD. Assays involving material collected from animal studies were performed three to five times. Data were analyzed by one-way ANOVA followed by Dunnett test for multiple group comparisons (GraphPad, Inc.). Differences of $P \leq 0.05$ were considered to be significant.

Section 3: Results

Lactational exposure to isoflavones induces Leydig cell proliferation, and alters serum and testicular steroid hormone production in PND 22 male rats

Lactational exposure of male rats to a combination of genistein and daidzein in the form of a whole soybean diet significantly increased progenitor Leydig cells proliferation compared to control ($P < 0.01$), as determined by [^{3}H] thymidine uptake (Fig. 1A). Western blot analysis demonstrated that increased cell proliferation was related to greater cyclin D3 protein expression (Fig. 1B) ($P < 0.05$). Results of radioimmunoassays (RIA) indicated that serum estradiol and testosterone levels were significantly decreased in prepubertal male rats (PND 22) lactationally exposed to the maternal whole soybean diet than in control animals (Fig. 2A and Fig. 2B, respectively) ($P < 0.05$ and $P < 0.01$, respectively). Conversely, testosterone production significantly increased in testicular explants collected from 22-day old male rats exposed to the daidzin and whole soybean diets (Fig. 3A) ($P<0.01$). Furthermore, Leydig cell testosterone production was decreased in prepubertal male rats exposed to the genistin ($P<0.01$), daidzin ($P<0.01$), and whole soybean ($P<0.01$) diets (Fig. 3B). Table 4 summarizes the previously-mentioned effects of lactational exposure to soy isoflavones on testicular function and steroid hormone production in male rats at 22 days of age.

Lactational exposure to isoflavones induces proliferation and alters testicular steroid hormone production in male rats 35 days of age

Immature Leydig cell proliferation increased in 35-day-old male rats lactationally exposed to the whole soybean diet (Fig.4) ($P < 0.05$). There were no differences in serum testosterone and estradiol levels in pubertal male rats (Fig. 5A and B). However,

testicular testosterone concentrations were increased in animals 35 days of age in the soybean diet group (Fig. 6A) ($P < 0.05$). In contrast, steroidogenic capacity was decreased in Leydig cells from 35-day-old male rats exposed only to the daidzin diet (Fig. 6B) ($P < 0.05$). Table 5 summarizes the effects of lactational exposure to soy isoflavones on testicular function and steroid hormone production in 35-day-old male rats.

Lactational exposure to soy isoflavones impairs steroidogenic capacity in adult male rats

At 96 days of age, serum estradiol levels were unchanged in male rats exposed to the isoflavones-containing diets (Fig. 7A). However, serum testosterone levels were decreased in the whole soybean diet group (Fig. 7B) ($P < 0.05$). Although testicular testosterone concentrations were unaffected in animals exposed to the soy isoflavone diets (Fig. 8A), Leydig cell testosterone production was decreased in male rats exposed to the genistin ($P<0.01$), daidzin ($P<0.01$), and whole soybean ($P<0.01$) diets, as shown in Fig. 8. Furthermore, the adverse effects of soy isoflavone exposure on steroidogenic capacity in Leydig cells was reflected in augmented expression of StAR (Fig. 9A) ($P < 0.01$), and enzyme protein expression [i.e., HSD3B (Fig. 9C) ($P < 0.01$), and CYP17A1 (Fig. 9D) ($P < 0.01$)], except for CYP11A1 (Fig. 9B), which remained unchanged, and HSD17B3, which was decreased (Fig. 9E) ($P < 0.05$). Table 6 summarizes the effects of lactational exposure to soy isoflavones on testicular function and steroid hormone production in adult male rats. The possibility of isoflavones action is reinforced by observations of increased in ESR1 expression (Fig. 10) ($P < 0.01$). However, there were

no differences in AR and LHR expression in adult male rats exposed to soy isoflavones in the diet (Fig. 11 and Fig. 12, respectively).

Soy isoflavones regulate MIS expression in progenitor Leydig cells

Lactational exposure of 22-day-old male rats to the daidzin and whole soybean diet decreased MIS expression in Sertoli cells (Fig. 13A) ($P < 0.05$ and $P < 0.01$, respectively). In addition, MISRII levels were increased in prepubertal male rats exposed to the genistin ($P < 0.01$), daidzin ($P < 0.01$), and whole soybean ($P < 0.01$) maternal diets, as shown in Fig. 13B. Together, these observations suggest that MIS plays a role in isoflavone-mediated regulation of Leydig cell function in male rats 22 days of age.

Section 4: Discussion

In the present study, there were three main objectives: (1) to determine the importance of the lactational period as a critical window of exposure to soy isoflavones, (2) to examine the individual actions and possible mixture effects of soy isoflavones genistin and daidzin on testicular function, and (3) to investigate the potential for soy isoflavones to disrupt the paracrine relationship between testicular Leydig and Sertoli cells. Results showed that lactational exposure to a combination of genistein and daidzein increased Leydig cell proliferative activity in 22- and 35-day-old male rats. We also observed that exposure to daidzein, alone or in combination with genistein, decreased expression of MIS, a paracrine hormone demonstrated to inhibit postnatal Leydig cell proliferation. In this regard, mice that overexpress human MIS are known to have reduced Leydig cell numbers [32]. In contrast, MISKO mice develop Leydig cell hyperplasia [138]. Therefore, it is possible that the high concentrations of soy isoflavones suppressed MIS expression, and subsequently contribute to the induction of Leydig cell proliferation. MIS has also been indicated to mediate testosterone production. For example, plasma testosterone concentrations were diminished in 60-day-old MISKO male mice [139]. Thus, observations of reduced serum testosterone levels in 22- and 35-day-old male rats when exposed to genistein and daidzein together may be a consequence of decreased MIS expression, at least in part.

In the present study, testicular testosterone production increased in prepubertal male rats exposed to genistein and daidzin together, and in pubertal rats exposed to daidzein alone or with genistein during lactational period. Testicular testosterone levels

primarily depend on two factors: (1) the number of Leydig cells and (2) testosterone production per Leydig cell. Although we observed decreased Leydig cell testosterone production in 22- and 35-day-old rats exposed to genistein and/or daidzein, greater Leydig cell numbers may explain the overall increase in testicular testosterone production.

Results also showed diminished serum 17β-estradiol concentrations in prepubertal male rats exposed to both genistein and daidzein during the lactational period. Therefore, diminished 17β-estradiol levels are possibly the result of reduced testosterone availability. The decrease in 17β-estradiol levels may be due a decrease in aromatase P450, the enzyme responsible for converting testosterone to 17β-estradiol. Although not explored in the present study, other studies have shown that isoflavones regulate aromatase expression. For instance, phytoestrogens, including genistein and daidzein, inhibit aromatase expression in human granulosa-luteal cells [142]. Moreover, it is possible that impairment to aromatase in these male rats may have adversely affected spermatogenesis, steroidogenesis, and fertility, as seen in transgenic aromatase-KO mice [46, 65].

Interestingly, most of the disturbances to testicular function and steroid hormone production in pre-pubertal male rats resulted from exposure to genistein and daidzein together, suggesting a dose-additive behavior when both isoflavones are administered to neonates. Also, lactational exposure to a combination of genistein and daidzein had similar outcomes in comparison to our previous perinatal (i.e., gestational and lactational periods) exposure studies. For example, perinatal exposure to 1000 ppm isoflavones also induced proliferation [141], and increased serum testosterone levels

[127] and decreased Leydig cell testosterone production [141]. It is possible that differences in testicular function and steroid hormone production are related to duration of exposure to dietary isoflavones. Overall, lactational exposure to soy isoflavones is a critical time of exposure to soy isoflavones in neonatal male rats.

The endocrine disruptor hypothesis states that early developmental exposure to exogenous estrogenic chemicals can disrupt male reproductive development and impair fertility at later stages of life. In the present study, the isoflavones-induced changes observed before and during puberty were still apparent into adulthood. Indeed, serum testosterone levels remained decreased in 96-day-old male rats exposed to both genistein and daidzein during lactational period. It is interesting to note that previous studies show that perinatal exposure to genistein and daidzein together increased serum testosterone levels [127, 141] in contrast to observations in the present study in which lactational exposure to both isoflavones decreased serum testosterone production in adult male rats. Nevertheless, changes in androgen secretion and serum testosterone levels may be linked to ESR1-mediated activity, as ESR1 expression was increased in adult male rats exposed to a combination of genistein and the daidzein. Because ESR1KO mice display elevated serum testosterone levels [70], enhanced ESR1 protein expression likely renders Leydig cells more sensitive to the action of soy isoflavones, which affects steroid hormone production. The results also demonstrated that lactational exposures to soy isoflavones affected differentiation of Leydig cell steroidogenic capacity, as evidenced by altered expression of proteins involved in androgen biosynthesis (i.e., StAR and steroidogenic enzymes). Although we observed increased StAR protein levels and yet a decrease in Leydig cell testosterone production,

it is suggested that augmented StAR expression is due to a decrease in phosphorylated StAR, which is required to make cholesterol available for androgen biosynthesis [141, 143]. Therefore, decreased androgen biosynthesis likely resulted from disruption of StAR phosphorylation after exposure to both genistein and daidzein during the lactational period. In addition, 3βHSD and CYP17A1 protein expressions were increased. Increased steroidogenic enzyme activity is probably the result of homeostatic adjustment to decreased cholesterol availability. However, there was a marked decrease in 17βHSD protein expression. The 17βHSD enzyme is involved in the final step in androgen biosynthesis; that is, converting androstenedione to testosterone. Moreover, reports associate suppressed MIS activity to inhibition of *Hsd17b* gene expression in adult MISKO mice [139]. Therefore, reduced MIS expression may contribute to decreased 17βHSD activity, ultimately impairing androgen biosynthesis in adult male rats exposed to both genistein and daidzein during the lactational period. However, it is also possible that the other enzymes in the androgen biosynthetic pathway are subject to interference by isoflavones-mediated MIS activity. For example, the observed increase in CYP17A1 expression may be also due to decreased MIS expression [32]. Thus, lactational exposure to both genistein and daidzein soy isoflavones may exert differential effects on steroidogenic enzyme capacity in Leydig cells.

There was evidence demonstrating that exposure to both isoflavones during the lactational period interferes with Leydig-Sertoli cell paracrine action, ultimately resulting in abnormal Leydig cell function. In this regard, I believe that the next direction on this topic includes a further evaluation of Sertoli cell action on testicular function . Indeed, in

addition to MIS, Sertoli cells secrete numerous products, including androgen-binding protein, which increases testosterone concentration in the seminiferous tubules to stimulate spermiogenesis. Because of its extensive regulation of sperm development, studies investigating the effects of lactational exposure to isoflavones on the expression of Sertoli cell products are warranted.

Section 5: Conclusion

Based on observations from the present study, the lactational period is a critical window of exposure to soy isoflavones. Indeed, Leydig cell proliferative activity and steroidogenesis in male rats were affected after lactational exposure to soy isoflavones in the maternal diet. Also, impairment of Leydig cell steroidogenesis persisted into adulthood because adult Leydig cells from animals exposed to soy isoflavones produced lesser testosterone amounts than control. Importantly, more parameters were affected by exposure to the whole soy bean diet (SOY) than was seen in the genistein (GEN) and daidzein (DAID) diet groups implying that soy isoflavones may act in a dose-additive manner. Moreover, results suggest that lactational exposure to soy isoflavones may interfere with paracrine signaling mechanisms between Sertoli and Leydig cells because MIS and MISRII protein expression was altered after exposure to soy isoflavones, respectively. Overall, given the homology in reproductive physiology among mammalian species, soy-based diets have the potential to disrupt the endocrine function of the mammalian testis especially following early-life exposures. However, additional studies are required to describe the long-term effects, if any, of the use of soy infant formulas in the population.

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Table 1: Experimental Protocol. Time-bred, Long Evans dams were fed diets containing casein, 516ppm genistin (GEN), 484 ppm daidzin (DAID), or soybean meal containing 516ppm genistin with 484 ppm daidzin (SOY; 1000 ppm total isoflavone) from PND1 until PND22.

Group 1: **Soy-free,** **casein-based diet** **(Control; n=9)**	**Group 2:** **516 ppm genistin diet** **(n=10)**
Group 3: **484 ppm daidzin diet** **(n=10)**	**Group 4:** **Soybean meal diet** **516 ppm genistin &** **484 ppm daidzin** **(1000 ppm total)** **(n=11)**

Ingredients (g/Kg)	Experimental Diets			
	Control Diet	Genistin Diet	Daidzin Diet	Soybean Diet
Wheat	380	380	380	271
Corn	354.39	353.57	353.59	250.39
Corn Gluten Meal	50	50	50	-
Casein	106.5	106.5	106.5	-
Genistin	-	0.823	-	-
Daidzin	-	-	0.796	-
Soybean Meal (48%)	-	-	-	372

Table 2: Crude composition of experimental diets. Soybean diet contains 516ppm genistin and 484ppm daidzin (total isoflavones content:1000ppm genistin and daidzin). Dashes indicate absence of the respective ingredient.

