Effects of combined genistein and di-(2-ethylhexyl) phthalate on male reproductive development and function

by

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Dedication

I dedicate this thesis to my family

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List of Abbreviations

ABP	androgen binding protein
AGD	anogenital distance
ALC	fetal Leydig cell
AMH	anti müllerian hormone
AP-2γ	activator protein-2γ
AR	androgen receptor
ART	assisted reproductive technologies
AUC	area under the curve
BPA	bisphenol A
BTB	blood-testis barrier
cAMP	cyclic-AMP
CSF-1	colony stimulating factor-1
D	diplotene
DAZL	deleted in azoospermia-like
DBP	dibutyl phthalate
DC	dendritic cell
DEHP	di-(2-ethylhexyl) phthalate
DHH	desert hedge-hog
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
E	estradiol
ECM	extracellular matrix
ED	endocrine disruptor
EGF	epidermal growth factor

ER	estrogen receptor
FGF	fibroblast growth factor
FLC	adult Leydig cell
FSH	follicle stimulating hormone
GATA	transcription factor 4
GCNA1	germ cell nuclear antigen 1
GD	gestational day
GEN	genistein
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
ICSI	intracytoplasmic sperm injection
IGF	insulin-like growth factor
IHC	immunohistochemistry
INSL3	insulin-like growth factor 3
IVF	in-vitro fertilization
L	leptotene
LH	leuteinizing hormone
LHCGR	leuteinizing hormone/choriogonadotropin receptor
LHr	leuteinizing hormone receptor
LHX9	lim homeobox gene 9
LIF	leukemia inhibitory factor
LXR	liver X receptor
MBP	monobutyl phthalate
MC	mast cell
MIS	mullerian inhibiting substances

MR	mineralocorticoid receptor
NGN3	neurogenin
OR	odds ratio
P	pachytene
PBR	peripheral-type benzodiazepine
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PGC	primordial germ cell
PKA	protein kinase A
PKC	protein kindase C
PLZF	promyelocytic leukemia zinc finger (PLZF),
PMC	peritubular myoid cell
PND	postnatal day
PPAR	peroxisome proliferator-activated receptor
PVC	polyvinyl chloride
PXR	pregnane X receptor
qPCR	quantitative polymerase chain reaction
RA	retinoic acid
RIA	radioimmunoassay
ROS	reactive oxygen species
SCP1	synaptonemal complex protein
SPG	spermatogonia
SRY	sex-determining region
SSC	spermatogonial stem cell
StAR	steroidogenic acute regulatory protein

STF	stem cell factor
STRA8	stimulated by retinoic acid gene 8
T	testosterone
TCDD	2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD)
TDS	testicular dysgenesis syndrome
TGCC	testicular germ cell cancer
TGF	transforming growth factor
TSPO	translocator protein
WT1	Wilms tumor 1
Z	zygotene

English Abstract

Early life exposure to endocrine disruptors (EDs) is thought to contribute to reported declines in male reproductive potential and increased incidence of male reproductive abnormalities such as testicular cancer, crytorchidism and hypospadias. These aberrations, collectively termed testicular dysgenesis syndrome (TDS), are thought to arise from impaired development and reprogramming of progenitor cells during the developmentally sensitive fetal and neonatal windows. The hormonal dependence and developmental intricacy of the fetal period, in combination with a comparatively permeable fetal skin surface, minimal detoxifying capacity and lack of blood-testis barrier, make the developing gonads particularly sensitive to EDs capable of crossing the placenta.

Although EDs have been extensively studied in the context of single high dose chemical exposures, few studies have examined the additive or synergistic effects of ED co-exposure during critical periods of development. In reality, humans and animals are exposed not to one, but a myriad of potentially harmful substances throughout their lifetimes. Two common early life exposures are those of genistein (GEN), a natural phytoestrogen found at high levels in soy based infant formula and other soy-derived products, and di-(2-ethylhexyl) phthalate (DEHP), a ubiquitous plasticizer used in the manufacturing of polyvinyl chloride (PVC) plastics.

We hypothesized that in utero co-exposure to genistein and DEHP, at low doses relevant to human exposure could induce alterations in testis development different or potentially more harmful than exposure to a single chemical at the same dose. Such changes could lead to long term effects on fertility.

Multiple rodent toxicological models demonstrated the ability of genistein and DEHP to present a long term male reproductive risk, acting in an additive or synergistic manner, below previously reported thresholds of toxicity, and involving different mechanisms of toxicity from perinatal ages through adulthood. Several vulnerable cell types were identified in perinatal and adult periods, along with potential mechanisms of toxicity.

The present thesis challenges the long running doctrine of assessing chemical safety in the context of high dose, single exposure animal and cell based models. Results also highlight how short term studies may not be predictive of long term effects. The notion that these EDs do not follow classical dose-response effects with consistent mechanisms of toxicity from perinatal ages to adulthood further stresses the importance of assessing impacts across a range of doses during appropriate windows of exposure and at different ages. These are very pertinent concepts in the context of chemical risk assessment and the ability of regulatory agencies to determine safe exposure thresholds.

Resumé

Il est maintenant reconnu qu'une exposition, en bas âge, à des perturbateurs endocriniens (PE) contribuerait au déclin observé de la fertilité masculine et de l'incidence accrue de certaines anomalies telles que le cancer du testicule, la cryptorchidie et l'hypospadias. Ces aberrations forment ensemble le syndrome de dysgénésie testiculaire ou TDS (de l'anglais testicular dysgenesis syndrome). Ce syndrome surviendrait après une altération du développement et de la reprogrammation des cellules progénitrices lors des périodes feotales et néonatales, périodes spécifiquement critiques aux changements développementaux. La relative perméabilité de la surface de la peau fœtale, la capacité minimale de détoxification ainsi que l'absence de barrière hémato-testiculaire, sont des facteurs rendant les gonades en développement particulièrement sensibles aux PE qui ont la capacité de traverser la barrière placentaire.

Bien que les PE aient été largement étudiés dans le cadre d'études utilisant des expositions de composés seuls et à de très fortes doses, très peu d'études ont examiné les effets additifs ou synergiques lors d'une co-exposition de PE durant les périodes critiques du développement testiculaire. En réalité, les humains et les animaux sont exposés, non pas à une seule, mais bien à une myriade de substances potentiellement dangereuses et cela tout au long de leur vie. Parmi les substances fréquemment présentes lors d'expositions néonatales, on retrouve la génistéine et le phthalate de bis(2-éthylhexyle) ou DEHP (de l'anglais, di-(2-ethylhexyl) phthalate). La génistéine (GEN) est un phytoestrogène naturellement retrouvé à une forte concentration dans les préparations de laits de remplacement pour nourrissons à base de soja, ainsi que dans les produits contenant des proteins dérivées du soja. Quant au DEHP, il est un agent plastifiant omniprésent dans la production des plastiques fait à base de polychlorure de vinyle ou PVC (de l'anglais polyvinyl chloride).

L’hypothèse de notre étude était que la co-exposition à la génistéine et au DEHP à une dose comparable à une exposition humaine pourrait induire des altérations du développement du testicule différentes ou potentiellement plus nocives qu’une exposition à un seul de ces produits chimiques a la même dose. De tels changements pourraient conduire à des effets à long terme sur la fertilité masculine.

A travers de multiples modèles toxicologiques chez les rongeurs, mes études ont démontré la capacité de la génistéine et du DEHP à induire un risque à long terme sur la reproduction masculine. Les deux substances semblent agir d’une manière additive ou synergique et en-dessous des seuils de toxicité habituellement rapportés. De plus, ils impliqueraient différents mécanismes de toxicité pour les âges périnataux et pour l’âge adulte. Plusieurs types cellulaires vulnérables ont été identifiés au cours des périodes périnatales et adultes ainsi que les mécanismes potentiels de toxicité.

La présente thèse remet en question la doctrine validant le traitement de modèles animaux ou cellulaires avec des doses élevées de produits chimiques isolés pour déterminer leur l’innocuité. Nos résultats soulignent également le fait que des études à court terme ne peuvent prédire les effets à long terme. De plus, le fait que les effets de ces PEs ne suivent pas des dose-réponse classiques et utilisent des mécanismes de toxicité différents en fonction de l’âge démontre l’importance d’évaluer leurs impacts pour une gamme de doses et différentes fenêtres d’exposition, et de déterminer leurs effets à des âges différents.

Ces concepts sont très pertinents dans le contexte de l’évaluation des risques que représentent les produits chimiques, dont les organismes de réglementation devraient tenir compte lors de la détermination des seuils sécuritaires d’exposition.

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Preface

Originality of scholarship and distinct contributions to knowledge

A combination of in-vivo, ex-vivo and in-vitro rodent toxicological models demonstrated for the first time, the ability of low dose genistein and DEHP to present a long term male reproductive risk, acting in an additive or synergistic manner, below previously reported thresholds of toxicity, and involving different mechanisms of toxicity from perinatal ages through adulthood. Several vulnerable cell types were identified in perinatal and adult periods, along with potential mechanisms of toxicity. These findings are novel and relevant for the fields of reproduction, endocrinology, toxicology and public health:

- One of few studies addressing mixtures of chemicals acting on different hormonal processes at doses relevant to human exposure
- Results support a paradigm shift in risk assessment
- Highlight inadequacy of conventional toxicology assessment for chemicals acting as endocrine disruptors

My original work has resulted in two published first author manuscripts and a third article currently under revision.

Contributions of authors to manuscripts

Chapter 2: Disruption of rat testis development following combined in utero exposure to the phytoestrogen genistein and antiandrogenic plasticizer di-(2-ethylhexyl) phthalate. Jones, S., Boisvert, A., Duong, T.B., Francois, S., Thrane, P. and Culty, M. 2014. Biology of reproduction 91, 64.

- Steven Jones: research design, performed experiments, analyzed data and wrote manuscript
- Annie Boisvert: animal handling, gross measurements and tissue collection
- Tam Duong: Assisted in quantitative PCR (qPCR)
- Sade Francois: Assisted in qPCR and immunohistochemistry (IHC)
- Pater Thrane: Assisted in qPCR
- Martine Culty: Research design, analyzed data and wrote manuscript

Chapter 3: In utero exposure to di-(2-ethylhexyl) phthalate induces testicular effects in neonatal rats that are antagonized by genistein cotreatment in utero. Jones, S., Boisvert, A., Francois, S., Zhang, L. and Culty, M. 2015. Biology of reproduction 93, 92.

- Steven Jones: research design, performed experiments, analyzed data and wrote manuscript
- Annie Boisvert: assisted in animal handling, gross measurements, tissue collection and RIA
- Sade Francois: assisted in qPCR and IHC
- Liandong Zhang: assisted in animal handling and tissue collection
- Martine Culty: research design, analyzed data and wrote manuscript

Chapter 4: Stimulatory effects of combined endocrine disruptors on MA-10 Leydig steroid production and lipid homeostasis. Jones S., Boisvert A., Naghi A., Hullin-Matsuda F., Greimel P., Kobayashi T., Papadopoulous V. and Culty M. 2015. Submitted to Toxicology.

- Steven Jones: research design, performed experiments, analyzed data and wrote manuscript
- Annie Boisvert: assisted in RIA
- Andrada Naghi: assisted in qPCR
- Francoise Hullin-Matsuda: assisted in training Steven Jones in lipid analysis techniques
- Peter Greimel: assisted in training Steven Jones in lipid analysis techniques
- Toshihide Kobayashi: research design and host principal investigator at RIKEN insitute
- Vassilios Papadopoulos: research design and mansucript editing
- Martine Culty: research design, analyzed data and wrote manuscript

Chapter I

“Introduction”

1. Introduction to thesis– Global trend of concern

Pioneering work by Skakkebaek in the early 1990s reported a global trend towards decreased male sperm counts and increased occurrence of male reproductive and developmental abnormalities, including testicular germ cell cancer (TGCC), cryptorchidism and hypospadias [1]. Indeed, it is estimated that 15-20% of couples experience infertility and that a male cause can be attributed in up to 50% of those cases [2, 3]. A decline in male reproductive capacity is reaffirmed by register data indicating that the proportion of children born from assisted reproductive technologies (ART), including in-vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) and donor insemination is increasing [4]. Globally, an estimated 6.2% of children were born by means of ART is recent years. ICSI, a technique involving guided injection of a single sperm into an egg, is used to circumvent poor semen quality and was employed in up to 4% of live births.

Apart from few well characterized genetic disorders associated with impaired male reproductive development, such as Klinefelters Syndrome, the root causes of these concerning trends remain unknown. The term testicular dysgenesis syndrome (TDS) was coined by Skakkebaek, encompassing a collection of male reproductive aberrations influenced by multiple genetic, lifestyle and environmental factors.

Evidence suggests that reproductive impairment in adulthood has a fetal origin and that late onset effects are the results of early deviations from normal physiological development. This notion is a derivation of the "developmental origins of disease hypothesis" which states that adverse events or influences early in life can lead to permanent changes in physiology and predispose adults to disease [5, 6]. With no indication of increased occurrence of genetic predisposition, the focus was placed on environmental factors potentially affecting hormonal control during a sensitive

development window. Normal male reproductive development is highly hormone dependent; a "masculinization window" driven most notably by fetal testis androgens and other Leydig/Sertoli derived factors, allows for development of the male reproductive tract, formation of the external genitalia and ultimately fertility in adulthood [7].

The number of chemicals used in our everyday lives has increased exponentially over the past century. Although they have contributed to many modern conveniences, the unfortunate reality is that relatively little safety information exists on the potential health hazards of these substances prior to entering the market. Of concern is that several ubiquitous environmental pollutants, including derivatives of plastics, paints and resins, as well as some natural soy derived phytoestrogens, have been shown to possess endocrine properties, mimicking, antagonizing or altering the effects of endogenous hormones. Endocrine disruptors (EDs) capable of crossing the placenta during developmentally intricate fetal period are believed to be contributing to concerning trends in male reproduction function [8-12].

Two common early life exposures, the plant derived phytoestrogen, genistein, found in soy based infant formula and the antiandrogenic plasticizer, Di-(2-ethylhexyl) phthalate (DEHP), primarily used in the production of polyvinyl chloride plastics (PVC), are suspected EDs and male reproductive and developmental toxicants. The objective of the current thesis is to address the potential long term male reproductive risk and mechanisms of toxicity of these two substances (alone or in combination) following environmentally relevant exposures during key windows of male reproductive development.

2. The male reproductive system

The male reproductive system comprises organs required for gamete production and accessory organs supporting reproductive processes (Fig. 1). Spermatogenesis occurs in the seminiferous tubules of the testis under complex regulation by endocrine and paracrine signals and direct cell contact. Accessory organs necessary for the subsequent maturation, concentration and transit of sperm include the excurrent duct system (efferent ducts, vas deferens and epididymis), glands (seminal vesicles, prostate and bulbourethral) and supporting structures (spermatic cords, penis, scrotum, arteries, nerves and lymphatic vessels) [13, 14].

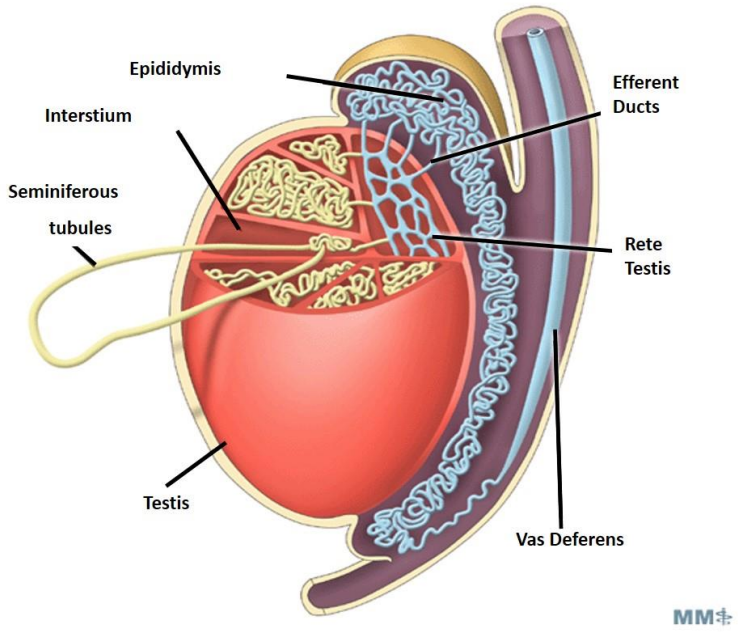

Figure 1.1. Schematic representation of the mammalian male reproductive system

Although germ cells production occurs in the seminiferous tubules of the testis, it is during transit through the excurrent duct system that sperm gain motility and fertility [15]. Testicular sperm are collected by the rete testis before entering into the efferent ducts and remainder of the excurrent duct system. The epididymis connects the efferent duct to the vas deferens. Sperm transit through

the epididymis is mediated by peristaltic contractions and pressure created by testicular fluids. During transit, sperm mature through interaction with epididymal epithelium and region specific (caput (head), corpus (body) and cauda (tail)) luminal microenvironment. Mature sperm are ultimately concentrated and stored in the cauda epididymis prior to entry in the vas deferens and ejaculatory ducts.

2.1 Testis

The principle functions of the testis are the development of viable gametes and the production of hormones and testicular factors [16, 17]. Spermatogenesis is a highly complex and dynamic process, principally regulated by factors produced by Sertoli cells within the seminiferous tubules, themselves dependent on the gonadotropin Follicle Stimulating Hormone (FSH) and on Leydig cell-produced testosterone (T).

Early masculinization and proper development of the male reproductive tract is highly hormone dependent, driven principally by fetal T production beginning at gestational day GD14.5 in the rodent. Shortly after birth, fetal Leydig cell (FLC) androgen production begins to decline and fetal Leydig cells gradually disappear. A second population of adult Leydig cells (ALC) derived from independent precursors appear at puberty and acquire steroidogenic capacity under the influence of anterior pituitary luteinizing hormone (LH). Thereafter, T production is sustained throughout adulthood, interacting with Sertoli cells alongside FSH to maintain optimal male fertility.

Sertoli cells play an essential role in early germ cell development, differentiation of Leydig precursors and the formation of the testis architecture and male reproductive structures through the production of growth factors (stem cell (SCF), transforming (TGF), insulin-like (IGF), fibroblast (FGF) and epidermal growth factors (EGF)) and hormones (Mullerian Inhibiting Substance (MIS)

and inhibin). Sertoli cells also provide factors that fuel germ cell metabolism such as lactate and transferring, and androgen binding protein (ABP) which regulates the availability of androgens within the testis and excurrent duct system [18-20]. Mature Sertoli cells play an additional critical role in the formation of the blood-testis barrier (BTB). Sertoli-Sertoli junctions within the BTB provide an immune privileged environment, physically separating compartments within the seminiferous epithelium and also contributing to the maturation and movement of germ cells [21].

A key series of early embryonic events are critical in the development program of testicular germ and somatic cell precursors. Any deviation from normal physiology can have profound consequences on male reproductive function later in life.

2.2 Embryonic and fetal development

Testis development within the mammalian embryo is a highly complex and interactive multicellular process. Gonad development initiates when primordial germ cells (PGCs), the first identifiable germ cells in the embryo, migrate to the genital ridge around 5 weeks gestation, corresponding to gestation day GD7.5-13 in the rodent [22, 23]. Male and female gonads are morphologically indistinguishable in early embryonic development; commitment to the male phenotype is dependent on the expression of the sex-determining region (SRY) region of the Y chromosome. SRY-induced sex determination of the fetus results in the proliferation and differentiation of bi-potential genital ridge mesenchymal precursors into fetal Sertoli cells, a key initiating step in subsequent somatic and germ cell development. Under the influence of transcription factors SRY and SOX9, Sertoli cells initiate the differentiation of Leydig precursors and the creation of a niche suitable for migrating PGCs. Other somatic cells that will ultimately comprise essential components of testis architecture and reproductive function, including blood

endothelial cells, peritubular myoid cells (PMCs), macrophages and nerve cells, develop in parallel.

Human somatic Sertoli cells critical for germ cell development and differentiation of Leydig cells from their progenitors are functional at 8 weeks gestation. Fetal Sertoli cells produce Müllerian inhibitory substance/Anti Müllerian Hormone (MIS/AMH) which drives the female Mullerian ducts regression in male embryo. In the absence of MIS, the female Mullerian ducts will persist and develop into the fallopian tubes and uterus, while the Wolffian ducts will fail to give rise to secondary male reproductive structures (vas deferens, seminal vesicles and epididymis). FLC development initiates androgen production at 9 weeks gestation, corresponding to GD14.5-15 in rodents, as well as Insulin-like factor 3 (INSL3), which act together on testicular decent. All these events need to be carefully timed and regulated to allow for sequential normal physiological development of the testis and future establishment of male reproductive potential.

2.3 Sertoli cell development and function

Prior to testis development in the rodent testis, the gonadal primordium is comprised of migrating PGCs and somatic progenitors expressing transcription factors (Steroidogenic factor 1 (SF1), Wilms tumor 1 (WT1) and GATA transcription factor 4 (GATA4) and Lim homeobox gene 9 (LHX9)[24]. Progenitors expressing both SF1 and SRY develop into Sertoli cells [24-26]. SRY activation of pre-Sertoli cells, thought to originate from the coelomic epithelium of the genital ridges, is an essential step in the formation of the primordial gonad. SRY promotes pre-Sertoli proliferation and expression of an endogenous differentiating factor, SOX9. Shortly after SOX9 expression, pre-Sertoli cells begin to aggregate around small groups of PGCs within the genital ridges, forming primitive cords that will ultimately become seminiferous tubules. This

process, serving a dual purpose of early segregation of spermatogenic and androgen producing testicular compartments and protection of developing germ cells, begins around GD10.5 in the mouse. Aggregates are driven by cell-cell interactions and conformational changes in cell surface that increases adhesion. Once aggregated, pre-Sertoli cells are considered Sertoli cells. Paralleling the early formation of primitive cords is the emergence of peritubular myoid cell (PMC) layers separated by extracellular matrix that will ultimately form the lamina propria, a basal contractile muscle layer surrounding mature seminiferous tubules. Continued expansion of Sertoli cells and strengthening of Sertoli-germ cell interactions allows for cord elongation. Sertoli cells mature to possess taller epithelial morphology, increasing cord diameter to make room for germ cell expansion.

2.4 Germ cell development

2.4.1 Fetal-neonatal phase

The first phase of germ cell development occurs in the feta/neonatal period and involves the generation of self-renewing spermatogonial stem cells (SSCs) from gonocytes, themselves derived from PGCs. Germ cells do not have the same embryonic origin as somatic testicular cells; PGC progenitors present in the proximal epiblast of the extra-embryonic ectoderm are first identifiable at GD5.5 in the mouse [27, 28]. Progenitors become PGCs under the influence of bone morphogenic proteins and beginning at approximately GD7.5, corresponding to 3 weeks in human, PGCs are clearly visible and begin to migrate to extra embryonic mesoderm [27, 29]. PGCs eventually migrate towards the genital ridge and become resident in the developing gonads. PGC migration occurs under the influence of *steel* (Stem Cell Factor or c-Kit ligand), beginning at approximately GD11 in rodents and 4-5 weeks in human [30]. PGCs proliferate during migration,

but do not differentiate. They also undergo erasure of their parental DNA methylation profile, allowing for subsequent remethylation and establishment of parental imprints [27].

Once resident in the gonads and surrounded by Sertoli cells in early testicular cords, PGCs are now termed gonocytes, identifiable by the expression of germ cell nuclear antigen (GCNA1), alkaline phosphatase and Stella and HSP90α, which is retained throughout fetal and neonatal periods [31, 32]. Subsequent gonocyte developmental stages comprise distinguishable periods; fetal mitotic (M), quiescent or transitional 1 (Q/T1) and a final transitional period comprising mitosis, migration to the periphery of the tubule and differentiation to spermatogonia (also referred as T2) [27, 33]. Rodent gonocytes undergo two periods of active mitotic activity separated by a quiescent period during which DNA methylation takes place. In the rat, gonocytes increase in numbers until GD17.5 and then become mitotically arrested until PND3 [27, 34]. Although not fully characterized, evidence suggests the involvement of retinoic acid (RA) in inducing simultaneously apoptosis and proliferation in fetal gonocytes, while activin A and androgens act as negative regulators of fetal gonocyte proliferation [35, 36]. In contrast, human data suggests the occurrence of a single proliferative gonocyte period followed by overlapping quiescent and transitional periods [34, 200].

Following a period of quiescence, rodent gonocytes again become mitotically active, suggested to be regulated by RA, platelet-derived growth factor (PDGF), 17β-estradiol and leukemia inhibitory factor (LIF), an interleukin cytokine [33]. This proliferation is concurrent with a migratory period where gonocytes descend to the basement membrane of the seminiferous tubules and differentiate to form a critical pool of SSCs that will sustain spermatogenesis, as well as the spermatogonia of the first wave. In rodent, this process begins asynchronously shortly after birth (PND3 in rat, 1 to 2 days earlier in mouse) likely under the influence of dynamically regulated Sertoli junctional interactions, c-Kit and PDGFRβ [37-39]. Cells that fail to migrate are removed by apoptosis,

possibly as a mechanism to balance Sertoli-germ cell numbers or as a developmental checkpoint preventing the progression of aberrant cells [40-44]. Improper SSC formation could lead to infertility, testicular cancer or allow the transfer of an altered genome or epigenome to subsequent generations. In that regard, gonocytes are a sole source of SSC precursors and therefore a critical mediator of future male reproductive function.

Differentiation of gonocytes to SSCs involves significant morphological changes; cells are transformed from centrally located large round cells to semicircular and compact peripheral cells [27, 33, 40]. RA has been shown once again to be a key mediator in this process, promoting differentiation and transcriptional changes including a decrease in GDNF receptor GFRα1 and increases in c-KIT and Stimulated by retinoic acid gene 8 (STRA8) [33, 45]. Many other SSC markers, including OCT-4, promyelocytic leukemia zinc finger (PLZF), THY-1, FOXO1, Ret, neurogenin 3 (NGN3) and ID4 have been identified, while pluripotency markers such as the activator protein-2γ (AP-2γ) and Nanog are down-regulated in SSCs but retained in seminoma, the most common testicular cancer [27, 46].

2.4.2 Second Phase – Spermatogenesis

The second phase of germ cell development involves the formation of mature sperm from a self-renewing pool of SSCs. This involves a complex series of events where round progenitors are transformed into cells with condensed nuclei and a functional flagellar apparatus (Fig. 2) [17, 47].

Spermatogonia (SPG) first increase in numbers through successive mitotic divisions, allowing for a greater number of cells to enter the subsequent meiotic phase [17, 48]. Several types of SPG have been characterized according to their morphology and chromatin architecture; Type A single comprise not only resident SSC but also undifferentiated early progenitor cells, followed by

undifferentiated mitotic type Apaired and Aaligned, and successive differentiating type A1,A2,A3, A4, intermediate and finally type B spermatogonia [49-52]. Similar to gonocytes, SPG undergo genetically programed and hormonally regulated apoptosis, drastically reducing (~ -75%) the theoretical number of meiotic spermatocytes [53-55]. Differentiating SPG can be identified by known markers, including STRA8 and the RNA binding proteins Deleted in Azoospermia-like (DAZL) and VASA [33, 56].