	Molecular Weight (kDa)	Catalog no.	Dilution factor
ACTB	42	sc-1616	1:2000
AR	110	sc-815	1:2000
Cyclin D3	35	sc-182	1:500
CYP11A1	60	sc-18043	1:500
CYP17A1	55	sc-46081	1:10000
ESR1	66	ab2746-50	1:1000
17βHSD	35	sc-66415	1:1000
3βHSD	42	sc-28206	1:1000
LHR	79	sc-26342	1:1000
MIS	65	sc-6886	1:1000
MISRII	63	sc-67287	1:2000
StAR	30	sc-25806	1:2000

Table 3: Compilation of primary antibodies recognizing respective proteins. Ab= Abcam, Inc., sc= Santa Cruz biotechnologies.

Lactational Exposure to Genistein and Daidzein Together Induced Progenitor Leydig Cell Proliferation

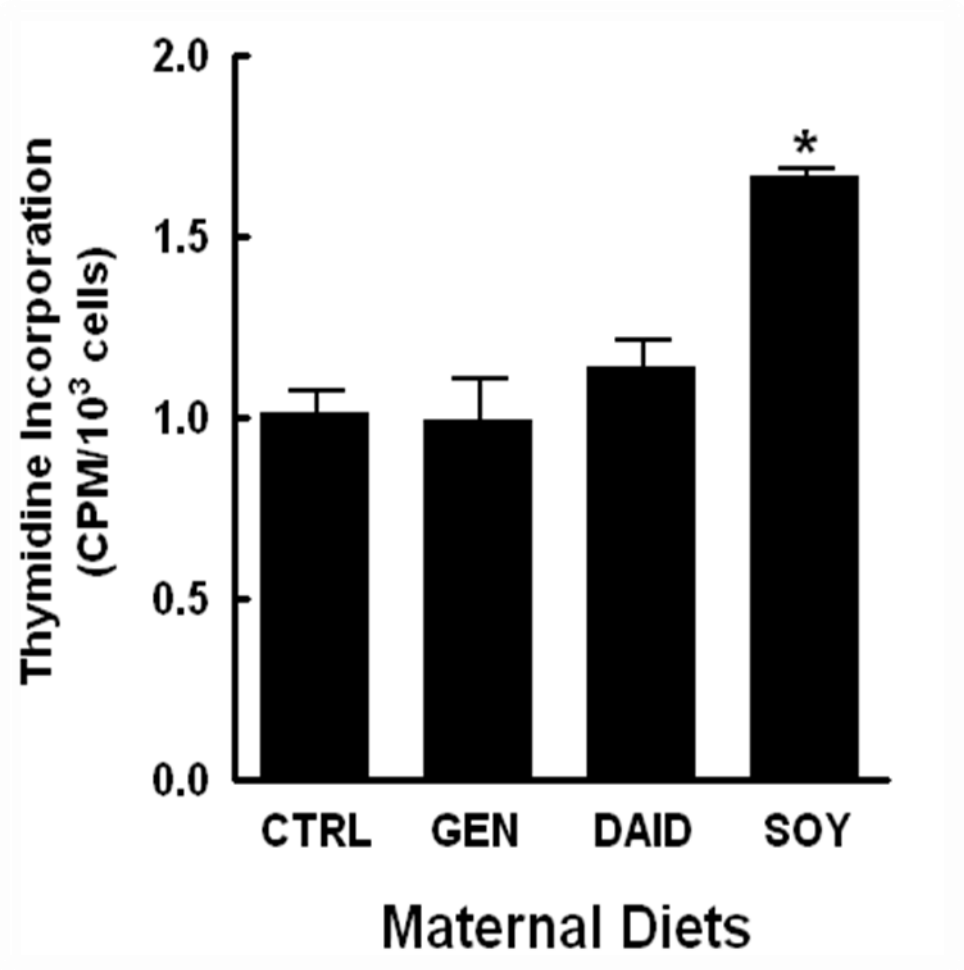

Figure 1A: Proliferative activity of progenitor Leydig cells after lactational exposure of neonatal male rats to dietary isoflavones. Progenitor Leydig cell proliferative activity was determined by tritium-labeled thymidine incorporation assay followed by scintillation counting. * P< 0.01 versus control.

Lactational Exposure to Genistein and Daidzein Together Increased Cyclin D3 Protein Expression in Progenitor Leydig Cells

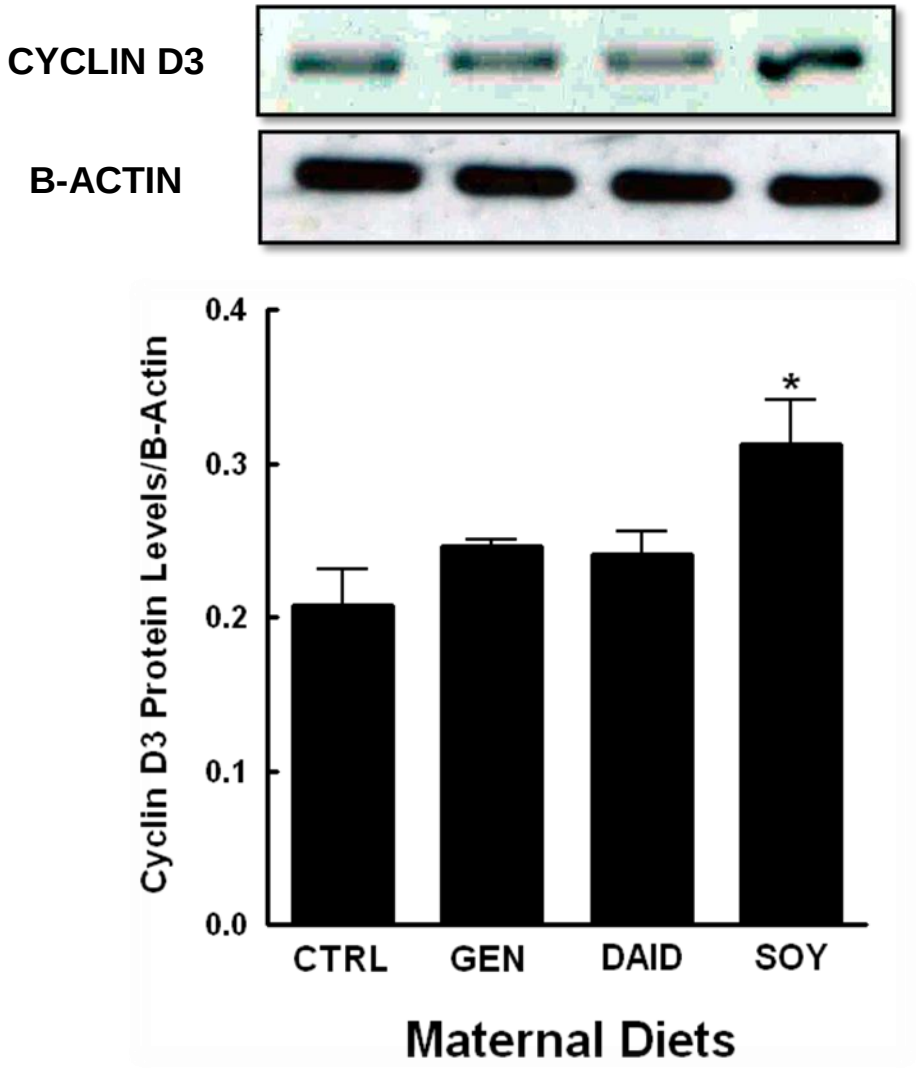

Figure 1B: In order to confirm proliferative activity in progenitor Leydig cells isolated from neonatal male rats, progenitor Leydig cell expression of cell-cycle protein cyclin D3 was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of three Western blots. Cyclin D3= 35 kDa, B-Actin= 42 kDa. *P < 0.05 versus control.

Lactational Exposure to Genistein and Daidzein Together Decreased Serum Estradiol Levels in Neonatal Male Rats (PND 22)

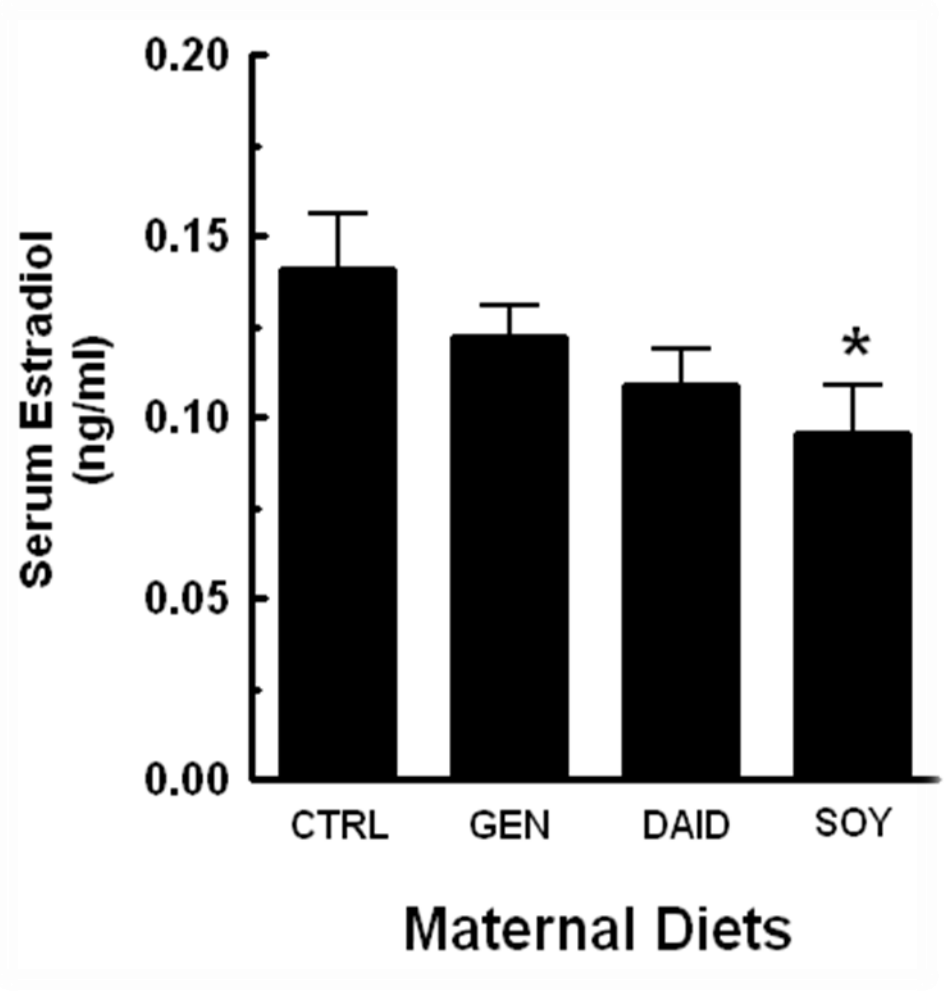

Figure 2A: Serum estradiol levels in neonatal male rats after lactational exposure to dietary isoflavones. Serum was separated from blood, which was collected at sacrifice. Serum estradiol levels were measured by radioimmunoassays. * P< 0.05 versus control.