The differentiation of Type B SPG yields primary spermatocytes, starting with pre-meiotic pre-leptotene followed by subsequent meiotic phases, during which genetic material is segregated to ultimately yield haploid round spermatids [17]. The first step involves the initial differentiation of diploid (2N DNA content) Type B SPG to preleptotene spermatocytes (Pl). Pl enter the S phase of the cell cycle, with DNA replication resulting in cells containing 46 chromatids (4N DNA). This genetic material is divided in subsequent meiotic divisions, ultimately contributing to a 4-fold increase in germ cell numbers. After S phase, Pl enter prophase which can be subdivided into five stages, identifiable by cell morphology and by their respective gene markers; Leptotene (L), Zygotene (Z), Pachytene (P) Diplotene (D) and Diakinesis. L have a round appearance, condensed unpaired chromosomes and express SPO11. Homologous pairing of chromosomes in Z is visible by light microscopy and along with P, these cells express synaptonemal complex protein (SCP1). P are identifiable be their enlarged nuclei and also by expression of a shortened form of SOX17 and LDHC. Nonsister chromatid crossing over and recombination occurs during P phase, leading to new chromosomes with unique parental combinations. Metaphase, anaphase and telophase follow prophase, completing the first meiosis. Secondary spermatocytes formed in this process enter into a second phase of meiosis, ultimately yielding four haploid (1N DNA content) round spermatids, identifiable by the presence of SP10, for every initial diploid cell.

The final stage of spermatogenesis involves a complex series of morphological changes yielding elongated spermatozoa [48, 57]. Cytoplasm is eliminated through the formation of the cytoplasmic droplet and residual body. This process, along with significant nuclear compaction permissible by the replacement of somatic and testis-specific linker histones with protamines, contributes to the formation of the sperm head. Reorganization of the Golgi apparatus through fusion of vesicles and capping of nucleus precedes acrosomal formation during sperm head elongation. Prior to final release of elongated sperm in the lumen of the seminiferous tubules, a flagellum is created [57]. This process, allowing for the eventual acquisition of motility and fertility, is mediated by centrosome driven migration of paired centrioles [48, 57, 58].

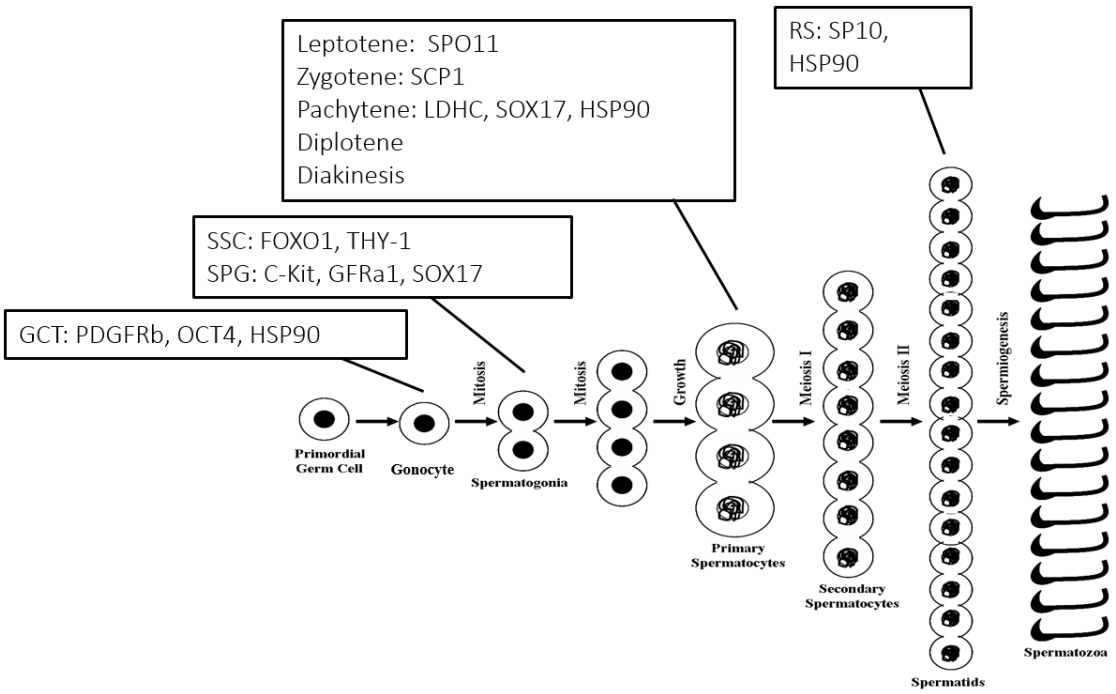

Figure 1.2. Schematic representation of rat spermatogenesis. Adapted from Danton O'Day (Formation of the Male Sex Cells: Spermiogenesis, ©Copyright 1998-2010 Danton H. O'Day)

2.5 Leydig cell development and function

ALC do not develop from FLC, but are thought to originate from an independent pool of neonatal mesenchymal precursors also present during the fetal period [59, 60]. In that regard, there are two 'waves' of Leydig cell development, with FLC first driving early masculinization and a second adult population responsible for developing male secondary sexual characteristics and maintenance of reproductive potential in adulthood (Fig. 3).

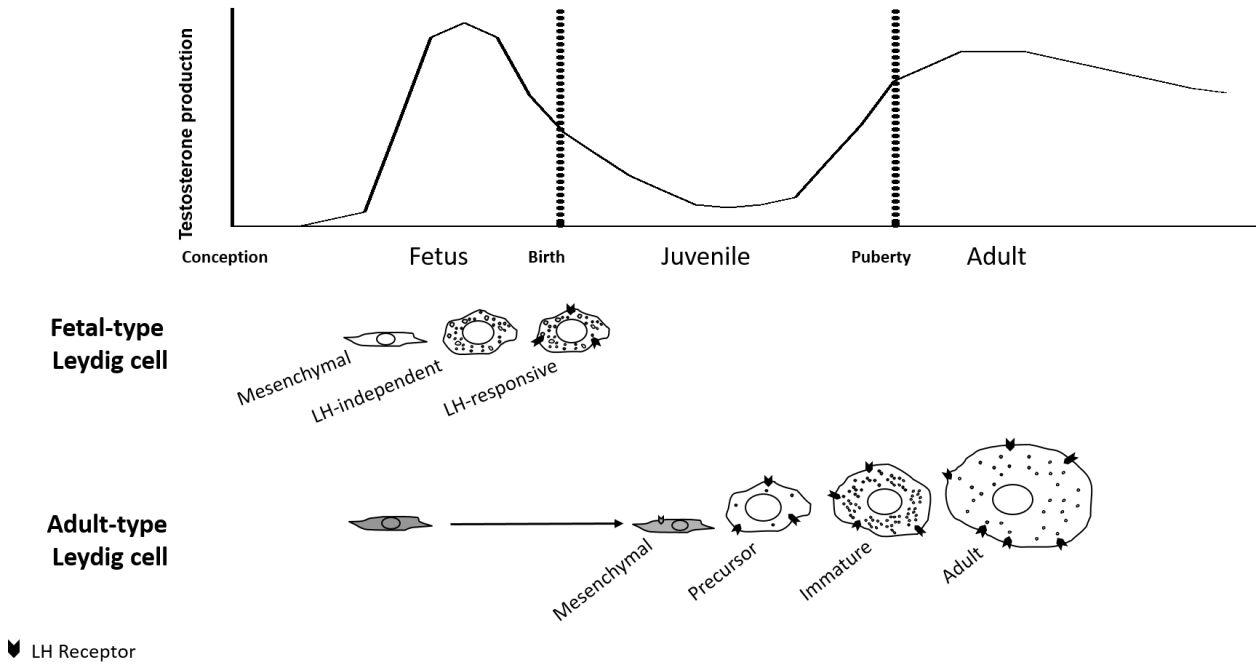

Figure 1.3. Fetal and adult Leydig cell testosterone production and LHr expression

The precise embryonic origins of FLC have not been confirmed. Several studies suggest possible derivation from the mesonephrous, neural crest or coelomic epithelium [24, 61]. Active FLC have been shown to arise 24 hrs after the appearance of Sertoli cells [24]. Interestingly, FLC do not express SRY and SOX9. Differentiation and proliferation is therefore dependent on paracrine regulation by Sertoli cells. Development of both FLC and ALC has been regulated by

Sertoli cell factors desert hedgehog (DHH) and platelet-derived growth factor A (PDGFA). Ptch1 (PTC1) and PDGFRA, receptors for DHH and PDGFA, are expressed in Leydig cells and are essential for FLC and ALC establishment and reproductive function. Interestingly, FLC differentiation as well as Sertoli cell proliferation and mesonephric cell migration are affected in embryonic PDGFRA knockouts [24, 62].

Other Sertoli cell secreted growth factors, including IGF1 and fibroblast growth factor 9 (FGF9) have been shown to regulate Leydig cell function [63]. FGF9 is downstream of SRY and SOX9 and is thought to feedback to maximize the number SOX9 positive supportive cells, possibly also contributing to FLC development [61, 64]. Within Leydig cells, Notch signalling has been proposed to be involved in restricting FLC differentiation by promoting progenitor cell fate [65]. In addition to the aforementioned factors, ALC differentiation and steroidogenic function has been shown to be regulated by pituitary LH and other proteins produced outside the reproductive tract, including bone derived osteocalcin [66, 67]. The androgen receptor (AR), expressed principally in PMCs during the fetal period and in Sertoli, Leydig and PMCs cells in adulthood, is required for ALC, but not FLC development [68].

Masculinization during the fetal period occurs principally through FLC production of androgens and insulin-like hormone 3 (INSL3). T or its derivative dihydrotestosterone (DHT), mediates development of the Wolffian ducts and external genitalia, testicular descent and masculinization of the male brain [61, 69, 70]. INSL3 deficiency leads to impaired testicular descent (cryptorchidism) and feminization [7, 69, 71]. Interestingly, initial fetal masculinization occurs prior to the expression of the LH receptor (LHCGR), demonstrating an independence from the hypothalamic pituitary gonadal axis (Fig. 4) [72, 73].

2.6 Steroidogenesis

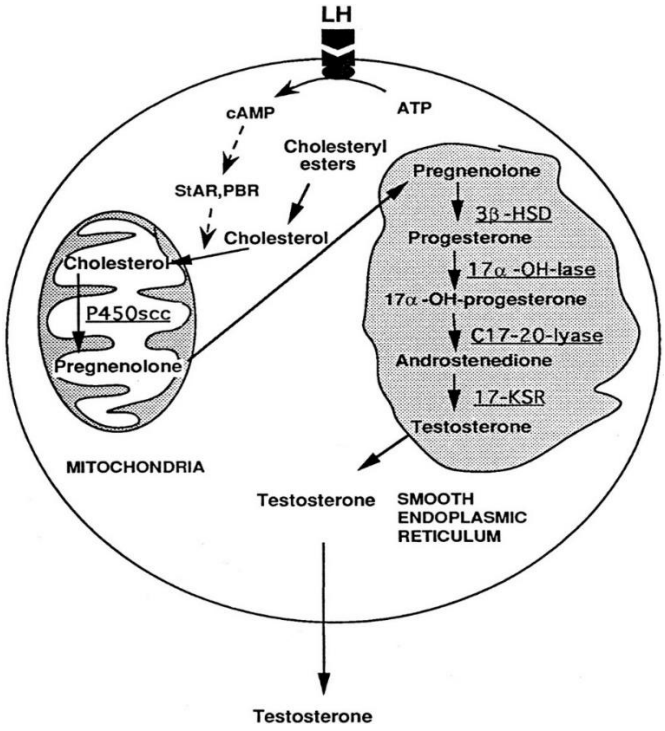

Figure 1.4. Leydig steroidogenesis [74]

Steroidogenesis in ALC requires activation by pituitary derived LH of the Luteinizing Hormone/Choriogonadotropin Receptor (LHCGR) on Leydig cell surface. LH release from the pituitary is initiated by pulsatile hypothalamic gonadotropin releasing hormone (GnRH). Binding of LH to LHCGR triggers a cascade of events, including increased intracellular cyclic AMP (cAMP) and activation of protein kinases (PKA and PKC). These processes ultimately lead to increased expression and acute phosphorylation of the steroidogenic acute regulatory protein (StAR), which along with translocator protein (TSPO), formerly known as the peripheral-type benzodiazepine (PBR) receptor, facilitates transport of cholesterol from the outer to the inner mitochondrial membrane. Cholesterol is the precursor of not only androgens,

but all steroids in the body. Over 50% of cholesterol stored in its free form or as cholesteryl esters, is formed from endogenous *de novo* synthesis [75, 76]. Delivery of free cholesterol derived from lipid droplets, plasma membrane and other cellular sources is an initial rate limiting step in steroidogenesis. Following transport of cholesterol to the inner mitochondrial membrane, a series of cytochrome P450 reactions catalyze the formation of T, beginning first with the conversion of cholesterol to pregnenolone by the side cleavage enzyme CYP11A1 [77]. Pregnenolone is able to diffuse freely from the mitochondria to the smooth endoplasmic reticulum where sequential reactions involving 3β reduction to progesterone (3βHSD), cleavage and hydroxylation (CYP17A - 17α-OH-lase/C17-20-lase) and final conversion of the ketosteroid, androstenedione (17-KSR) ultimately yielding T.

In pubertal and adult testis, T diffuses into the seminiferous tubules where it acts on Sertoli cells. T is also converted to DHT, considered to be a more potent androgen with a higher affinity for AR, within the epididymis and excurrent duct system. Except in the mouse, the majority of circulating androgens within the testis and excurrent duct system are not free, but remain bound to ABP [20].

Although androgens are the principal sex steroids mediating male reproductive function and development, estrogens have also been demonstrated to play an important role. This was first shown in male estrogen receptor (ER) ablation animal models which developed infertility [78, 79]. CYP19A1, an aromatase responsible for the conversion of T to estradiol (E), is expressed in the endoplasmic reticulum of several testicular cell types, including Sertoli, Leydig and germ cells. Additionally, ERs (ERα/ESR1 and ERβ/ESR2) mediating the effects of E are expressed in all mouse testicular cell types during fetal development [80, 81]. Contrarily in the rat,

although ERβ is expressed in all fetal testis cell types, ERα is only expressed in fetal Leydig cells [82, 83].

2.6.2 Transcriptional regulation of steroidogenesis

Transcriptional activation of steroid biosynthesis mediators involves the conversion of signaling pathways initiated by tropic hormones into genomic responses. This process involves the activation transcription factors capable of directly binding the promotor region of target genes. Among steroidogenic genes, StAR regulation remains the best characterized; in addition to StAR being phosphorylated in the presence of trophic hormones, expression is also increased upon stimulation [63, 84]. Regulation of StAR involves a coordinated response between transcription factors already present in Leydig cells and those that they themselves will be upregulating.

Proteomic and microarray approaches in MA-10 Leydig cells stimulated with hCG revealed the differential expression of 79 gene targets, including StAR, nuclear receptor Nr4a1 (Nur77) and JunB, a negative transcriptional regulator of StAR [85]. Nr4a1 has been demonstrated to activate promoters for *StAR*, *Cyp17a1*, *Hsd3b1* and *Insl3* [86-88]. Other reported inducible transcription factors include C/EBPβ and cJun [63].

A complex network of other transcription factors expressed in Leydig cells under basal conditions have also been demonstrated to regulate StAR expression, including SF1 (NR5A1), COUPTF-II (NR2F2), GATA4, SP1, CREB, MEF2 and AP1 family members (cFos and cJun) [63, 86]. Leydig-specific SF1 -/- mice have reduced expression of steroidogenic genes and INSL3, highlighting an essential role in gonadal function [89]. GATA4 is also a critical regulator of steroidogenic genes and FLC cell differentiation [90]. MEF2 has been demonstrated to be expressed in Sertoli and Leydig lineages [91]. In Leydig cells, it has been shown to regulate NR4A1, along with genes

implicated in testis function and development, steroidogenesis and cellular defense [91-93]. Pre-pubertal, but not adult ablation of NR2F2 results in impaired steroidogenesis, highlighting an important role in progenitor Leydig differentiation [94]. In addition to StAR, NR2F2 has also been demonstrated to regulate INSL3 [95, 96].

2.7 Adult Testis

In addition to Sertoli and Leydig cells, other somatic cell types of the testis, including PMCs, mast cells (MC), macrophages and dendritic cells (DC), contribute to the SPG niche and spermatogenesis (Fig. 5).

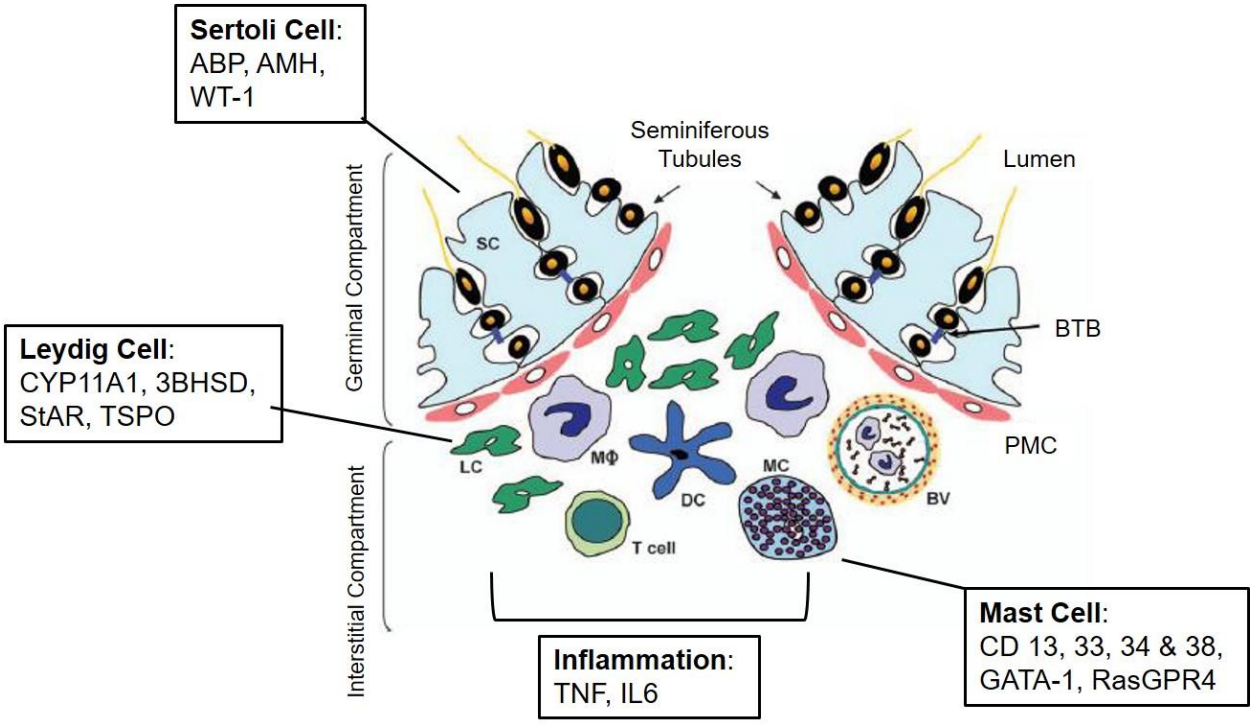

Figure 1.5. Schematic representation of the adult testis and cellular markers

PMCs on the outer edge of the seminiferous tubules mediate sperm movement towards the

excurrent duct system. PMCs contain abundant actin filaments and other cytoskeletal proteins including myosin, vimentin, and alpha-actin [97]. In addition to their contractile and scaffolding functions, PMCs secrete extracellular matrix components (fibronectin, collagens and proteoglycans) as well as cytokines (inflammatory IL-6 and MCP-1) and growth factors (TGFβ, IGF-1, activin A, GDNF) regulating other testicular cell types, including Sertoli cells [17, 97].

MC mediate allergic and inflammatory responses through the release of basophilic granule contents (histamine, serotonin, proteoglycans and serine proteases), cytokines (TNFα and several ILs) and chemoattractant proteins (MCP-1). These cells act in a paracrine fashion on collagen producing fibroblasts and other inflammatory mediators. Derived from the myeloid lineages, several MC types can be identified by the expression of transmembrane receptors (CD13, 33, 34 and 38), transcription factors (GATA-1) and Ras signalling proteins (RASGPR4) [98-102].

Surprisingly, macrophages comprise approximately 25% of the cells in the interstitium of the rat testis [103]. These cells are the principal phagocytic cell in the testis, playing a key role cellular defence and disposal of cellular debris. Macrophages have also been demonstrated to regulate SPG proliferation and differentiation through the expression of colony-stimulating factor 1 (CFS1) and enzymes involved in RA synthesis; experiments involving macrophage depletion lead to impaired SPG differentiation [104]. Infiltration of both MC and macrophages is a hallmark of testicular fibrosis and inflammatory events, both of which are associated with male infertility [105].

Lastly, DC of the testis interstitium are an additional contributor to the immune privileged environment [106]. The principal role of DC is to detect antigenic material and present it to T lymphocytes (T-cells) of the adaptive immune system. Within the rat testis, DC were significantly

increased in an experimental model of autoimmune orchitis, suggesting an important role in testis inflammation and autoimmunity [107].

3. DEHP

Phthalates are used in a diverse array of industrial and commercial application, but most notably in the production of PVC plastics. They increase durability and impact flexibility, accounting for up to 40% of an total plastic weight [108]. DEHP is among the most commonly used plasticizers in industry, accounting for over half of all plasticizers used in recent years [109]. As such, it can be found ubiquitously in numerous products, including cosmetics, shampoos, deodorants, hairsprays, adhesives, solvents and many others [110, 111]. Notably, a number of medical devices, including medical tubing and blood bags used in haemodialysis, transfusions and transplants, are essentially made entirely from plasticized PVC and contain high levels of DEHP. Of concern is that DEHP is not chemically bound to the materials it is used in. Leaching can occur, contaminating the environment and leading to unintentional human and wildlife exposure. A recent study demonstrated the ability of DEHP along with a purported new 'green' plasticizer DINCH, to migrate from PVC medical devices at rates up to 20x higher than other commercially plasticizers [112]. Approximately $1/8^{th}$ of the DEHP initially contained in the PVC leached after 24hrs, highlighting a potential exposure risk for patients, and particularly pregnant women and neonates undergoing medical intervention.

3.1 DEHP structure and metabolism

Raw DEHP is a colorless, viscous liquid soluble in mineral oil, dimethyl sulfoxide (DMSO), benzene and ethanol, but not water. It is a diester with the chemical formula $C_8H_4(C_8H_{17}COO)_2$

formed from phthalic acid and 2-ethylhexanol. The two long branched chains of DEHP result in many metabolites. Upon ingestion, DEHP undergoes rapid hydrolysis by esterases and lipases in the gut to form a catabolite mono (2-ethylhexyl) phthalate (MEHP) with a higher bioactivity (10x) in-vitro [113, 114]. MEHP is subsequently metabolized by Phase 1 detoxifying enzymes to yield over 15 more water soluble metabolites [113]. Metabolites undergo further Phase II modification by glucuronidation and sulfation, increasing hydrophilicity and subsequent excretion in the feces (biliary) and urine.

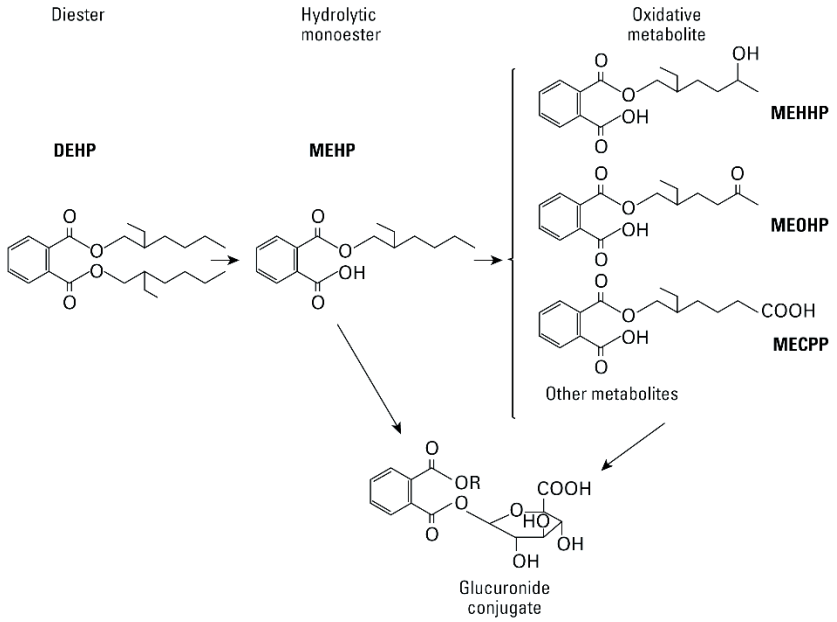

Figure 1.6. DEHP metabolism [115]

DEHP and its metabolites do not bioaccumulate, even following repeated exposures; healthy male volunteers administered ring-deuterated DEHP (DEHP-D4) demonstrated a terminal half-life of 4.3-6.6 hrs [116]. Consistent with other reports, the majority of DEHP-D4 and MEHP-D4 were cleared within 24hrs. Interestingly, normalized MEHP-D4 area under the curve (AUC) values were up to 8.1x higher than DEHP-D4. This ratio is 50x higher than comparative studies in rats,

suggesting a possible increased bioavailability of active MEHP in humans. Sprague-Dawley rats exposed orally with doses up to 1000 mg/kg bw labelled DEHP demonstrated a slightly longer half-life for DEHP (~13 hrs) and comparable values for MEHP (~6hrs) [117].

3.2 DEHP exposure levels

Accidental exposure of DEHP can occur through ingestion of contaminated food (particularly fatty food) or dust or through inhalation and dermal exposure during the use of DEHP containing products. Conventional fetal exposure occurs indirectly via maternal exposure of similar routes. Postnatally, common sources of DEHP exposure include breast milk, formula, baby food and water. Indeed, biomonitoring studies have reported the presence of DEHP and its metabolites in amniotic fluid, cord blood and neonatal blood and urine [8-10, 12, 118]. Averages from several studies indicate that normal daily DEHP human exposure is in the range of 0.003-0.03 mg/kg. Neonates undergoing aforementioned medical interventions however, have been reported to receive up to 6 mg/kg/day, often through a direct intravenous injection [119]. These are concerning numbers given the relatively small body surface area, weight and toxicological vulnerability of neonates [9, 11, 119-123].

3.3 DEHP epidemiology

Several correlative retrospective or cross sectional studies have demonstrated an association between early life or adult DEHP exposure and adverse male reproductive outcomes. Cross sectional studies involve observational characterization of disease prevalence at a defined point in time. They provide a ‘snapshot’ of a particular population and allow for correlative identification of risk factors.

Concerned with the possible effects of phthalate exposure, a Chinese reproductive center recently enrolled 1040 men, collecting semen and urine samples for reproductive parameter and phthalate exposure measurements, respectively [124]. Results demonstrated a dose dependent relationship between urinary MEHP levels and the percentage of DEHP excreted as MEHP with the occurrence of abnormal sperm heads. Following multivariable adjustments, linear regression analysis also demonstrated a positive association (Odds ratio (OR) 2.01 and 1.80) of monobutyl phthalate (MBP) with sperm parameters below reference sperm concentration and absolute sperm numbers. Interestingly, participants had multiple phthalate burden, possibly indicating additive or synergistic effects of multiple exposures.