Lactational Exposure to Genistein and Daidzein Together Decreased Serum Testosterone (T) Levels in Neonatal Male Rats (PND 22)

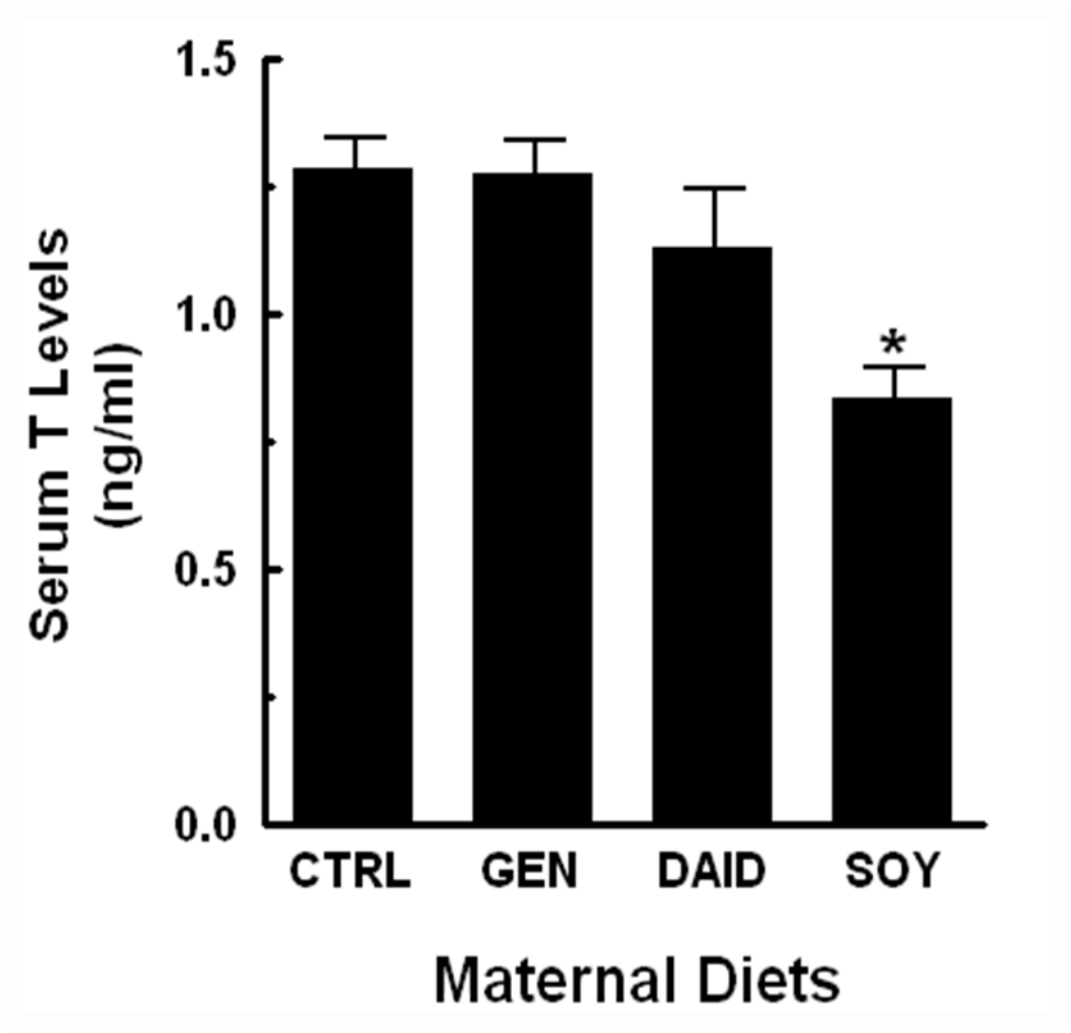

Figure 2B: Serum testosterone (T) levels in neonatal male rats after lactational exposure to dietary isoflavones. Serum was separated from blood, which was collected at sacrifice. Serum T levels were measured by radioimmunoassays. * P< 0.01 versus control.

Lactational Exposure to Daidzein Alone or with Genistein Increased Testicular Testosterone (T) Production in Neonatal Male Rats (PND 22)

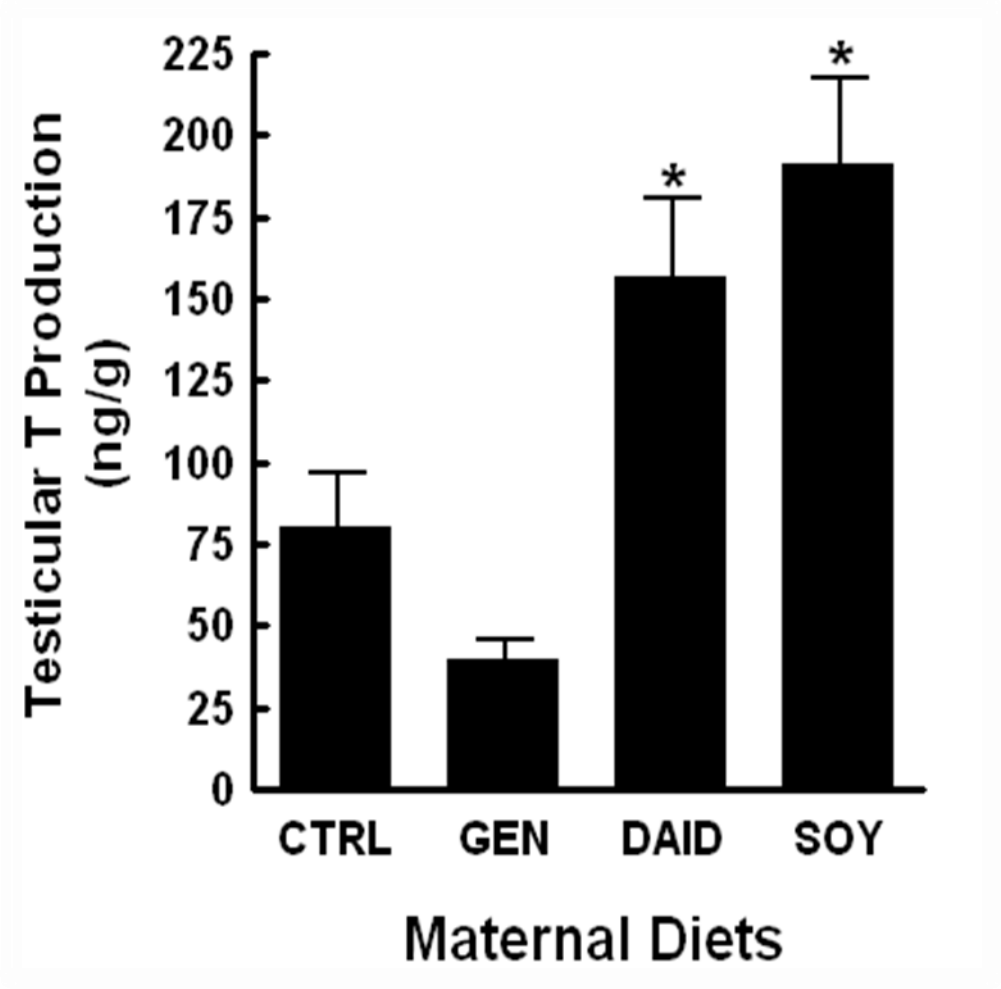

Figure 3A: Testosterone (T) production per gram of testicular explants collected from testes of neonatal male rats after lactational exposure to dietary isoflavones. Testicular T production was measured in aliquots of spent media after 3 h incubation by radioimmunoassays. * P< 0.01 versus control.

Genistein and Daizdzein, Acting Singly or Together, Decreased Progenitor Leydig Cell Testosterone (T) Production after Lactational Exposure (PND 22)

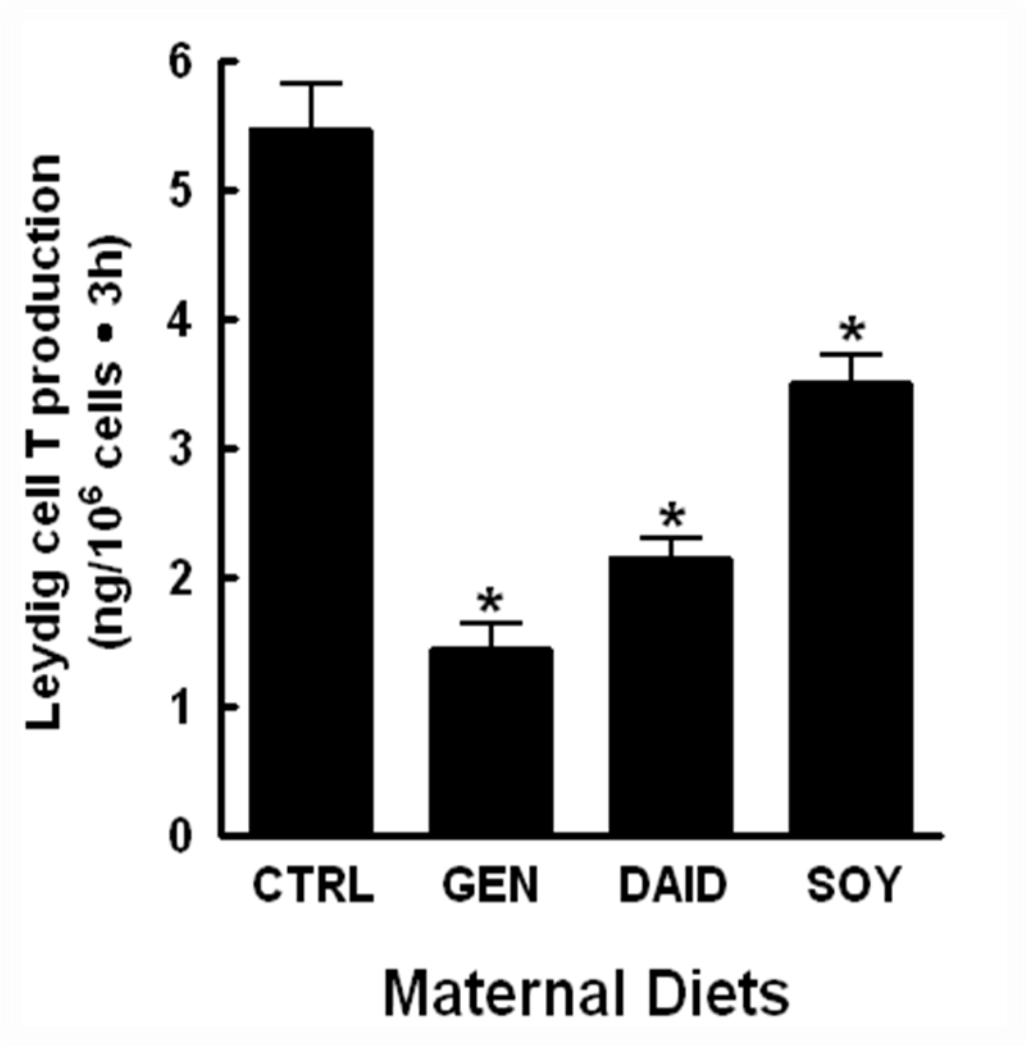

Figure 3B: Testosterone (T) production by progenitor Leydig cells collected from testis of neonatal male rats after lactational exposure to dietary isoflavones. Leydig cell T production was measured in aliquots of spent media after 3 h incubation by radioimmunoassays. * P< 0.01 versus control.

Table 4: Summary of the effects of lactational exposure to dietary isoflavones on endocrine parameters in neonatal male rats. ND = No significant difference compared to control

	Genistein	**Daidzein**	**Genistein & Daidzein**
Leydig cell Proliferation	ND	ND	⇧
Estradiol Levels	ND	ND	⇩
Serum T Levels	ND	ND	⇩
Testicular T Production	ND	⇧	⇧
Leydig Cell T Production	⇩	⇩	⇩

Lactational Exposure to Genistein and Daidzein Together Increased Immature Leydig Cell Proliferation in Prepubertal Male Rats (PND 35)

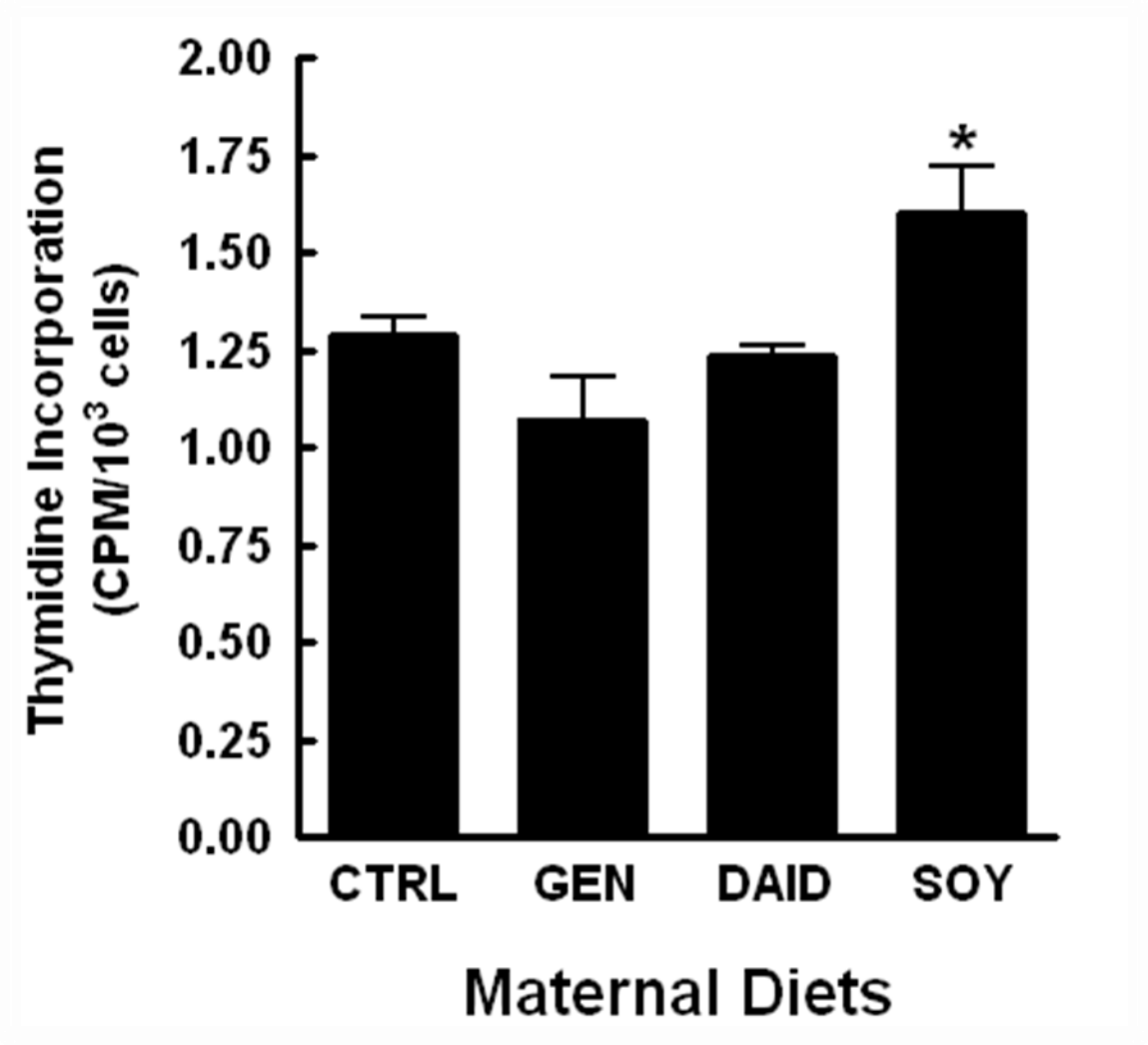

Figure 4: Proliferative activity of immature Leydig cells after lactational exposure of prepubertal male rats to dietary isoflavones. Immature Leydig cell proliferative activity was determined by tritium-labeled thymidine incorporation assay followed by scintillation counting. * P< 0.05 versus control.

Serum Estradiol Levels Were Similar in Prepubertal Male Rats (PND 35) with and without Lactational Exposure to Soy Isoflavones

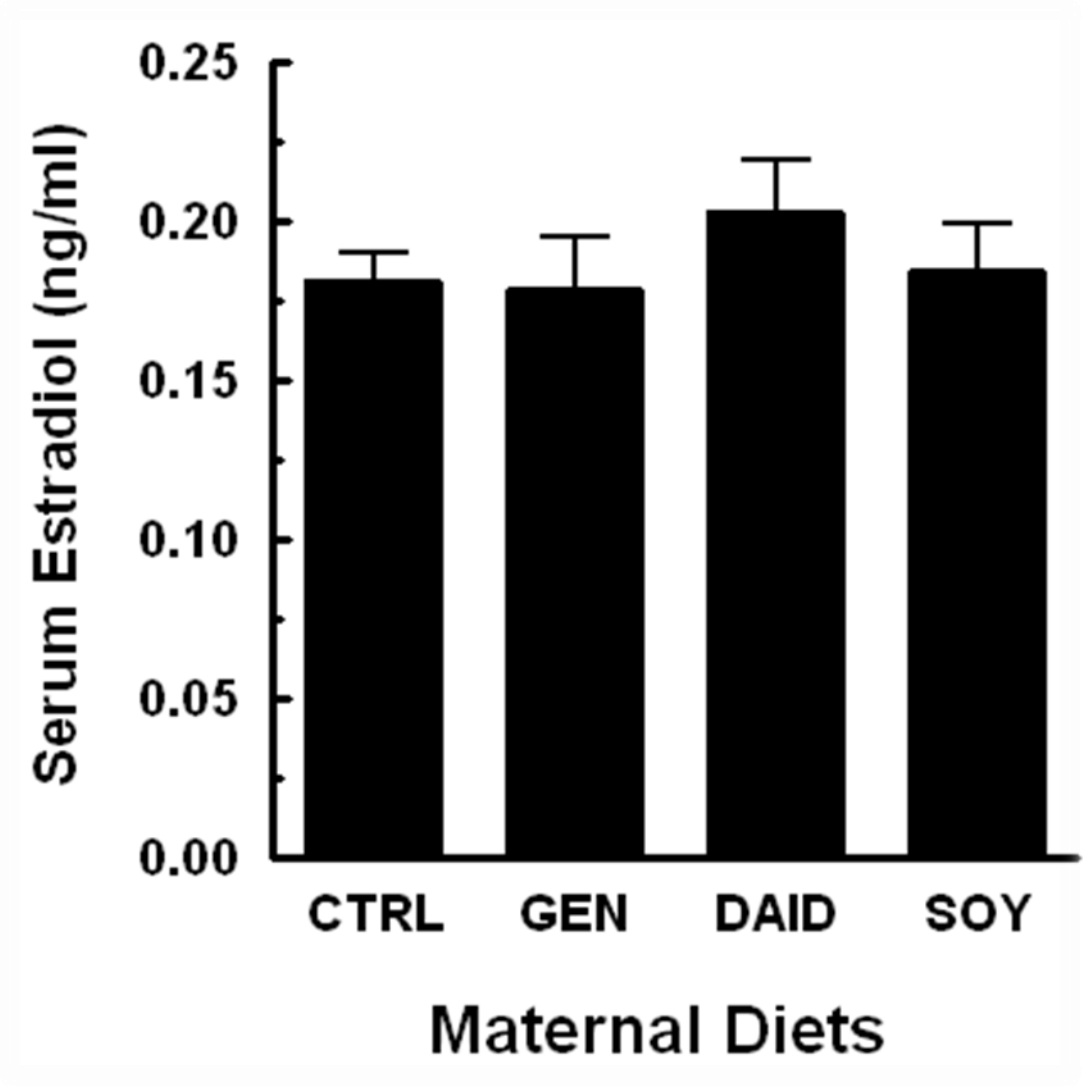

Figure 5A: Serum estradiol levels in prepubertal male rats after lactational exposure to dietary isoflavones. Serum was separated from blood, which was collected after sacrifice. Serum estradiol levels were measured by radioimmunoassays.

Serum Testosterone (T) Levels Were Similar in Prepubertal Male Rats (PND 35) with and without Lactational Exposure to Soy Isoflavones

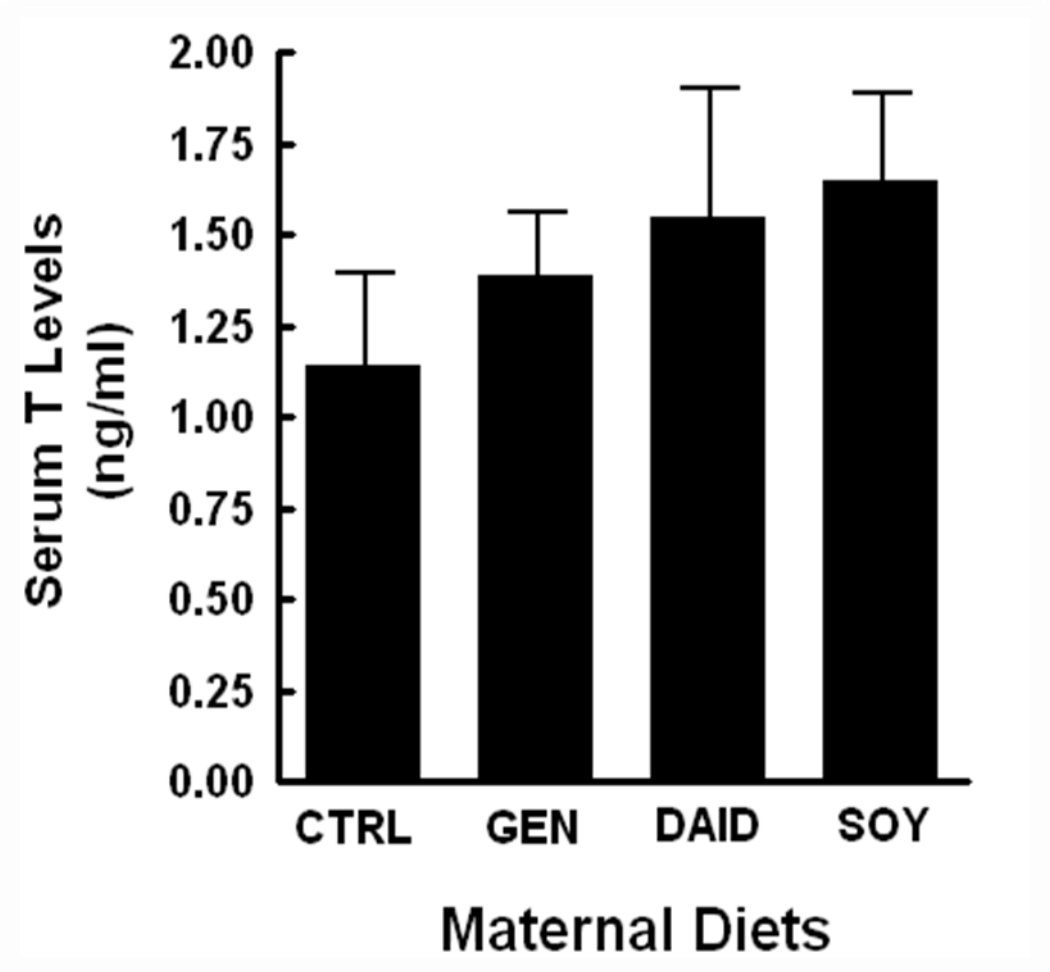

Figure 5B: Serum testosterone (T) levels in prepubertal male rats after lactational exposure to dietary isoflavones. Serum was separated from blood, which was collected after sacrifice. Serum T levels were measured by radioimmunoassays.

Lactational Exposure to Genistein and Daidzein Together Increased Testicular Testosterone (T) Production in Prepubertal Male Rats (PND 35)

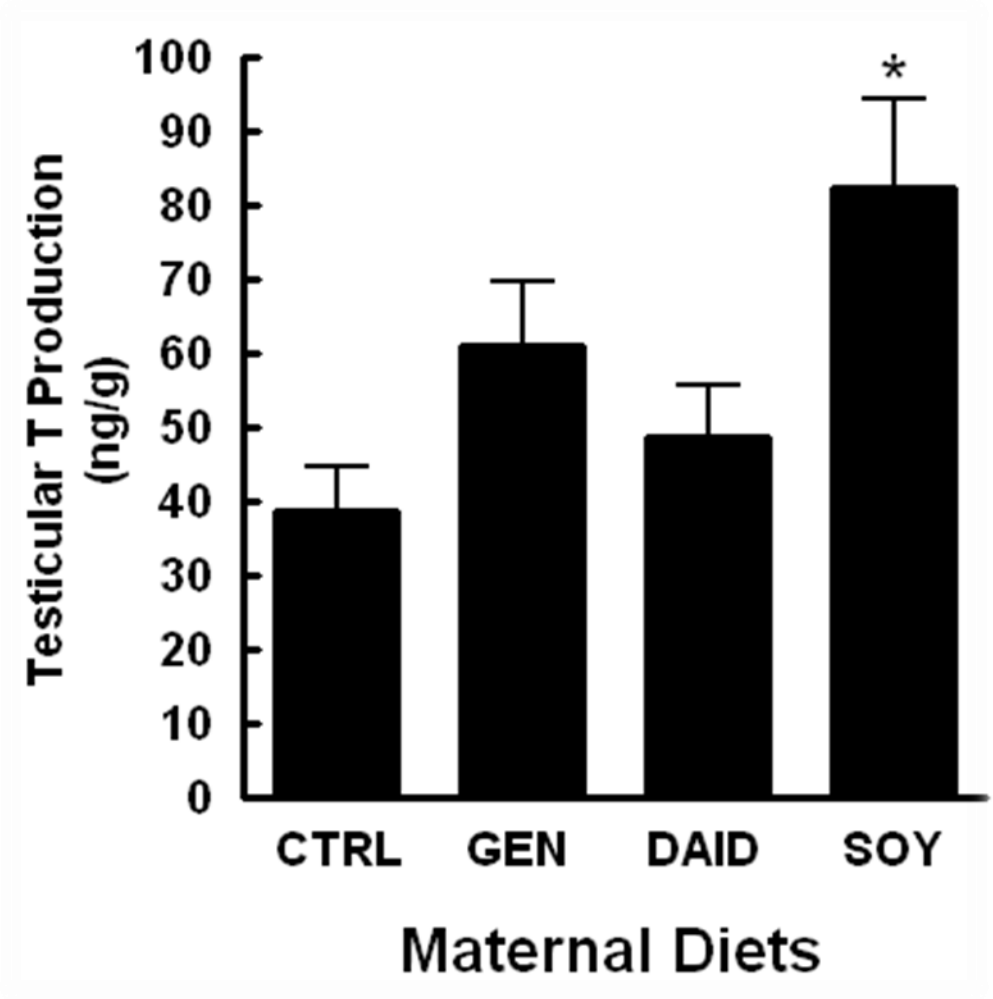

Figure 6A: Testosterone (T) production per gram of testicular explants collected from testes of prepubertal male rats after lactational exposure to dietary isoflavones. Testicular T production was measured in aliquots of spent media after 3 h incubation by radioimmunoassays. * P< 0.05 versus control.