A Swedish study recruited a smaller number of healthy young men (314), collecting semen, urine blood and semen samples, and measuring DEHP metabolites in relation to detailed reproductive and hormonal parameters [125]. DEHP metabolite levels were found to be significantly associated with decreased sperm motility. Further stratification revealed that the highest quartile of MEHP levels had 27% higher DNA stability, a marker of sperm immaturity, compared to those in the lowest quartile, again highlighting a potential dose response relationship.

Consistent with these findings, lower testosterone was reported in occupationally exposed PVC flooring manufacturers, subject to high levels of inhaled DEHP [126]. Interestingly, DEHP exposure was also associated with reduced sex drive in menopausal women, suggesting that hormonal effects may not be limited to adult males [127].

The limitations of this study design, particularly in the case of EDs, are that they provide little information on previous exposure. Given the current hypothesis that male infertility and testicular cancer are late onset effects of early life exposure, and that EDs do not bio-accumulate and are

rapidly metabolized, a high phthalate burden in adulthood may not necessarily correlate with disease outcomes. This has been reinforced by numerous toxicological animal models which have demonstrated a particular sensitivity to DEHP and its metabolites during the fetal and neonatal windows. These findings contrast with studies on per-pubertal and adult periods in rodents, in which a much higher exposure of DEHP is needed to compromise androgen production and testicular function. These types of studies are likely most valuable in the context reproductive outcomes in relation to acute ED exposure.

Although more challenging to orchestrate, early life cross sectional studies examining reproductive markers or retrospective/prospective fetal and early life exposure studies are more informative. A recent Japanese study provided insight into human fetal risks; 514 pregnant women were assessed for MEHP blood levels between 23-35 weeks gestation along with key cord blood hormonal, Sertoli and Leydig parameters [128]. Adjusted linear regression analysis demonstrated a positive association between maternal MEHP levels and lower T, E, progesterone, Sertoli secreted inhibin B and Leydig INSL3. These findings correlated with extensive animal toxicity data, highlighting Sertoli and Leydig cells as early reproductive targets.

Longer term studies have also been conducted correlating higher maternal DEHP levels at 12 weeks gestation, a sensitive masculinization period, with lower semen volume in adolescence [129]. Anogetinal distance (AGD), a developmental parameter correlated with fetal testosterone levels [130], was also negatively associated with fetal exposure to four phthalates, suggesting feminizing effects [131].

3.4 DEHP animal studies

Compelling evidence in multiple animal species has conveyed a reproductive and developmental risk of DEHP and its principal metabolites. High dose (>234 mg/kg/day) fetal exposure to DEHP from GD14 to parturition suppressed fetal and adult T biosynthesis [132, 133]. Interestingly, results highlighted mechanistic differences between fetal and adult periods; whereas fetal T reductions were correlated with a reduction in steroidogenic enzymes Cyp11a1 and Cyp17a1, effects in adults were unrelated to changes in steroidogenic machinery. Late effects on adult Leydig cell function, long after the initial chemical exposure, highlighted the potential targeting and reprogramming of somatic progenitors during the fetal period. Numerous other studies reported dose dependent effects on Sertoli and Leydig numbers and function, sperm parameters (concentration, motility) and germ cell atrophy in high dose fetal exposure models [134, 135].

Rodent testis organ culture systems have demonstrated the ability of 10μM MEHP, but not DEHP to reduce fetal gonocyte (GD14.5) proliferation and Sertoli AMH production [136]. In contrast, studies treating human fetal testis explants with doses up to 100μM have demonstrated no effects on basal or LH stimulated testosterone production [137]. Independent of hormonal changes, apoptosis of germ cells was observed at doses of 10μM.

3.5 DEHP proposed mechanisms of action

Despite many years of phthalate toxicity experiments, the precise mechanisms of action remain to be elucidated. Evidence from AR deficient mice have shown that DEHP is an AR-independent anti-androgen [138]. Much attention has been directed towards the proposed involvement of peroxisome proliferator-activated receptors (PPAR α/γ), mediators of lipid, cholesterol and fatty acid homeostasis. Indeed, MEHP has been shown to activate rodent PPARα (EC50 = 0.6μM) and

PPARγ (EC50 = 10.1μM) [139]. DEHP has also been shown to induce apoptosis in rat testis via PPARγ [140]. However testis effects were only partially blocked in PPARα (-/-) mice, suggesting the involvement of other nuclear receptors [141]. New evidence suggests the ability of MEHP to act as a Liver X (LXR) or Pregnane X (PXR) receptor (EC50 = 7μM) agonist [142, 143]. Other proposed mechanism involve mineralocorticoid receptor (MR) mediated downregulation of T biosynthesis and early epigenetic alterations in germ and somatic testis cell types [144-146].

4. Genistein

In contrast to synthetic phthalates, phytoestrogens are natural isoflavones found principally in soybean derived foods such as tofu, soy milk and flour, textured vegetable protein and miso, a traditional Japanese seasoning derived from fermented soybeans [122, 123]. Vegetarians are therefore among the highest adult soy consumers. However, reports indicate that soy protein is increasingly being used as an additive in up to 60% of conventional consumer products, including cheese, meat, poultry, fish, cereal and baked goods. Concentrated phytoestrogen supplements are also commonly sold in natural health food stores and pharmacies claiming health benefits. An important source of phytoestrogens in young infants is soy-based infant formula; hospital records in the United States indicate that approximately 25% of infants are fed soy formula [122, 147]. Other early studies report comparable rates between 18-23% [148, 149]. Commonly used baby formulas have been shown to contain phytoestrogen levels up to 47 mg/L, resulting in high infant exposure levels [150, 151].

The primary isoflavones derived from soy are genistein and daidzein, and to a lesser extent glycitein. These derivatives are called phytoestrogens in light of their demonstrated ability to bind ERs. The majority of isoflavones (>94%) found in raw soy preparations are glycosylated;

conjugated parent compounds are called genistin, daidzin and glycitin. The aglycones genistein, daidzein and glycitein generally referred to in the literature are the bioactive metabolites rapidly formed in the gut following digestion. Among the three phytoestrogens, genistein has been reported as the most predominant in soy-based formula at approximately 60% [150, 151].

4.1 Genistein structure and metabolism

Genistein has a non-steroidal ring structure, but a phenolic ring that allows for ER binding (Fig. 7). It is considered the principle bioactive metabolite. Genistein is ingested as a β-D-glycoside, genistin that needs to be hydrolyzed by β-glycosidases in the gastrointestinal tract prior to entering systemic circulation.

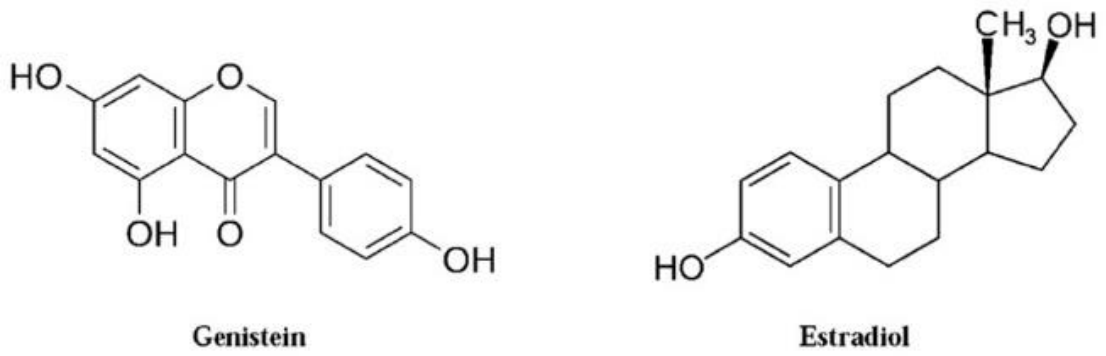

Figure 1.7. Comparison of genistein and estradiol chemical structures

The majority of genistein is also conjugated through glucuronidation and sulfation in the intestine and liver, greatly limiting the actual bioavailability of the active aglycone.[123, 152]. Studies in healthy adults have reported a genistein bioavailability in the plasma of 1-3% following oral administration up to 16 mg/kg bw [152, 153]. Free genistein is also cleared

rapidly, with average half-lives reported being approximately 9hr. Similar results were found for genistein when it was ingested alongside other isoflavones in soy foods [154].

Adult Sprague Dawley rats orally administered 4 mg/kg bw of radiolabeled genistein demonstrated a comparable half-life of 9-12 hrs [155].This was in contrast to earlier reports in involving higher oral dose (50mg/kg bw) half-lives of 3-4 hrs, suggesting saturable kinetics at elevated doses [156]. Average percentages of bioavailable genistein in rodents are below 10%, slightly higher than human data [123, 157].

Unfortunately, little information exists on the kinetics of genistein during the human fetal or neonatal periods. Studies in pregnant Sprague Dawley dams demonstrated however, that while fetal levels of total genistein were 20-fold lower that maternal plasma, active aglycone levels were only 5-fold lower [158]. This related to an up to 34% fetal bioavailability of active genistein, suggesting a high rate of placental transfer.

4.2 Genistein exposure

Unlike DEHP, foods containing soy and phytoestrogens are intentionally ingested, leading to much higher levels. Exposure rate is proportional to the quantity of soy based foods and additive consumed. Certain population, including many Asian countries consume high soy diets; Japanese men and women are reported to have the world's highest average circulating genistein levels at ~0.5μM [159, 160]. In contrast, Europeans range from 0.00143-0.378μM and Americans have very low (0.0017μM) blood levels [161].

In infants, a limited amount of genistein exposure occurs via ingestion of breast milk. Levels can be higher in vegetarian and vegan mothers, but are generally low in the range of several μg/L [150, 151]. It has been well documented that the highest early life exposure occurs in

infants fed soy-based infant formula. Indeed, infants fed soy formula have blood levels of genistein between 1-10μM [162]. These levels are up to 20-fold higher than Japanese men and women, hundreds of times higher than non-vegetarian and up to 20,000-fold higher than circulating endogenous E levels [150, 157, 162]. Given the comparatively small body size and surface areas of newborns, exposures through soy-based formula translate into 2.3-9.3 mg/kg bw total phytoestrogens and 1.3-6.2 mg/kg bw of aglycone equivalents [122].

4.3 Genistein epidemiology

A short term retrospective study was conducted in male partners of infertile couples [163]. Surveying of soy-based food consumption over the previous three months was assessed in relation to semen quality parameters by linear and quantile regression; an inverse relationship between soy food consumption and sperm concentration was observed and this effect was more pronounced in overweight and obese men. Men with the highest soy consumption rates had on average 41 million fewer sperm/ml ejaculate than those who consumed very little. Other sperm parameters, included motility, morphology and ejaculate were not found to be associated with soy intake in any way. A cross-sectional study in Japanese men examining ED urine metabolites in male partners of subfertile couples found a similar inverse relationship between phytoestrogen exposure and sperm concentration [164].

Studies investigating the potential influences of soy intake on ART efficacy however, reported no association or a potential benefit; maternal dietary soy was positively associated with live birth probability in infertile couples undergoing ART [165]. Similarly, no association between paternal soy intake and fertilization, implantation, clinical pregnancy or live birth was observed in couples undergoing IVF [166]. These results are encouraging regarding the potential

hormonal interference of phytoestrogens in ART. They may also highlight the ability of ART to circumvent environmental causes of infertility. Techniques such as ICSI only require a single sperm and are employed in particularly poor fertility conditions. Despite the ability to fertilize and generate live births, questions arise as to whether 'unfit' sperm that would normally be screened out by endogenous mechanisms are being used to create offspring.

While no studies have investigated the effects of individual phytoestrogens during fetal and neonatal periods, several retrospective outcome studies have been conducted with a focus on soy based infant formula. These studies are inherently difficult to control however, estimating previous exposure through surveys that are subject to recall bias and other confounders.

Apart from a reported 5-fold increase in hypospadias in male offspring of vegetarian mothers [167], presumed to have elevated phytoestrogen exposure, a limited number of small sample size outcomes have not demonstrated male reproductive or developmental risks. Revisiting adults (male and female) that had previously participated in a controlled, nonrandomized infant formula trial demonstrated no difference in self-reported general health (height, weight, current condition) and reproductive history, between soy and cow's milk formula [149].

In contrast, early soy exposure was associated with a 25% increased risk of premature puberty (before 12 years old) in a relatively small sample of young women [168]. Similarly, high circulating isoflavone levels were positively associated (4-fold increased risk) with precocious purberty, as determined by breast budding and a GnRH stimulation tests, in 8-10 year old Korean girls [168].

4.4 Genistein animal studies

Several rodent studies have demonstrated the ability of early life genistein exposure to induce alterations in testicular function and external genitalia dysgenesis. Male rats sired from pregnant dams placed on genistein containing (5mg/kg feed) diet during gestation and lactation displayed decreased AGD [169]. Puberty onset was significantly delayed in male rats treated in utero with higher doses of genistein (25 mg/day) by maternal subcutaneous injection. Low dose (0.17 mg/kg bw) daily in utero exposure to genistein also led to a 25% occurrence of hypospadias in male mice [170], an effect that was correlated with impairment of tissue morphogenesis pathways, cell proliferation and cell survival at higher doses (500 mg/kg bw) [171].

Male rats exposed via gestational exposure to high dose genistein displayed late onset germ cell effects, including aberrant or absent spermatogenesis [172]. Organ culture studies using fetal testis from selective ER knockout mice (ERα -/- or ERβ -/-) demonstrated the ability of low dose genistein (10nM) to induce aberrations in prepubertal spermatogenesis, Leydig and Sertoli cell development and function in an ERα dependent manner [173]. Genistein also induced alterations in developing gonocytes, transiently altering intracellular signaling (PGDFRs, RAF1, MAPK) via ERβ, suggesting cell and receptor type specificity [32, 174].

4.5 Genistein proposed mechanisms of action

Genistein has been shown to possess broad biological activity, including activation of ERs, PPARs and LXR, direct or indirect antioxidant action, modulation of cellular signaling (protein tyrosine kinase (PTK) inhibition) and modulation of DNA methylation [175-177]. There is strong rationale to suggest that much of genistein's ED action is related to activation of ER gene targets. Indeed,

genistein has been demonstrated to be a full agonist for ERα and partial agonist for ERβ with EC50 of 0.1-1μM and 0.01-0.1μM respectively [178].