Lactational Exposure to Daidzein Decreased Immature Leydig cell Testosterone (T) Production in Prepubertal Male Rats

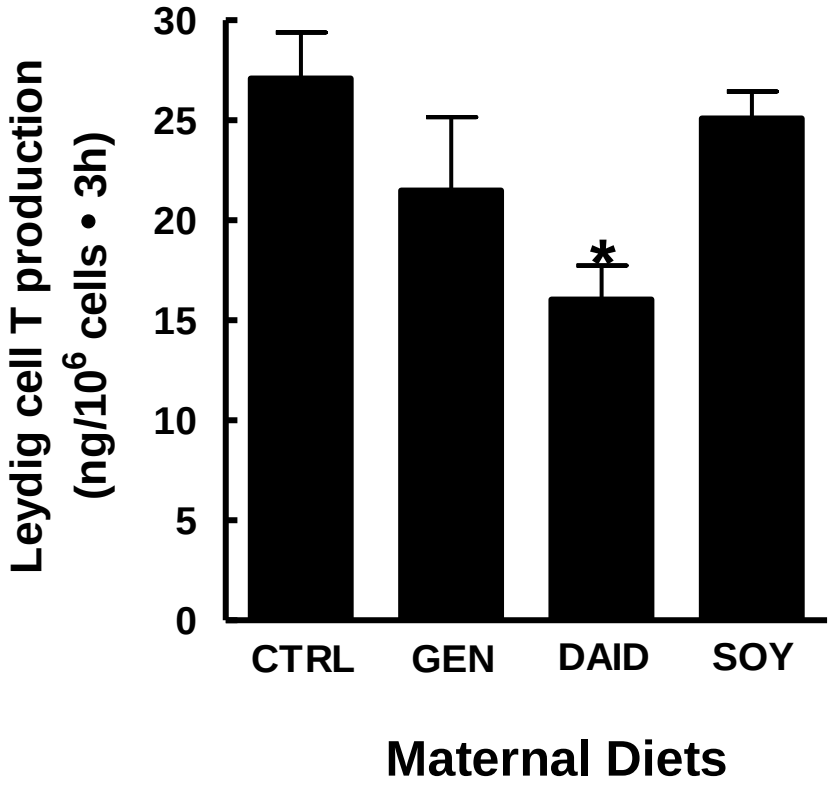

Figure 6B: Testosterone (T) production by immature Leydig cells collected from testis of prepubertal male rats after lactational exposure to dietary isoflavones. Leydig cell T production was measured in aliquots of spent media after 3 h incubation by radioimmunoassays. * P< 0.05 versus control.

	Genistein	Daidzein	Genistein & Daidzein
Leydig cell Proliferation	ND	ND	⇧
Estradiol Levels	ND	ND	ND
Serum T Levels	ND	ND	ND
Testicular T Production	ND	ND	⇧
Leydig Cell T Production	ND	⇩	ND

Table 5: Summary of the effects of lactational exposure to dietary isoflavones on endocrine parameters in male rats 35 days of age. ND = No significant difference compared to control

Serum Estradiol Levels Were Similar in Sexually-mature Male Rats (PND 96) with and without Lactational Exposure to Soy Isoflavones

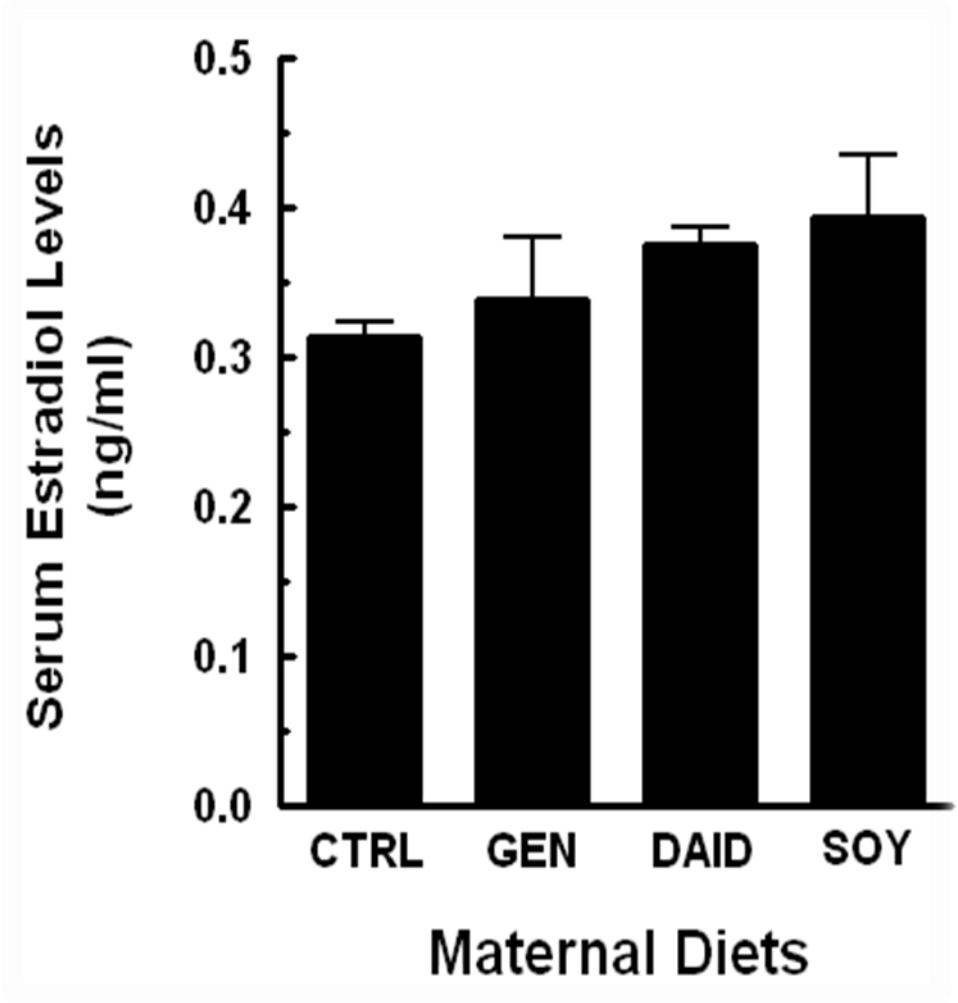

Figure 7A: Serum estradiol levels in sexually-mature male rats after lactational exposure to dietary isoflavones. Serum was separated from blood, which was collected after sacrifice. Serum estradiol levels were measured by radioimmunoassays.

Lactational Exposure to Genistein and Daidzein Together Decreased Serum Testosterone (T) Levels in Sexually-mature Male Rats (PND 96)

Figure 7B: Serum testosterone (T) levels in sexually-mature male rats after lactational exposure to dietary isoflavones. Serum was separated from blood, which was collected after sacrifice. Serum T levels were measured by radioimmunoassays. * P< 0.05 versus control.

Testicular Testosterone (T) Production Was Similar in Sexually-mature Male Rats (PND 96) with and without Lactational Exposure to Soy Isoflavones

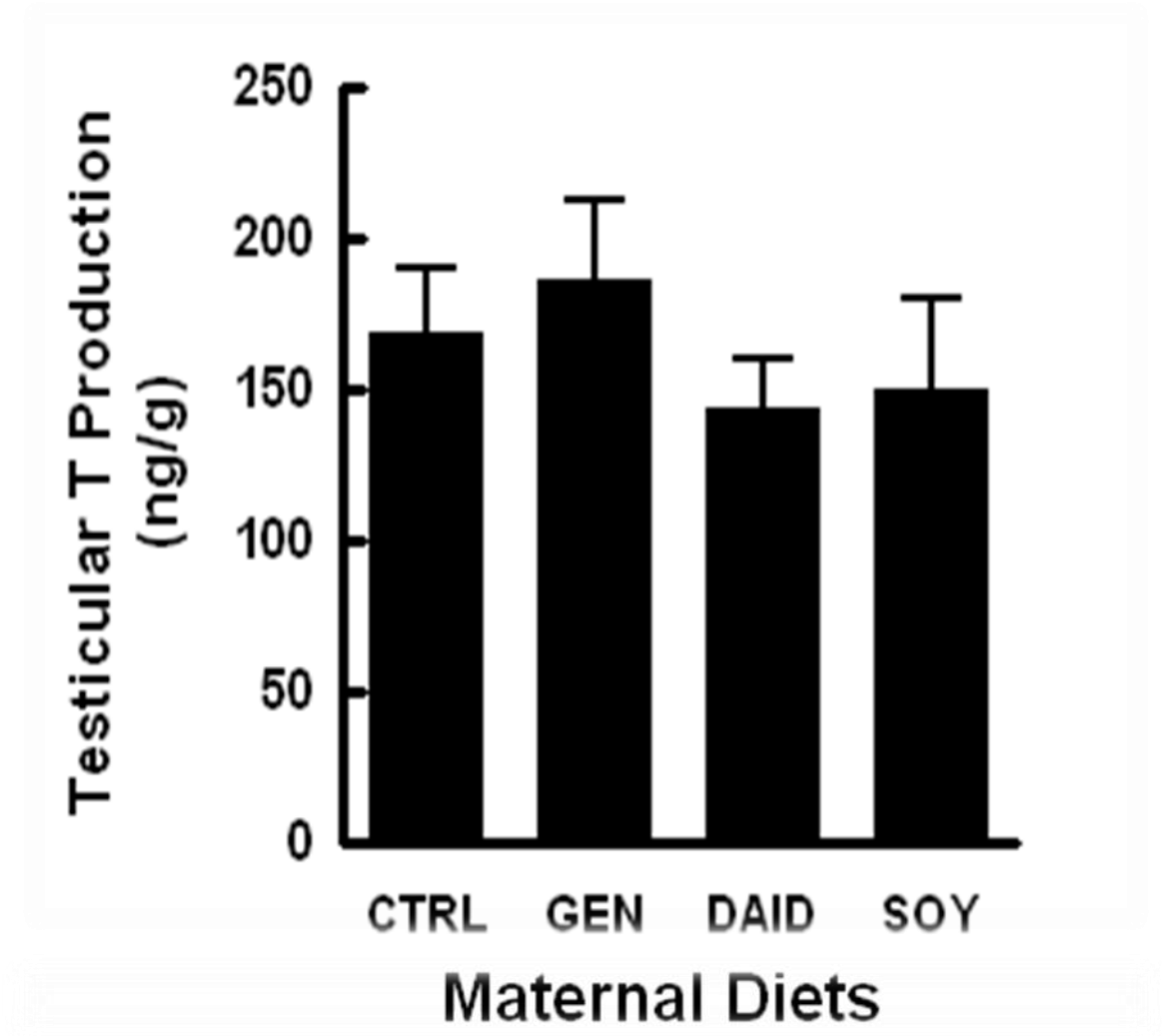

Figure 8A: Testosterone (T) production per gram of testicular explants collected from testes of sexually-mature male rats after lactational exposure to dietary isoflavones. Testicular T production was measured in aliquots of spent media after 3 h incubation by radioimmunoassays.