Other mechanism however may still be contributing in an additive or synergistic manner to observed reproductive effects; In-vitro evidence has identified genistein as a potent aromatase inhibitor, highlighting the potential disruption of endogenous estrogen levels or the androgen/estrogen balance during male reproductive development [179]. Interaction with other nuclear receptors with known involvement in male reproductive function and development, including PPAR, LXR and aryl hydrocarbon (AhR) receptor has also been proposed [29, 180, 181]. Numerous dietary studies have proposed the ability of genistein to act as a lipid modifier via LXR and PPAR in adipocytes [29, 180].

Genistein may also act via non-genomic pathways, including through PTK inhibitory activity. PTKs are signaling mediators downstream of numerous key growth factors including EGF, IGF, PDGF and NGF [182]. Although IC50 are generally high, ranging from 2.6 μM for EGF-R to 29.6 μM for v-SRC depending on the PTK substrate, PTK inhibition of important cellular pathways may be an additional component of genistein toxicity [182].

5. Studies involving ED mixtures

Historically, conventional chemical risk has been conducted on a single chemical basis. More recently, the potential cumulative actions of toxicants have been seriously considered by regulatory agencies. Although characterizing specific mechanism of toxicity and subsequent legislative decisions become increasingly challenging, assessing chemical risk in a cumulative manner is thought to better parallel real life human exposure involving a myriad of environmental substances.

This change in doctrine has been supported by a comparatively limited number of more recent toxicological studies demonstrating additive or synergistic effects of multiple chemical exposures, including EDs.

Pioneering work by the United-States Environmental Inspection Agency was conducted with the ultimate goal of generating a framework for cumulative chemical exposure risk assessment [183, 184]. Studies involved mixing known and proposed antiandrogens (pesticides vinclozolin, procymidone and linuron and phthalate esters with varying mechanisms of antiandrogenicity) at half of known ED50 for hypospadias and epididymal dysgenesis in rodents. A cumulative synergistic risk for male reproductive toxicity that well exceeded additive predictions was demonstrated for these multi-component mixtures. In contrast, a rodent study combining phthalate esters with a cholesterol lowering statin (Simvastatin) reported additive effects on fetal T production and gene expression despite possessing significantly different modes of action [185].

Reproductive toxicity of mixtures containing EDs with similar mechanisms of action has been demonstrated to occur in a predictive dose-additive manner; fetal exposure to 5 phthalate esters resulted in predicted reductions in fetal T levels, male reproductive tract malformations and correlated changes in gene expression (StAR, INSL3 and CYP11A1) [186].

Cumulative epigenetic effects of EDs have also been reported; fetal exposure to a mixture of plasticizers (bisphenol A (BPA), DEHP and dibutyl phthalate (DBP)) induced a transgenerational (F1-F3) risk for pubertal abnormalities, testis disease and primary ovarian insufficiency in females [187]. Effects in males were also correlated with numerous differential DNA methylation regions within the sperm epigenome. The limitation of this study however, was that only mixtures were

assessed. Cumulative risk can only be supported when mixtures are compared against individual exposures.

Despite considerable groundwork in the effects of ED mixtures, the translatability of findings to risk assessment are greatly limited by the doses used. Much of the ED mixture data, like previous single dose studies, are based on high dose (% of ED50) exposures well exceeding actual environmental levels. A growing body of evidence suggests that EDs do not follow linear dose response curves for many toxicological endpoints. The existence of non-monotonic or bi-phasic response curves has been the subject of numerous recent reviews and expert panel discussions [188-191]. It is therefore likely that low dose ED mixtures will also deviate from predictive toxicological principles.

6. Methodology

The following section discusses certain unconventional methodologies and experimental methods used in this thesis. Although each method is introduced briefly in the manuscripts of chapters 2 and 3, this section is intended to provide supplemental information, clarity and rationale for the use of these methods.

6.1 Gestational day (GD)-14 to parturition exposure model

Our studies employ a developmental exposure model in which pregnant Sprague Dawley dams are gavaged from GD14 to parturition with either control corn oil or corn oil + genistein, DEHP or combined genistein + DEHP, once daily at dose of 10 mg/kg per day adjusted for body weight (FIG. 8).

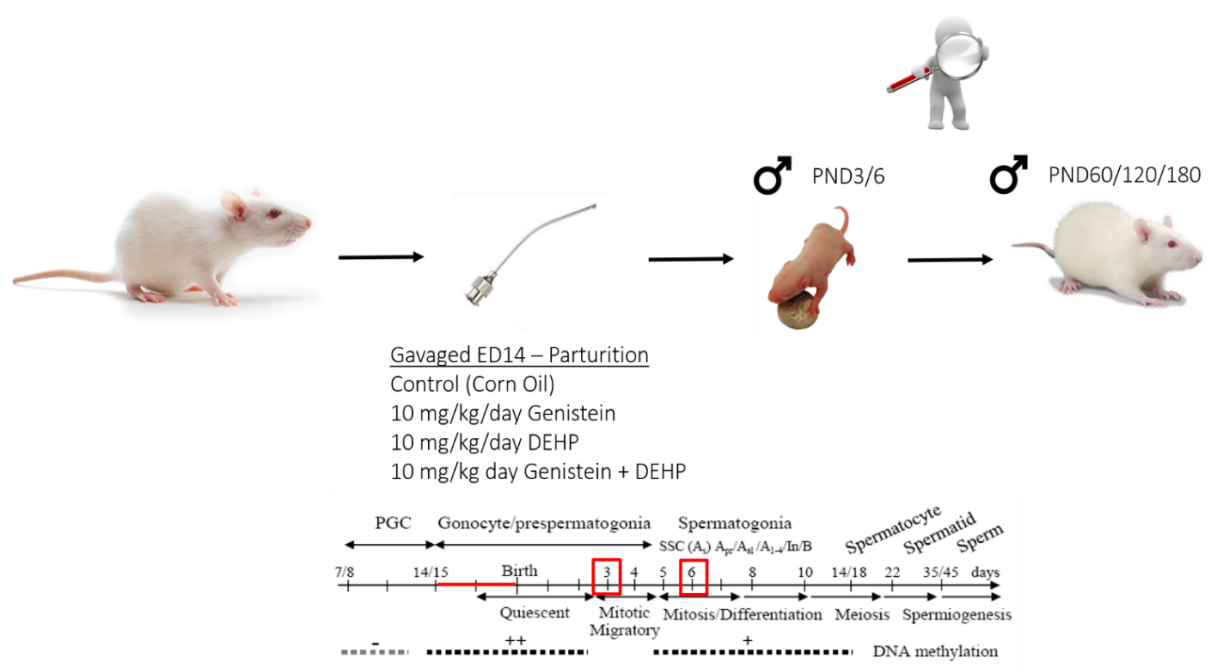

Figure 1.8. GD14 to parturition rat exposure model. Germ cell timeline adapted from Culty et al. 2009 [27]

In the context of male reproductive development and function, it is generally accepted that the fetal and neonatal period corresponds to a critical hormone dependent "masculinization" window. In the rodent, fetal T production begins at approximately GD14.5, peaks between GD17-18 and declines shortly after birth [192]. Although gestational trophic factors vary between humans and rodents, this masculinization window parallels a peak in human fetal androgen production occurring around 15 weeks gestation. The time sensitivity of this developmental widow was emphasized in previous studies involving phthalate exposed pregnant Sprague Dawley dams; gestational treatment for a little as 2 days in late gestation (GD16-18) was sufficient to induce detrimental reproductive effects in male offspring [193].

Although in reality human exposure to EDs starts at the time of conception and continues throughout the neonatal period via lactational and dietary exposure, treatment GD14-parturition

allows specific targeting of the masculinization window. This regimen minimizes additional variables and allows for more accurate characterization of toxicological mechanisms: beginning treatment post organogenesis at GD14 allows differentiation from secondary toxic effects, such as impaired heart development resulting in poor testicular blood flow that can also affect male reproductive development. Continual treatment of dams during lactation also creates additional toxicokinetic variables; lactational transfer of the active aglycone form of genistein is very limited in rats in comparison to humans [194]. Placental transfer is reported to be the principal route of exposure in developing rodents [158].

Oral gavage of pregnant dams, rather than intraperitoneal or intravenous injections was selected as the route of administration. This route was selected both to tightly regulate exposure doses and also mimic physiological metabolism, including activation of genistein and DEHP metabolites in the gut microflora, liver and kidney clearance and placental transfer that occur prior to human fetal exposure. Although EDs can also be incorporated into maternal food for subsequent consumption, study designs utilizing this method have a greater difficulty standardizing actual exposure levels.

The dose selected for the in-vivo experiments was considered to be in the upper end of human exposure. It was also selected based on previous studies demonstrating that 10 mg/kg was below previous reported thresholds of toxicity [32, 132, 133, 174, 195], allowing us to appropriately test our hypothesis pertaining to additive or synergistic effects between genistein and DEHP.

Gestationally treated male offspring were raised on a normal diet to defined ages, sacrificed and analyzed. Treatment doses were adjusted according to changes in dam weights. Male offspring were euthanized at specific postnatal ages to evaluate testis function and development, including fetal Leydig activity and the initiation of gonocyte proliferation/migration (PND3 and

differentiation to form a critical pool of SSCs (PND6) following in-utero exposure. Mature adult male offspring were also assessed to determine long term, permanent alterations in testis somatic and germ cell function. Testes were collected, weighed and either snap frozen for protein or gene expression analyses, fixed in paraformaldehyde for histology or immunohistochemistry, or dissected for organotypic cultures.

6.2 Testis organ culture

Organotypic PND3 testis cultures were performed both ex-vivo with in-utero treated animals and in-vitro using control testis in defined concentrations of genistein and MEHP, the principal bioactive metabolite of DEHP (FIG. 9).

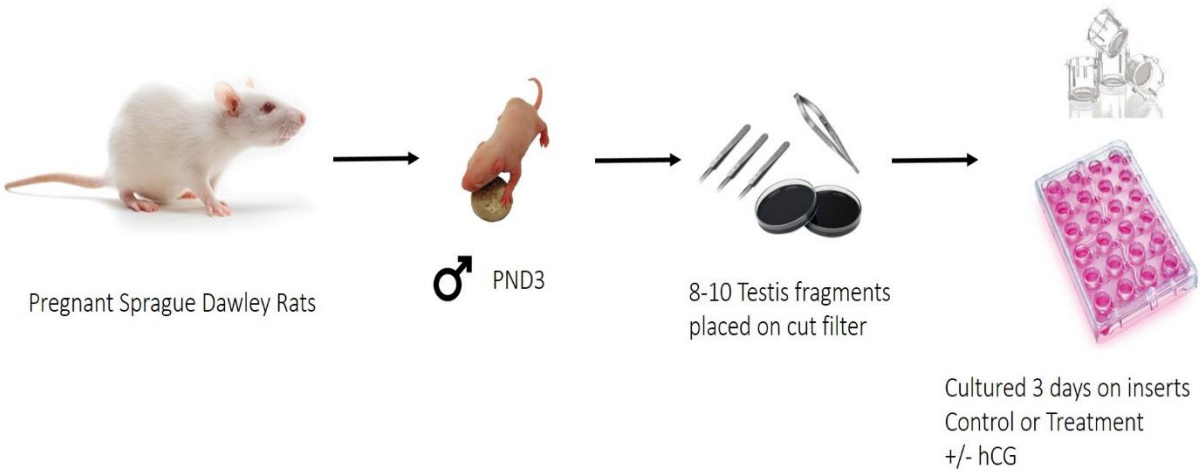

Figure 1.9. PND3 testis organ culture

The testis fragments maintain function over several days in culture, allowing for functional assessment of both somatic and germ cell types. In contrast to individual primary cultures or

cell lines, organ culture also has the added benefit of maintaining testis architecture and functional interaction between multiple cell types.

Although surrogate markers for testis androgen production can be easily evaluated through protein and gene analyses, actual intra-testicular T production is logistically challenging to measure. Excision of previously treated testis for organotypic cultures allows for direct assessment of basal and hormonally stimulated androgen production. In-vitro treatment of control testis also serves as an additional 'in-between' toxicological model. Despite being based on an artificial system, the in-vitro method allows for precise control of toxicant exposure levels and the ability to distinguish direct effects related to the presence of the chemicals from systemic toxicity.

Pre-treated or control PND3 testis were excised and cut into 8-10 small fragments, placed on a filter paper and cultured on trans-well inserts in normal medium, medium containing defined amounts (10 uM) of either control (0.2% DMSO), genistein, MEHP or genistein + MEHP, and in the presence or absence of hCG for three days. Supernatant containing steroids was collected daily and replenished with new medium. Testosterone levels in supernatant were measured by radioimmunoassay (RIA), expressed in ng/testis or fold change relative to control. After organ culture, testis fragments were collected, fixed and sent for embedding and cutting. Histological and morphological alterations were evaluated following hematoxylin and eosin counter staining.

Chapter II

“Manuscript I”

DISRUPTION OF RAT TESTIS DEVELOPMENT FOLLOWING COMBINED IN-UTERO EXPOSURE TO THE PHYTOESTROGEN GENISTEIN AND ANTI-ANDROGENIC PLASTICIZER DI-(2-ETHYLHEXYL) PHTHALATE

Running Title: Effects of genistein and DEHP on Male Reproduction

Summary Sentence: Combined in utero exposure to genistein and Di-(2-ethylhexyl) Phthalate induces long term alterations in male reproductive function in a manner that is different from individual compounds.

Key Words: Endocrine Disruptor, genistein, germ cells, Leydig, Phthalate, Sertoli, Testis, Toxicology

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ABSTRACT

Fetal exposure to environmental endocrine disruptors (EDs) is thought to contribute to reported idiopathic increases in adult male reproductive abnormalities. Although humans are exposed to a myriad of EDs from conception to adulthood, few studies have evaluated the effects of combined EDs on male reproduction. In the present study, we demonstrate that simultaneous gestational exposure to the phytoestrogen genistein and the anti-androgenic plasticizer Di-(2-ethyhexyl) phthalate (DEHP) induces long term alterations in testis development and function. Pregnant Sprague Dawley rats were gavaged from gestational day 14 to birth with corn oil, genistein, DEHP or their mixture at 10 mg/kg/day, a dose selected from previous dose-response studies using single chemicals for its lack of long term testicular effects. Hormonal and testicular end-points were examined in adult male offspring. Serum testosterone levels were unchanged. However, significant increases were observed in testis weight and in the expression of mast cell markers in testes from adult rats exposed gestationally to combined compounds. The ED mixture also altered the mRNA expression of Sertoli cell makers *Wt1* and *Amh*, and germ cell markers *cKit* and *Sox17*, measured by quantitative real time PCR (qPCR), suggesting long term disruption in testis function and spermatogenesis. Alterations in germ cell markers might reflect direct effects on fetal gonocytes or indirect effects via primary targeting of somatic cells, as suggested by differentially regulated Leydig cell associated genes (*Hsd3b*, *Anxa1*, *Foxa3* and *Pdgfra*), determined by gene expression array, qPCR and protein analyses. The two chemicals, when given in combination, induced long term reproductive toxicity at doses not previously reported to produce any conspicuous long term effects. Our study therefore highlights a need for a more comprehensive evaluation of the effects of ED mixtures.

INTRODUCTION

Reported increases in the incidence of male reproductive abnormalities are believed to result from endocrine disruptor (ED) induced perturbations of fetal programming and gonadal development [196-198]. Indeed, from the time of conception through adulthood, humans are exposed to countless anthropogenic and naturally occurring EDs. The androgen dependence and requirement of an adequate androgen/estrogen balance of the male reproductive tract creates a particular vulnerability to EDs possessing anti-androgenic or estrogenic properties [7, 199, 200]. Interestingly, several common environmental pollutants, including natural phytoestrogens, derivatives of plastics, paints, pesticides and other industrial processing chemicals have been shown to act as EDs and have been found in amniotic fluid, cord blood, breast milk and semen [8-12, 118].

The fetal period is a particularly sensitive and critical developmental window for the male reproductive system. Germ cell development is a highly controlled process that is regulated by a combination of endocrine and paracrine signals [16, 17, 48, 201]. During development, fetal primordial germ cells (PGCs) first migrate to the genital ridge and interact with somatic cells of the seminiferous cords and interstitium of the gonad primordia [202]. At this point, fetal germ cells are termed gonocytes [203]. While PGCs undergo erasure of their parental DNA methylation profile, the period of fetal to neonatal gonocyte development corresponds to active DNA remethylation and establishment of new paternal imprints [204]. Interstitial fetal Leydig cells produce testosterone necessary for differentiation of the urogenital tract and testis descent. Testosterone diffuses into the seminiferous tubules and, along with pituitary follicle stimulating hormone (FSH), interacts with Sertoli cells. Sertoli cells provide factors that fuel germ cell metabolism and male reproductive development [61].

Neonatal gonocytes proliferate and differentiate to form a critical pool of renewable spermatogonial stem cells (SSCs) that will support spermatogenesis. During spermatogenesis, testicular germs cells are transformed from round cells to cells with a unique shape, highly condensed nucleus and flagellar apparatus. The generation of spermatozoa from stem cells allows the passage of genetic material to offspring. Therefore, improper SSC formation and differentiation carries the risk of generating spermatozoa that could transfer an altered genome or epigenome to the next generation.

Genistein is a plant derived phytoestrogen reported to have broad biochemical interactions, including estrogen receptor and PPAR activation, direct or indirect antioxidant action, and modulation of important signaling molecules and DNA methylation [175-177]. Fetal exposure to genistein is predominantly via maternal ingestion of soy derived products and subsequent placental transfer, whereas neonatal exposure is highest in soy formula fed newborns [11]. Fetal genistein exposure impairs testosterone production via ERα, inducing aberrations in prepubertal spermatogenesis, Leydig and Sertoli cell development and function [173]. Fetal exposure has also been shown to transiently alter intracellular signaling (PGDFRs, RAF1, MAPK) in neonatal gonocytes and affect neonatal and prepubertal spermatogenesis [32, 174, 195].

Phthalates are plasticizing agents commonly used in the production of industrial and commercial products. They are primarily used in the production of polyvinyl chloride (PVC) plastics [108]. DEHP is the most common phthalate plasticizer, accounting for over 50% of market share in 2010 [109]. DEHP is not chemically bound to plastic and can therefore leach out into the environment and contact humans. Fetal exposure occurs via maternal ingestion, inhalation or dermal exposure. Neonatal exposure continues via contaminated breast milk, baby food and water, but is highest in neonates undergoing medical intervention using high phthalate

equipment [8-10, 12, 118]. Phthalates are well known reproductive toxicants with androgen receptor independent anti-androgen effects [138]. Fetal exposure to DEHP suppresses fetal and adult testosterone biosynthesis [132, 133]. Dose dependent induction of testicular atrophy, alterations in Leydig and Sertoli cell numbers and functions, changes in sperm concentrations and motility parameters and loss of spermatogenic cells types have been reported in numerous animal models for fetal phthalate exposure [134, 135]. DEHP, or its principal bioactive metabolite, MEHP, has also been demonstrated to activate PPARs, and alter DNA methylation status and estradiol-regulated proteins [145, 146, 205, 206].

Relatively few studies have evaluated the effects of combined exposures [186, 207], while animal studies often used doses exceeding environmental levels. We hypothesized that prenatal exposure to combined genistein and DEHP, at doses not previously reported to induce any conspicuous long term male reproductive toxicity, will pose a greater risk than individual compounds.

We report herein that two common endocrine disruptors induced long term alterations in testis function and development in a manner that is different from individual compounds. Thus, the present findings indicate that assessing reproductive risk based on single chemical effects might not faithfully represent the true risks of exposure to ED mixtures during critical periods of male reproductive development.

MATERIALS AND METHODS

Treatments and Tissue Collection

Pregnant Sprague Dawley rats were purchased from Charles River Laboratories, and kept on a 12L:12D photoperiod with ad libitum access to food and water. To address long term alterations in testis development and function, pregnant rats were treated daily from GD14 to parturition

by gavage with 1 ml of corn oil alone (control) or containing genistein, DEHP or a combination of genistein and DEHP, both used at a dose of 10 mg/kg (all from Sigma Aldrich, St. Louis, MO). This dose was selected from previous dose-response studies in which we had examined the short and long term effects of *in utero* exposure to genistein or DEHP used separately [32, 132, 133, 174, 195]. These studies had shown that a fetal exposure to 10 mg/kg/day genistein did not induce changes in circulating testosterone nor germ cell numbers in adult rats [195], while long term effects of DEHP were seen for much higher doses [132, 133].

The selected doses of genistein and DEHP correspond to 1.6-2.0 mg/kg/day in human equivalents [208]. Equivalent doses were calculated with the formula: human equivalent dose (in mg/kg) = rat dose (in mg/kg) x (rat K_m/human K_m), using human adult or child K_m and rat K_m, determined from the body surface area of both species [208]. Under normal conditions, human exposure to genistein and DEHP ranges from 0.01 to 0.2 mg/kg/day and 0.003-0.03 mg/kg/day, respectively. Neonates fed soy based infant formula or undergoing medical intervention however, can experience levels that are 10 to 100 fold higher than the general population [9, 11, 119-123].

Treatment doses were adjusted according to changes in dam weights. Gestationally treated male offspring were raised on a normal diet to defined adult ages, sacrificed and analyzed. Male offspring were euthanized at PND 60, 120 and 180 by CO_2 and weighed. Each end-point was determined using randomly selected offspring of 3 to 7 dams per treatment, where N is defined as the number of dams. In figure 1, N were 7, 4, 4 and 6 for controls, genistein, DEHP and genistein + DEHP treated rats respectively at PND120 and 5, 4, 3, 5 at PND180. Following the same order of treatments, in figures 2 to 4, N were 6, 4, 3, 5; and in figure 6 they were 5, 4, 3, 5 respectively. Testes were collected, weighed and either snap frozen or fixed in paraformaldehyde. Blood of male offspring was collected by percutaneous cardiac puncture. Serum testosterone and LH/FSH were

measured by radioimmunoassay and ELISA, respectively (data not shown). Animals were handled according to protocols approved by the McGill University Animal Care and Use Committee.

RNA Extraction and Quantitative Real-Time PCR

Testis RNA was extracted using QIAGEN RNeasy Plus Mini kit (Qiagen, Santa Clarita, CA). cDNA was synthesized from isolated RNA using the single-strand cDNA transcriptor synthesis kit (Roche Diagnostics, Laval, QC). Quantitative real-time PCR (qPCR) was performed as previously described using the LightCycler 480 Real-Time PCR System (LC480, Roche Diagnostics) [209]. Alpha tubulin was used as an endogenous control and for normalization of gene targets. A minimum of three male offspring from different litters were evaluated in triplicate. The comparative Ct method was used to calculate relative expression. Data are represented as mean relative mRNA expression in arbitrary units. Complete primer sequences for all gene targets can be found in Table 2.1.

Immunohistochemistry

Testis from male offspring were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were used for immunohistochemical analysis as previously described [195]. Briefly, tissue sections were dewaxed and rehydrated, followed by antigen retrieval using a DAKO solution. Primary antibodies, PDGFRa (Abcam, ab61219) and HSD3b (Abcam, ab65156), were incubated at 1/100 and 1/300 dilutions respectively overnight at 4^{o}C and revealed using species appropriate secondary antibodies (mouse or rabbit), HRP/colorimetric HRP chromogen (AEC) and hematoxylin counterstaining. Representative images are shown.

Gene Expression Array Analysis

Ultra-pure total RNA was extracted as described above from the testes of PND120 rats, using offspring from 3 different dams per treatment, and diluted to 100ng/µl following nanodrop analysis. Affymetrix 2.0 ST microarray analysis was conducted by Genome Quebec. Initial data analysis was performed by Dr. Jaroslav Novak as previously described [209, 210]. Briefly, data analysis contained quality control analysis, normalization, abbreviation and dispersion analysis, differential analysis of gene expression and gene set enrichment analysis. Data was normalized using PLIER and further filtered using FlexArray, only retaining entries whose fold changes were greater than 40% relative to control and p-values were less than 0.05 according to the Bayesian approach, and that were linked to a specific RefSeq. Pathway analysis (DAVIDS, Inginuity and Reactome) and key word searches in PubMed were used to identify candidate genes for subsequent validation and expression studies.

Statistical Analysis

Excluding microarray data, all statistical analysis was performed using unpaired two-tailed t-test with statistical analysis functions in GraphPad Prism version 5.0 software (GraphPad Inc., San Diego, CA). Asterisks indicate a significant change relative to control ($P \leq 0.05$, n varying from 3 to 7 per condition/end-point).

RESULTS

In Utero Exposure to Genistein and DEHP Alters Adult Testis Weights and the Expression of Inflammatory Mast Cell Markers

Examination of the body weights (Fig. 2.1A, C) and anogenital distances (data not shown) of PND120 and 180 male offspring revealed no significant differences between control and treated animals. At PND120 however, the testis weights were significantly increased in the combined genistein and DEHP treatment group (Fig. 2.1B), while there was an increasing trend for the same exposure at PND180 (Fig. 2.1D). Serum testosterone in PND60, PND120 and PND180, as well as PND60 and PND120 LH and FSH levels were not altered following in utero treatments (data not shown).

qPCR analysis of PND120 testes showed an increase in the expression of inflammatory mast cell markers *Cd13* (Fig. 2.2A), *Cd33* (Fig. 2.2B) and *Cd38* (Fig. 2.2C), observed only in the testis of PND120 animals treated with combined genistein and DEHP. The mRNA expression of the pro-inflammatory macrophage marker *Cd68* (Fig. 2.2D) and anti-inflammatory macrophage marker *Cd163* (Fig. 2.2E) demonstrated an increased trend in the combined exposure group, but the changes were not statistically significant. Extracellular matrix (ECM) markers Collagen I, IV, XVIII, Laminin and Fibronectin mRNA levels in testes were also quantified by qPCR, but were unaltered in any treatment group relative to control (data not shown).

In Utero Exposure to Genistein and DEHP Alters Adult Testis Expression of Key Somatic and Germ Cell Markers

The mRNA expression of known Leydig and Sertoli cell markers was examined by qPCR to gain insight into adult testis somatic cell function following in utero exposure to genistein and DEHP. Two Leydig specific genes were initially examined. *Cyp11a1*, a gene that catalyzes the initial conversion of cholesterol to pregnenolone and *Tspo*, a gene involved in cholesterol transport from the outer to the inner mitrochondrial membranes of steroidogenic Leydig cells [211]. While

Cyp11a1 expression was not altered in any treatment group (Fig. 2.3A), *Tspo* expression was significantly decreased in PND120 DEHP treated rats, but not by genistein or combined genistein and DEHP (Fig. 2.3B). Sertoli cell expressed *Amh* (Fig. 2.3C) and *Wt1* (Fig. 2.3D) were significantly decreased only in genistein + DEHP treated animals, whereas *Abp* was not altered by any treatment (data not shown). The mRNA expression of two key steroid receptors, *Ar* and *Esr1*, was also determined. *Ar* was significantly increased in PND120 animals treated with either genistein or genistein + DEHP, but not DEHP alone (Fig. 2.3E). *Esr1* was significantly decreased only in animals treated gestationally with the combined compounds (Fig. 2.3F).