Genistein and Daizdzein, Acting Singly or Together, Decreased Adult Leydig Cell Testosterone (T) Production after Lactational Exposure

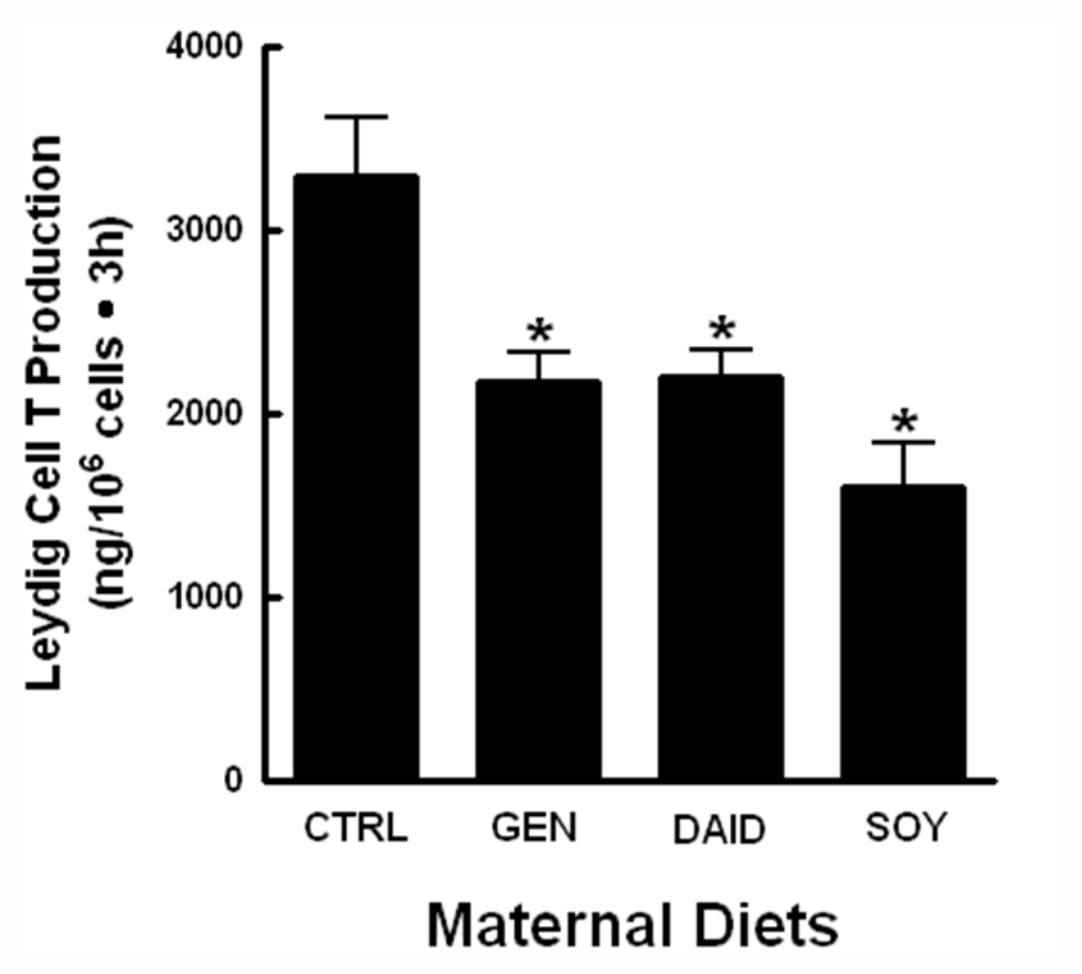

Figure 8B: Testosterone (T) production by adult Leydig cells collected from testis of sexually-mature male rats after lactational exposure to dietary isoflavones. Leydig cell T production was measured in aliquots of spent media after 3 h incubation by radioimmunoassays. * P< 0.01 versus control.

	Genistein	Daidzein	Genistein & Daidzein
Estradiol Levels	ND	ND	ND
Serum T Levels	ND	ND	⇩
Testicular T Production	ND	ND	ND
Leydig Cell T Production	⇩	⇩	⇩

Table 6: Summary of the effects of lactational exposure to dietary isoflavones on endocrine parameters in PND 96 male rats. ND = No significant difference compared to control.

Lactational Exposure to Genistein and Daidzein Together Increased Steroidogenic Acute Regulatory (StAR) Protein Expression in Adult Leydig Cells (PND96)

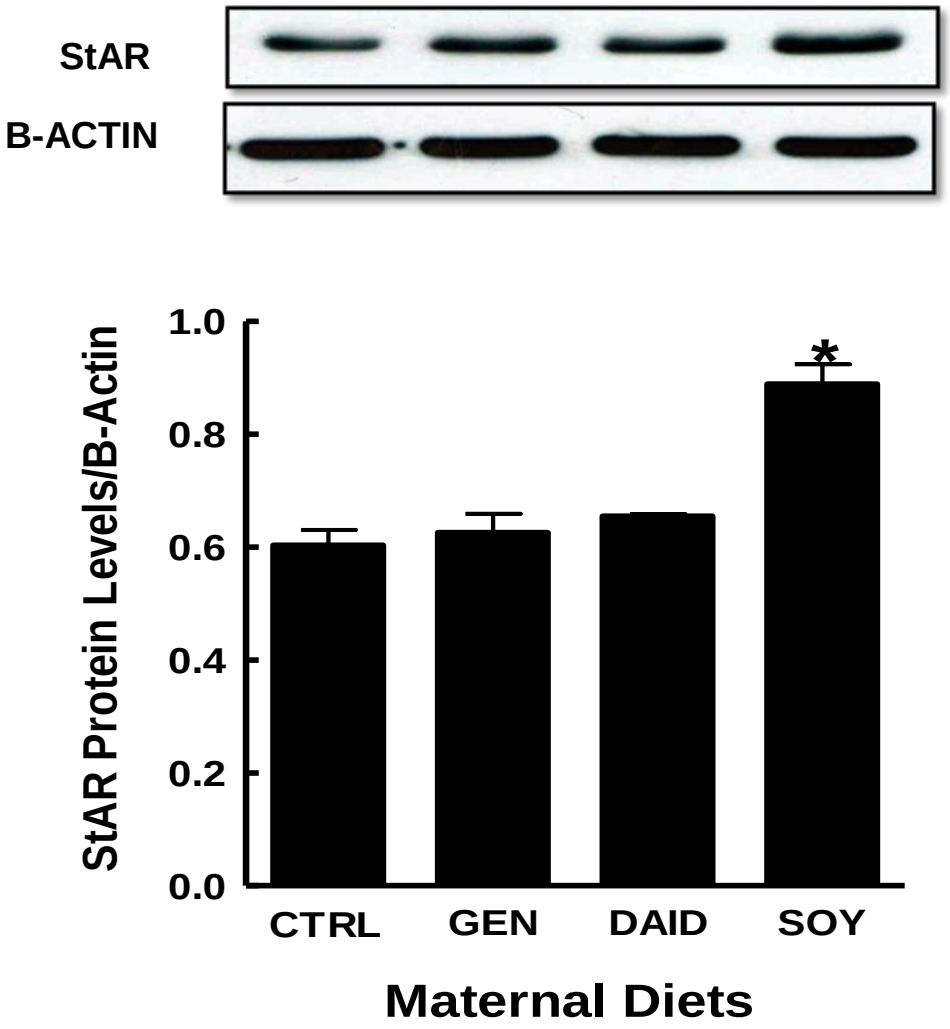

Figure 9A: Expression of StAR in adult Leydig cells from sexually-mature male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of three Western blots. StAR= 30 kDa, B-Actin= 42 kDa. *P < 0.01 versus control.

Cytochrome P450 Side Chain Cleavage (CYP11A1) Protein Expression Was Not Affected by Lactational Exposure to Soy Isoflavones in Sexually-mature Male Rats (PND 96)

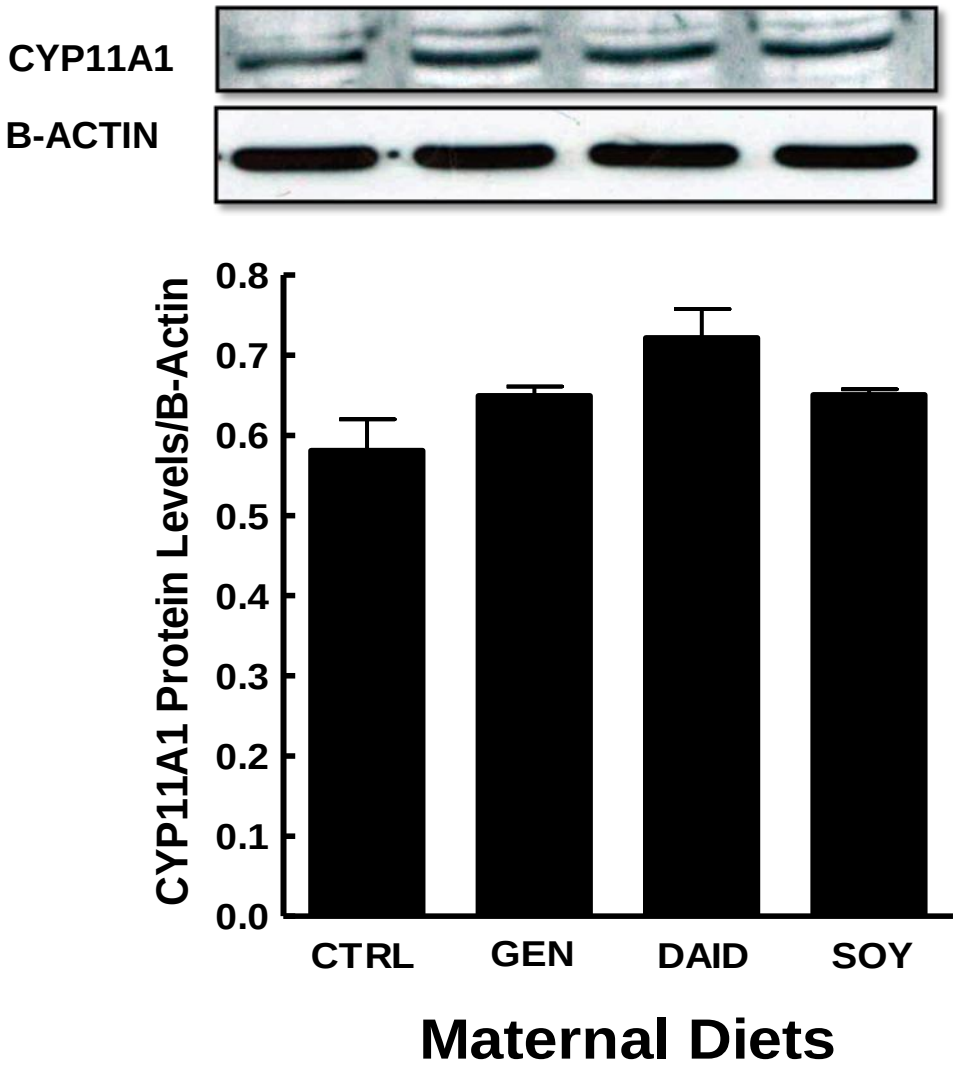

Figure 9B: Expression of CYP11A1 in adult Leydig cells from sexually-mature male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of two Western blots. CYP11A1= 60 kDa, B-Actin= 42 kDa.

Lactational Exposure to Genistein and Daidzein Together Increased 3-Beta Hydroxysteroid Dehydrogenase (3BHSD) Protein Expression in Adult Leydig Cells (PND 96)

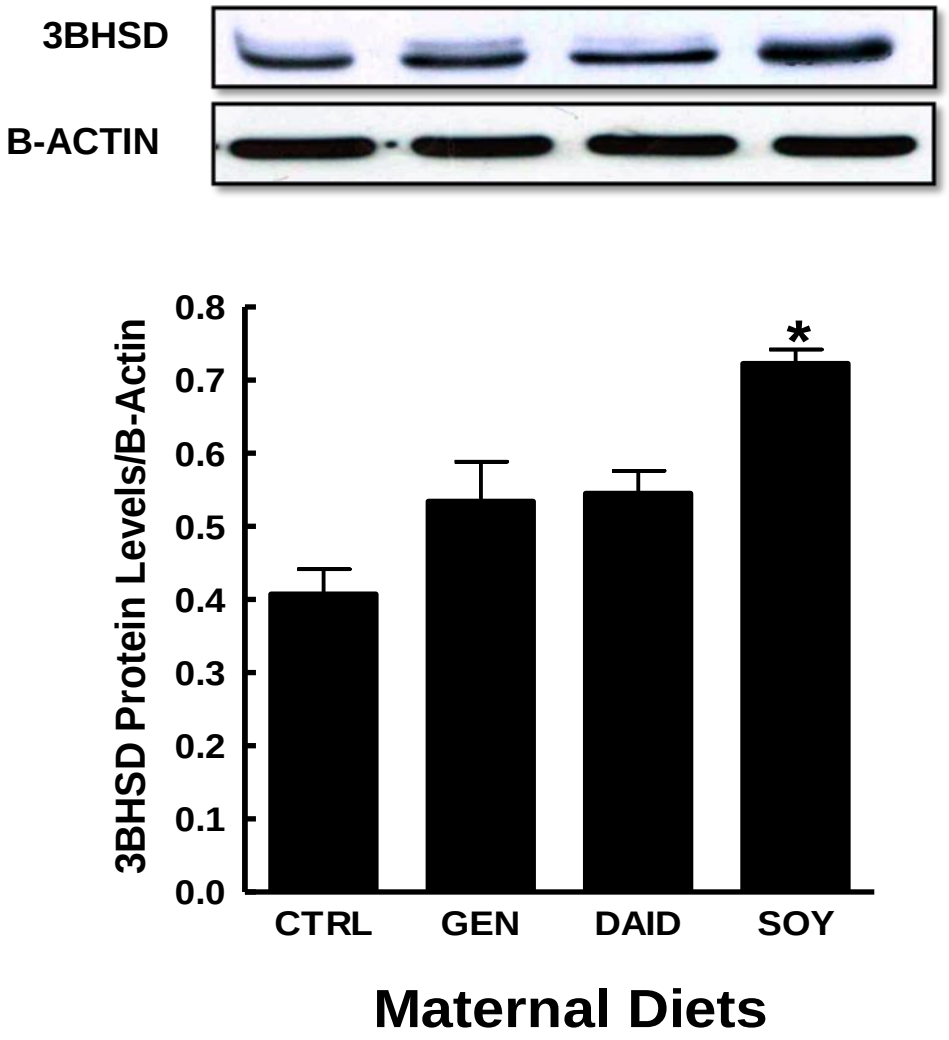

Figure 9C: Expression of 3BHSD in adult Leydig cells from sexually-mature male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of three Western blots. 3BHSD= 42 kDa, B-Actin= 42 kDa. *P < 0.01 versus control.

Genistein and Daizdzein, Acting Singly or Together, Increased Cytochrome P450 17α-hydroxylase (CYP17A1) Protein Expression in Adult Leydig Cells (PND 96) After Lactational Exposure

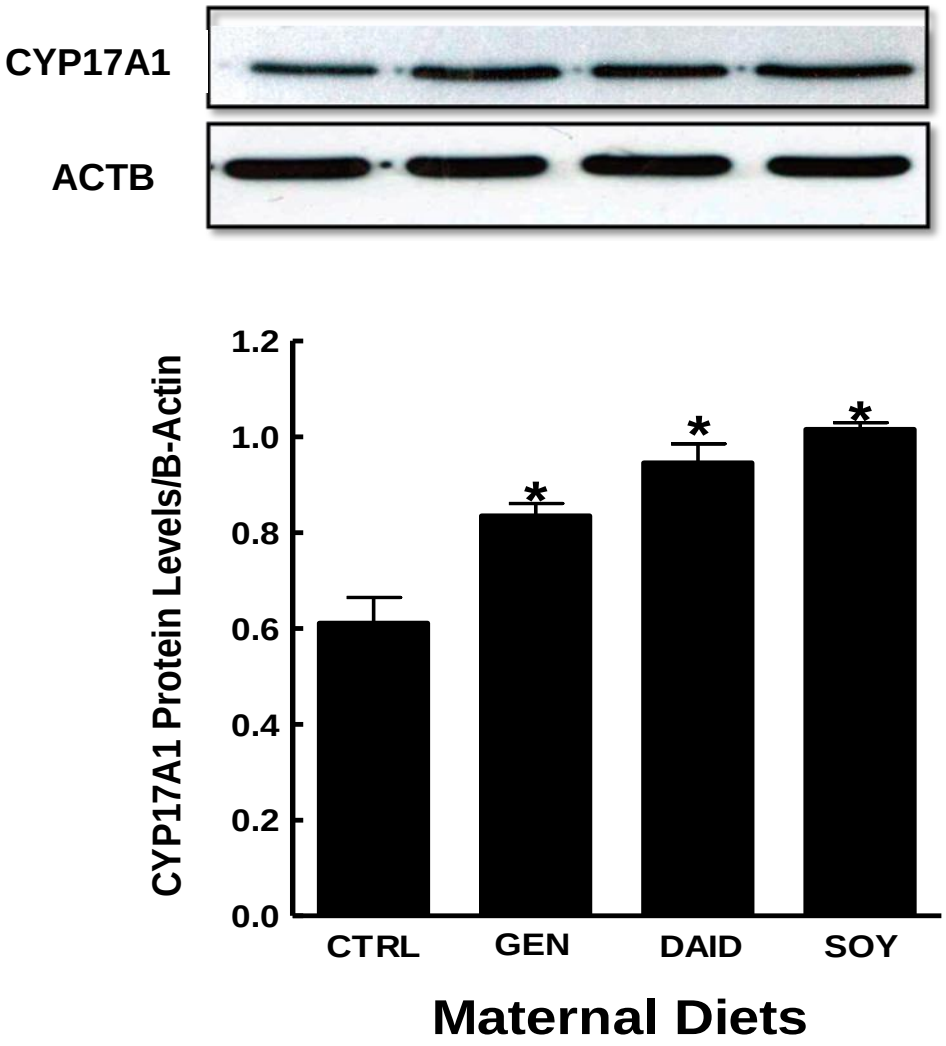

Figure 9D: Expression of CYP17A1 in adult Leydig cells from sexually-mature male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of three Western blots. CYP17A1= 55 kDa, B-Actin= 42 kDa. *P < 0.05 versus control.

Lactational Exposure to Genistein and Daidzein Together Decreased 17-Beta Hydroxysteroid Dehydrogenase (17BHSD) Protein Expression in Adult Leydig Cells (PND 96)

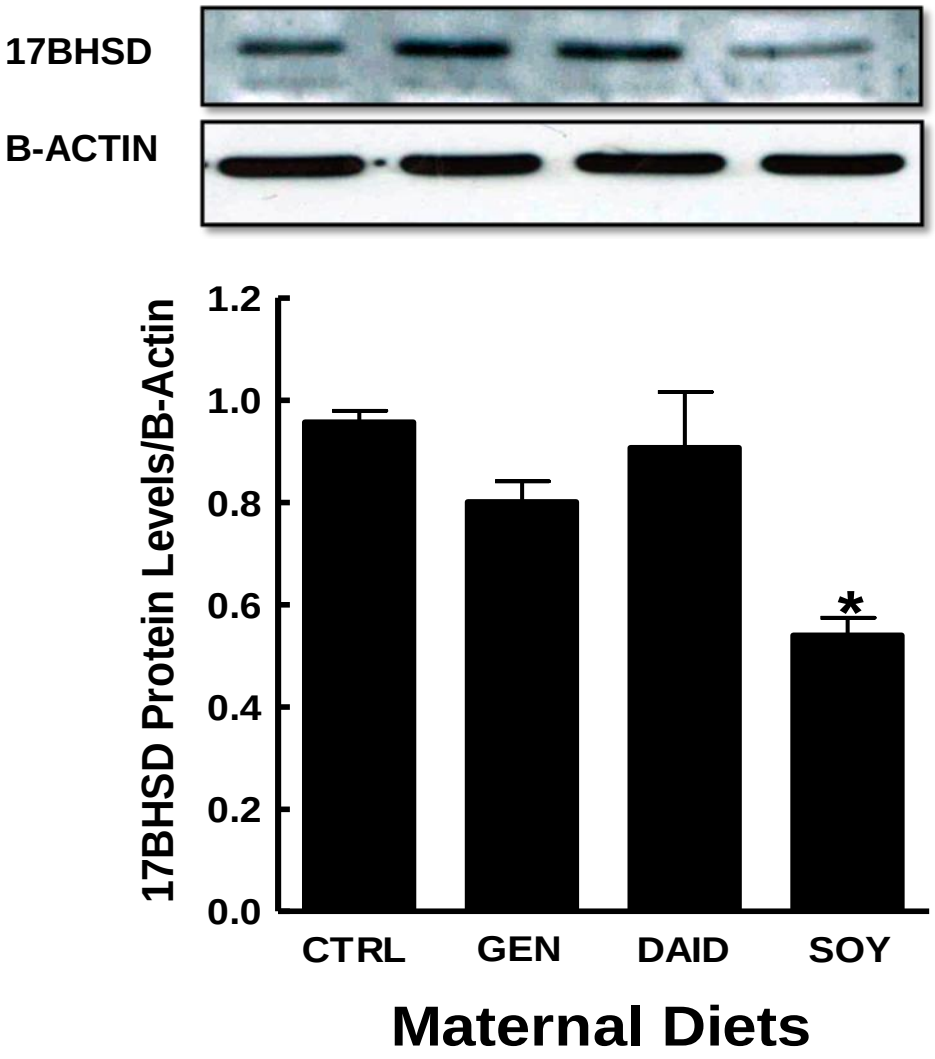

Figure 9E: Expression of 17BHSD in adult Leydig cells from sexually-mature male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of three Western blots.17BHSD= 35 kDa, B-Actin= 42 kDa. *P < 0.01 versus control.

Lactational Exposure to Genistein and Daidzein Together Increased Estrogen Receptor 1 (ESR1) Protein Expression in Adult Leydig Cells (PND 96)

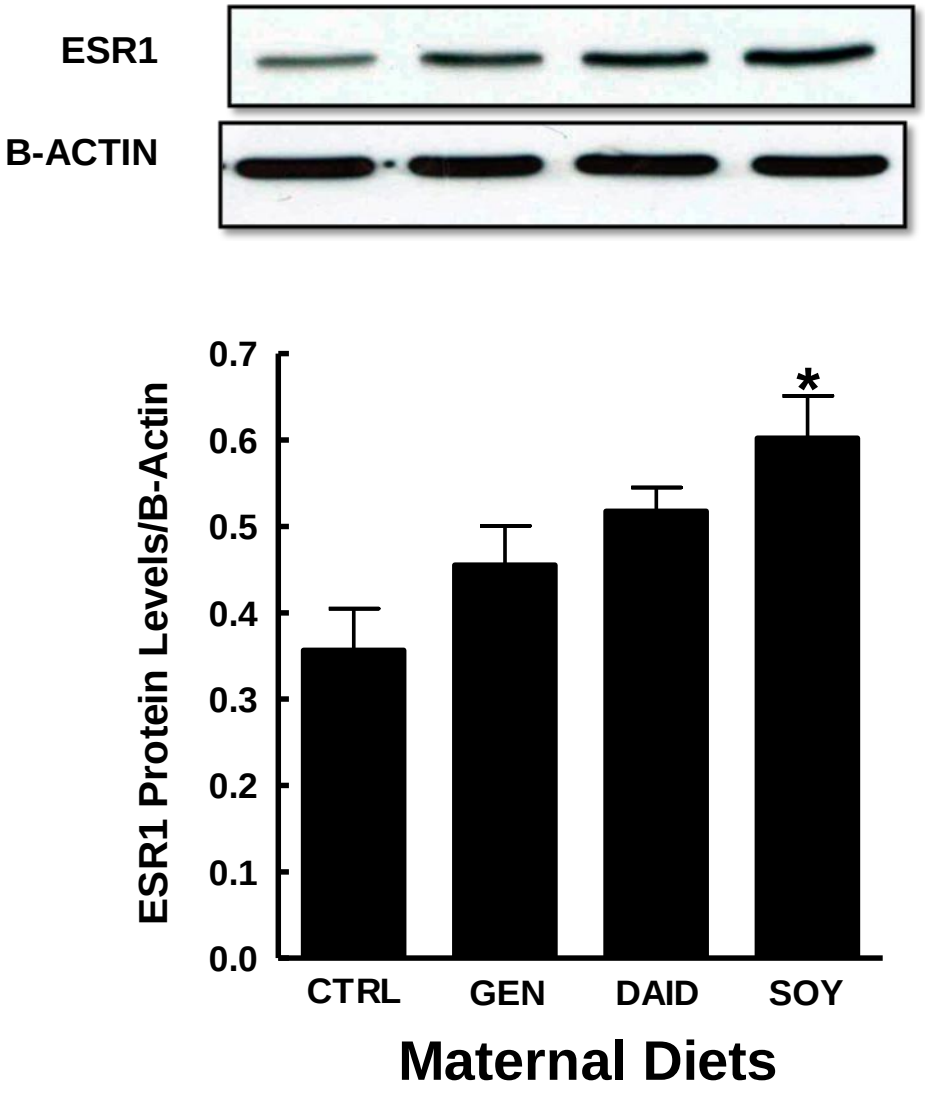

Figure 10: Expression of ESR1 in adult Leydig cells from sexually-mature male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of four Western blots. ESR1= 66 kDa, B-Actin= 42 kDa. *P < 0.01 versus control.

Androgen Receptor (AR) Protein Expression in Adult Leydig Cells (PND 96) Was Not Affected by Lactational Exposure to Soy Isoflavones

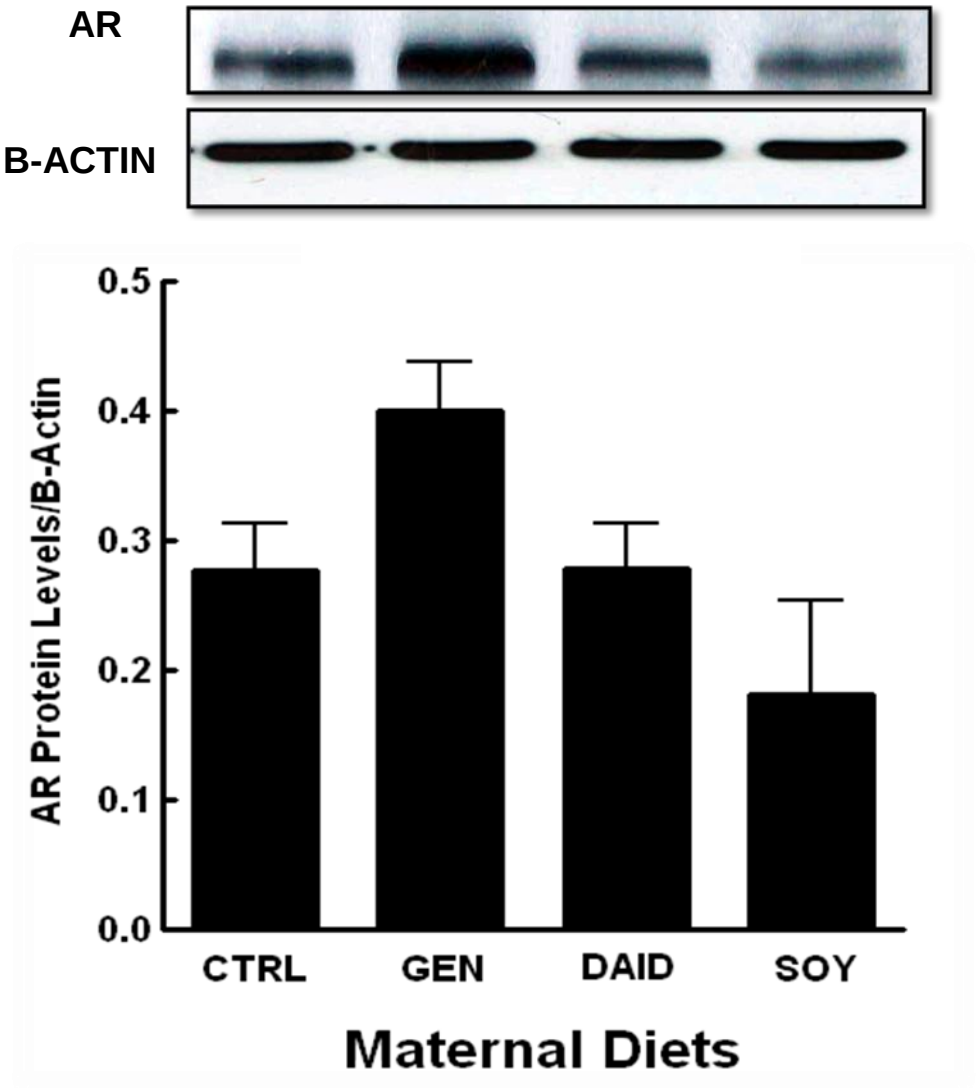

Figure 11: Expression of AR in adult Leydig cells from sexually-mature male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of four Western blots. AR= 110 kDa, B-Actin= 42 kDa.

Luteinizing Hormone Receptor (LHR) Protein Expression in Adult Leydig Cells (PND 96) Was Not Affected by Lactational Exposure to Soy Isoflavones

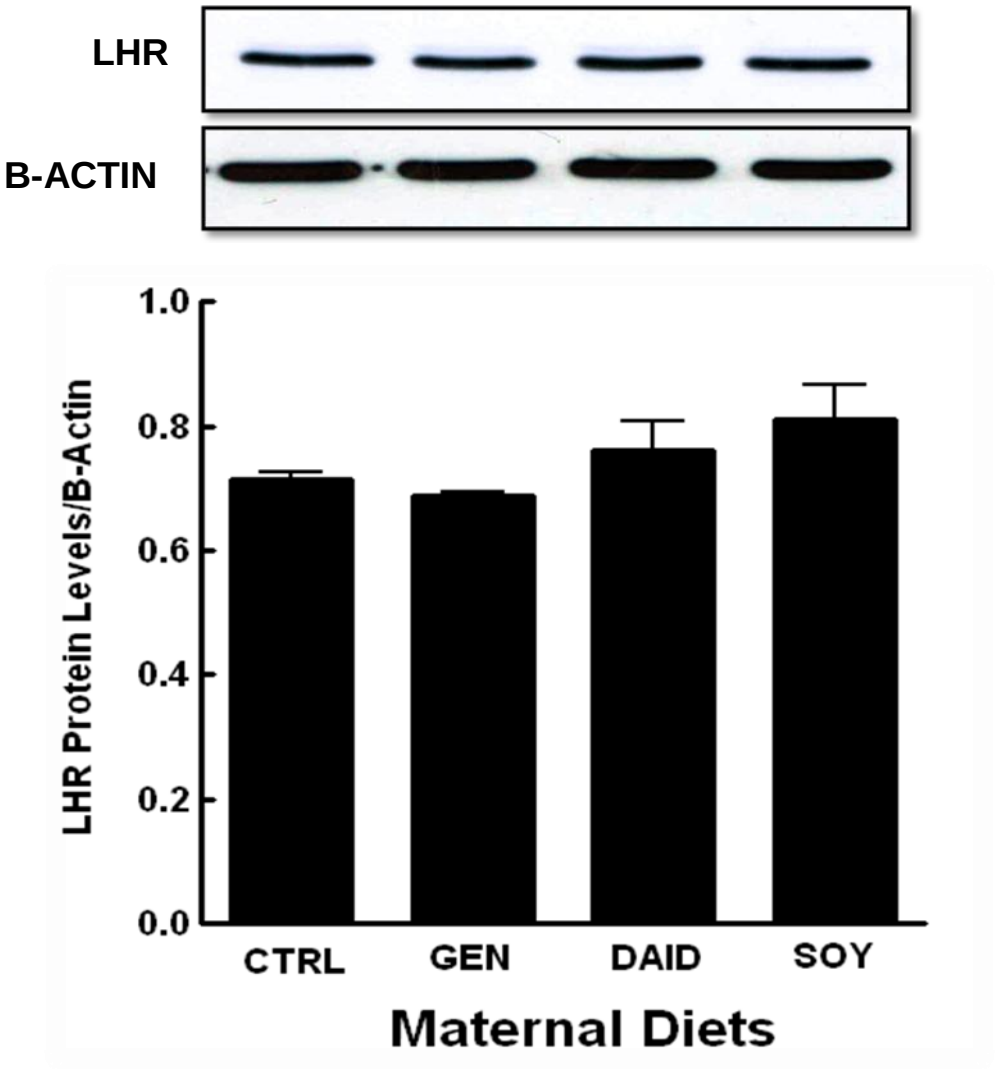

Figure 12: Expression of LHR in adult Leydig cells from sexually-mature male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of four Western blots. LHR= 79 kDa, B-Actin= 42 kDa.

Lactational Exposure to Daidzein Alone or with Genistein Decreased Mullerian-inhibiting Substance (MIS) Protein Expression in Sexually-mature Male Rats

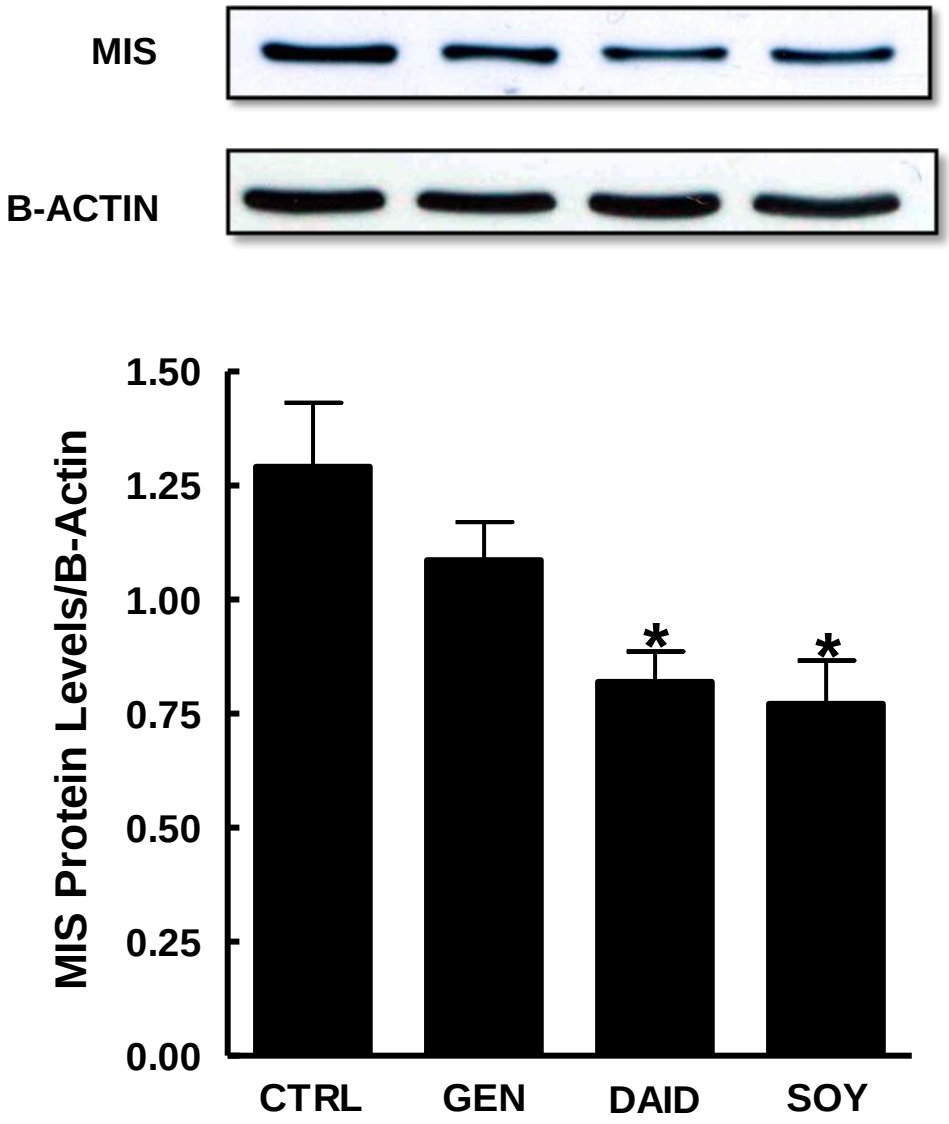

Figure 13A: Expression of MIS in testes from neonatal male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized B-Actin. Data represent results from densitometric analysis of four Western blots. MIS= 65 kDa, B-Actin= 42kDa. *P < 0.01 versus control.

Genistein and Daizdzein, Acting Singly or Together, Increased Mullerian-inhibiting Substance Type II Receptor (MISRII) Protein Expression in Adult Leydig Cells (PND 96) after Lactational Exposure

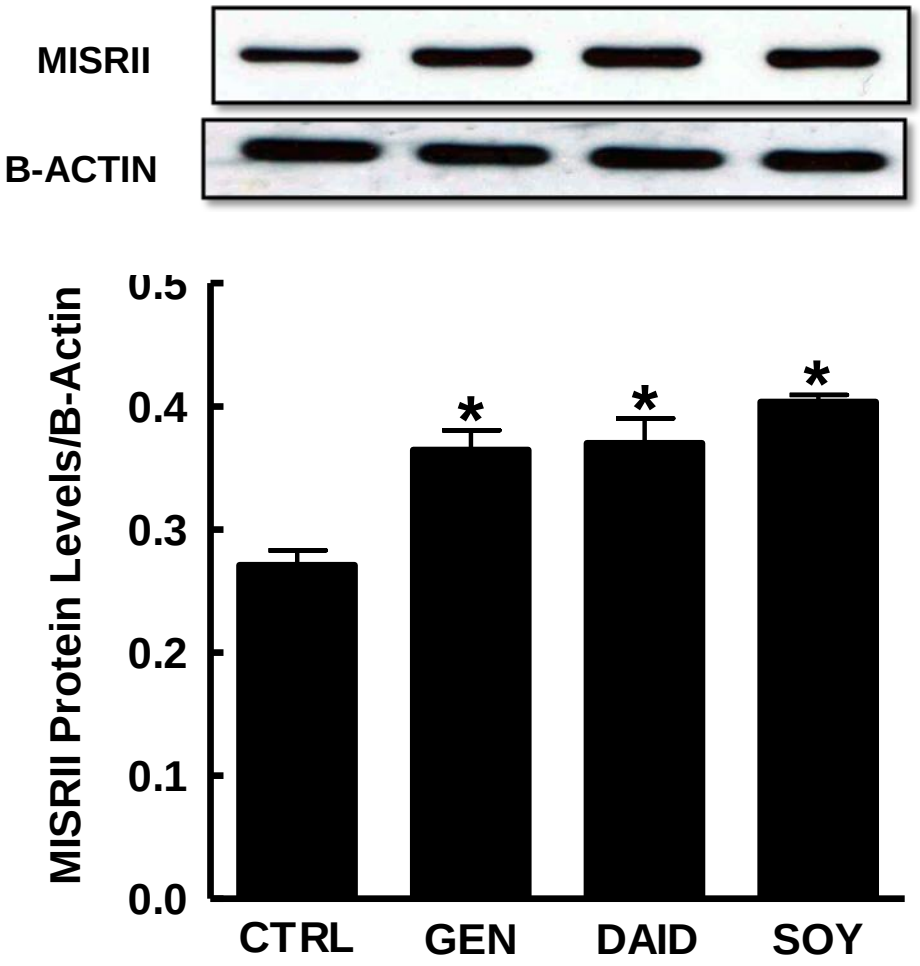

Figure 13B: Expression of MISRII in progenitor Leydig cells from neonatal male rats after lactational exposure to dietary isoflavones was analyzed by Western blotting and normalized to B-Actin. Data represent results from densitometric analysis of three Western blots. MISRII= 63 kDa, B-Actin= 42kDa. *P < 0.01 versus control.