The expression of cell specific germ cell markers [48, 212] was investigated to assess long term effects on spermatogenesis. The SSC/undifferentiated spermatogonia marker *Thy1* (Fig. 2.4A) was decreased in DEHP and combined treatment group, whereas differentiating spermatogonia marker *cKit* (Fig. 2.4B) was significantly decreased only in the combined treatment group. *Sohlh2*, a spermatogonial marker enriched in undifferentiated spermatogonia, was significantly increased in all treatment groups relative to control (Fig. 2.4C), whereas a late spermatogonia/pachytene marker *Sox17*, was significantly decreased only in animals treated with both genistein and DEHP (Fig. 2.4D). Lastly, *Scp1* (Fig. 2.4E), expressed in zygotene and *Sp10* (Fig. 2.4F), expressed in round spermatids, were not significantly altered in any treatment group.

Gene Expression Arrays Reveal Key Underlying Genetic Alterations in PND120 Testis Following In Utero Exposure to Genistein and DEHP

Gene expression array analysis of PND120 testes was conducted to mine underlying genetic aberrations following gestational exposure to genistein and DEHP. A combined total of 240 genes were significantly altered in all treatment groups relative to control (Fig. 2.5). Animals treated with

both genistein and DEHP had the greatest number of genes changes (90), followed by those treated with DEHP (88) and genistein (76) alone. Of the total 240 genes altered, 83 were up regulated and 171 were down regulated. Of note also is that there were a relatively low number of gene changes that were common between different treatments, emphasizing unique responses to individual or combined treatments.

Gene lists were sorted and blasted in PubMed to identify those implicated in male reproduction and development (Table 2.2). Genes related to extra cellular matrix (*Dcn*, *Gsn* and *Fn1*) and intercellular communication (*Gjb2* and *Gjb6*) were down and up regulated, respectively, in various treatment groups including combined genistein and DEHP. Of particular interest was the large number of Leydig cell-related genes (*Pdgfra*, *Hsd3b*, *Lhcgr* and *Stc1*) that were found to be significantly altered in various treatment groups, while two novel Leydig candidate genes, *Anxa1* and *Foxa3*, were significantly altered only in the combined treatment group.

Validation of Candidate Genes Identified by Expression Arrays

A subset of candidate genes identified by expression array analysis was further validated and examined by qPCR and IHC. Relative mRNA expression of *Pdgfra* was significantly reduced in PND120 testis of DEHP and combined genistein and DEHP treated animals (Fig. 2.6A), whereas *Hsd3b* mRNA was significantly reduced in all treatment groups relative to control (Fig. 2.6B). A novel candidate gene for endocrine disruption, *Foxa3*, was confirmed as being reduced only in response to the combined treatment (Fig. 2.6C). Lastly, consistent with expression array data, *Stc1* mRNA levels were significantly reduced only in DEHP treated animals relative to control (Fig. 2.6D).

Immunohistochemistry was done on PND120 testis tissue sections to investigate alterations in protein expression and localization. As previously reported in the literature, PDGFRa was expressed predominantly in interstitial Leydig cells (LC), blood vessels (BV) and myoid cells (MC) surrounding the seminiferous tubules in control PND120 testis (Fig. 2.7A). Consistent with qPCR and array results, interstitial localization of PDGFRa was slightly less intense in genistein (Fig. 2.7B) treated animals and considerably less in DEHP (Fig. 2.7C) and combined genistein and DEHP (Fig. 2.7D) animals compared to control. The steroidogenic enzyme HSD3b was also expressed in the intersitium, but was restricted to Leydig cells in control animals (Fig. 2.7E). Also in agreement with qPCR and array data, there was substantially less HSD3b immunopositive staining in interstitial Leydig cells of genistein (Fig. 2.7F), DEHP (Fig. 2.7G) and combined genistein and DEHP (Fig. 2.7H) treated animals.

DISCUSSION

The present study showed that combined exposure to two common endocrine disruptors, genistein and DEHP, induced long term alterations in testis function and development, in a manner that is different from individual compounds. A significant increase in testis weight and increases in mast cell markers, *Cd13*, *Cd33* and *Cd38* mRNA were observed only in PND120 testis animals treated gestationally with both compounds. CD13 is also expressed in granulocytes and monocytes and their precursors, as well as non-hematopoietic fibroblasts, whereas CD33 is expressed in myeloid progenitors, monocytes, granulocytes and mast cells [98-100]. CD38 expressing mast cells are also found in close proximity to collagen producing fibroblasts and are involved in the recruitment of inflammatory mediators [101]. Increases in mast cell and macrophage markers are hallmarks of inflammatory events in the testis, often

associated with fibrosis [213, 214]. Indeed, exposure to both genistein and DEHP has been linked to such phenotype. High circulating levels of estrogen are linked to testicular fibrosis and mast cell infiltration, inflammation and Leydig cell atrophy [215, 216]. The association with estrogen is also observable in mice overexpressing aromatase, that have increased testicular fibrosis, TNF-alpha signaling, mast cell and macrophage infiltration and Leydig cell atrophy [217-219]. Neonatal exposure to DEHP also induced eosinophilic inflammation, mast cell degranulation and eotaxin expression in humans, hallmarks of fibrosis in a rat air pouch model [220, 221]. Taken together, these findings indicate that combined *in utero* exposure to genistein and DEHP induce long term alterations in pro-inflammatory testicular cells, creating a condition favorable to the development of fibrosis.

The combined treatment altered the mRNA levels of the Sertoli cell makers *Wt1* and *Amh*, suggesting long term disruption in Sertoli cell function. Besides its role in gonad and seminiferous cord formation, WT1 has been shown to regulate PDGFa expression by Sertoli cells and to promote junctional complexes between Sertoli and germ cell, playing a critical role in spermatogenesis [222]. Although much of the literature has focused on the role of AMH in fetal and prepubertal periods, it is also expressed at significant levels in the adult testis and is dynamically regulated throughout spermatogenesis [223, 224]. Similarly, fetal exposure to the combination of genistein and DEHP led to reduced mRNA expression of several spermatogonial markers in adulthood, including *Thy1*, a glycoprotein highly expressed in SSC and early undifferentiated spermatogonia, the tyrosine kinase receptor *cKit*, marker of differentiating spermatogonia, and *Sox17*, a transcription factor present at maximal levels in spermatogonia, but also found in pachytene spermatocytes [225, 226]. Interestingly, the only significantly up regulated germ cell marker, *Sohlh2*, is reported as being enriched in undifferentiated

spermatogonia [227]. Together with recent studies highlighting the coexistence of SSCs and progenitor spermatogonia and the continuity between the successive steps of spermatogonial differentiation [228], the present results suggest that fetal exposure to the combined EDs leads to the long term disruption of spermatogonial genes and possibly altered kinetics of spermatogenesis.

Gene array analysis of *in utero* exposed adult testes revealed underlying genetic alterations and insight into mechanisms of toxicity. The majority of altered genes were down regulated and specific to a particular treatment group. Whereas some genes were uniquely affected by the ED combination, others showed exacerbated or normalized effects with the combination in comparison to single ED exposure, emphasizing the potential for additive, synergistic or inhibitory effects when exposed to ED mixtures. The alterations in germ cell markers might reflect direct effects on fetal gonocytes or indirect effects via primary targeting of somatic cells, as suggested by the large number of Leydig cell-associated genes altered in the adult testis. Indeed, *Hsd3b, Anxa1, Esr1, Foxa3, Pdgfra and Stc1* mRNAs, as well as HSD3b and PDGRFa protein expression, were all significantly depressed in response to treatment. HSD3b catalyzes the synthesis of progesterone from pregnenolone and decreased expression suggests a deregulation of a key steroidodogenic enzyme in the testis. Despite a significant reduction in HSD3b expression at the mRNA and protein level, global serum testosterone levels were not significantly altered in any treatment group. Further investigation is necessary to elucidate this discrepancy. A possibility is that circulating serum testosterone levels do not correlate with the elevated intratesticular levels observed in humans and rodents [229]. Indeed, rat intratesticular testosterone can be reduced by up to 60%, while sustaining normal spermatogenesis [229]. Interestingly, *Ar* mRNA expression was significantly increased in genistein and combined

treatment groups. Although a limited number of expression and conditional knockout studies have provided evidence for AR function in germ cells [230, 231], in the adult, AR is primarily expressed in Leydig, Sertoli and myoid cells [232], where it plays a key role in androgen response. An increase in expression may therefore be a compensatory response to decrease in intratesticular androgen levels and expression of Leydig cell-related genes.

Stc1, a calcium and phosphate regulating glycoprotein hormone with demonstrated autocrine and paracrine functions in numerous mammalian tissues, was identified as a DEHP specific target following in utero exposure [233]. Interestingly, *Stc1* has been reported as having sexually dimorphic expression during embryonic and early postnatal development, appearing in interstitial mesenchymal cells as of embryonic day 12.5 and later in developing gonocytes in the seminiferous chords of the mouse testis [234]. *Stc1* expression and testosterone production was also altered in isolated rat Leydig cells by exposure to the synthetic hormone, dexamethasone, and in early postnatal testis following gestational exposure to a known reproductive and development toxicant, 2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD), suggesting an important role in both normal testis development and endocrine disruptor mediated alterations [235, 236].

Two novel candidate genes, *Anxa1* and *Foxa3*, were depressed only in animals exposed to the combined treatment. ANXA1 is a Ca^{+2} dependent phospholipid binding protein expressed in Leydig cells [237]. It is suggested to have anti-inflammatory activity via phospholipase A2 inhibition, and therefore its reduction may also contribute to the observed inflammatory changes in adult testis [238]. FOXA3 is a testis specific winged-helix transcription factor expressed in Leydig cells and post-meiotic germ cells [239]. Interestingly, complete knockout and haploinsufficient mice display germ cell loss and Sertoli cell only syndrome. Additionally,

previously published microarray analysis of FOXA3 (-/-) mice identified subsequent gene alterations, including several interesting testis specific Kallikreins implicated in semen liquifaction and male fertility [239].

The effects on adult Leydig cells are particularly interesting when considering their origin; adult Leydig cells are not derived from fetal Leydig cells, but rather from neonatal mesenchymal precursors. [59, 60]. These results therefore suggest that there is a population of fetal cells that gives rise to the mesenchymal precursor of adult Leydig cells, either directly or indirectly disrupted in response to impaired fetal testis function. Indeed, the androgen sensitivity of adult Leydig precursors expressing chicken ovalbumin upstream promoter transcription factor II (Coup-tfII, NR2F2) [94] and AR was recently demonstrated in rodent ablation and regeneration model: fetal androgen deficiency, via AR KO or dibutyl phthalate exposure, reduced adult Leydig stem numbers resulting in compensated adult Leydig cell failure [240]. Interestingly, consistent epigenetic alterations were observed in both adult Leydig cells and fetal adult Leydig "stem" cells, suggesting a possible mechanism for impaired fetal Leydig programming. Studies involving platelet derived growth factor (PDGF) ligand and receptors (a/b) also reinforce this notion. PDGF is secreted by Sertoli cells and plays a key role in gonocyte proliferation and migration, fetal myoid cell migration and proliferation, and Leydig cell differentiation and proliferation [241, 242]. PDGFRa, which we have reported as being significantly depressed at the mRNA and protein levels in testicular interstitium, is expressed in both fetal and adult Leydig cells and their precursors, peritubular myoid cells, blood vessels and gonocytes [38, 174, 241, 243, 244]. Interestingly, deletion of the PDGFa ligand (-/-) in mice results in few adult Leydig cells, reduced testis size and spermatogenesis arrest at PND32, but normal fetal

Leydig cells, again suggesting altered adult Leydig cell development via targeting of a distinct stem-like Leydig population [38, 243, 244].

The present data suggest the ability of a combined fetal exposure of genistein and DEHP to induce long term alterations in important somatic and germ cell markers in adult testis, supporting the notion of chemical susceptibility of this critical prenatal window. The alterations in germ cell markers might reflect direct effects on fetal gonocytes or indirect effects via primary targeting of somatic mesenchymal precursors present during the period of exposure, as suggested by the large number of Leydig associated genes altered in the adult testis. A recent study reported cumulative effects of genistein and DEHP following gestational and lactational exposure, albeit at much higher doses not encountered in the environment and likely acting via different mechanisms of toxicity [245]. The doses used in our study however, were not previously known to induce any conspicuous long term reproductive toxicity in exposed animals. Moreover, these results suggest that in utero exposure to the combination of genistein and DEHP leads to long term alterations in various testicular cell types. These changes could sensitize the testes to subsequent environmental insults by jeopardizing their ability to maintain homeostasis in response to stressors, or by exacerbating inflammatory responses. Interestingly, consequences of EDs may also not be limited to F1 males, as epigenetic changes and subsequent gene alterations following fetal exposure have been demonstrated to persist until F3 via the paternal genome [187, 246, 247]. Thus, assessing reproductive risk based on single chemical effects might not faithfully represent the true risks of exposure to ED mixtures during critical periods of male reproductive development. We have identified several important candidate genes that will be the subject of further mechanistic studies. Future experiments will involve detailed analysis of early fetal and postnatal offspring of control and treated mothers. The aim of these

studies will be to elucidate early cellular and molecular events contributing to long term alterations in testis development and function. Of particular interest is investigating epigenetic aberrations, such as DNA methylation and histone modifications that may be driving long term perturbations in gene expression.

ACKNOWLEDGEMENT

We thank Dr. Jaroslav Novak for assistance with microarray data analysis and biostatistics.

FIGURES AND LEGENDS

Figure 2.1. Effects of in utero exposure to genistein (GEN) and DEHP on general and reproductive health parameters. PND 120 and 180 average body weight (g) (A, C) and testis weights (g) (B, D). Data are represented as mean measurements ± SEM of parameters measured in the offspring from 3 to 7 dams per treatment. Asterisks indicate a significant difference relative to control ($p \leq 0.05$).

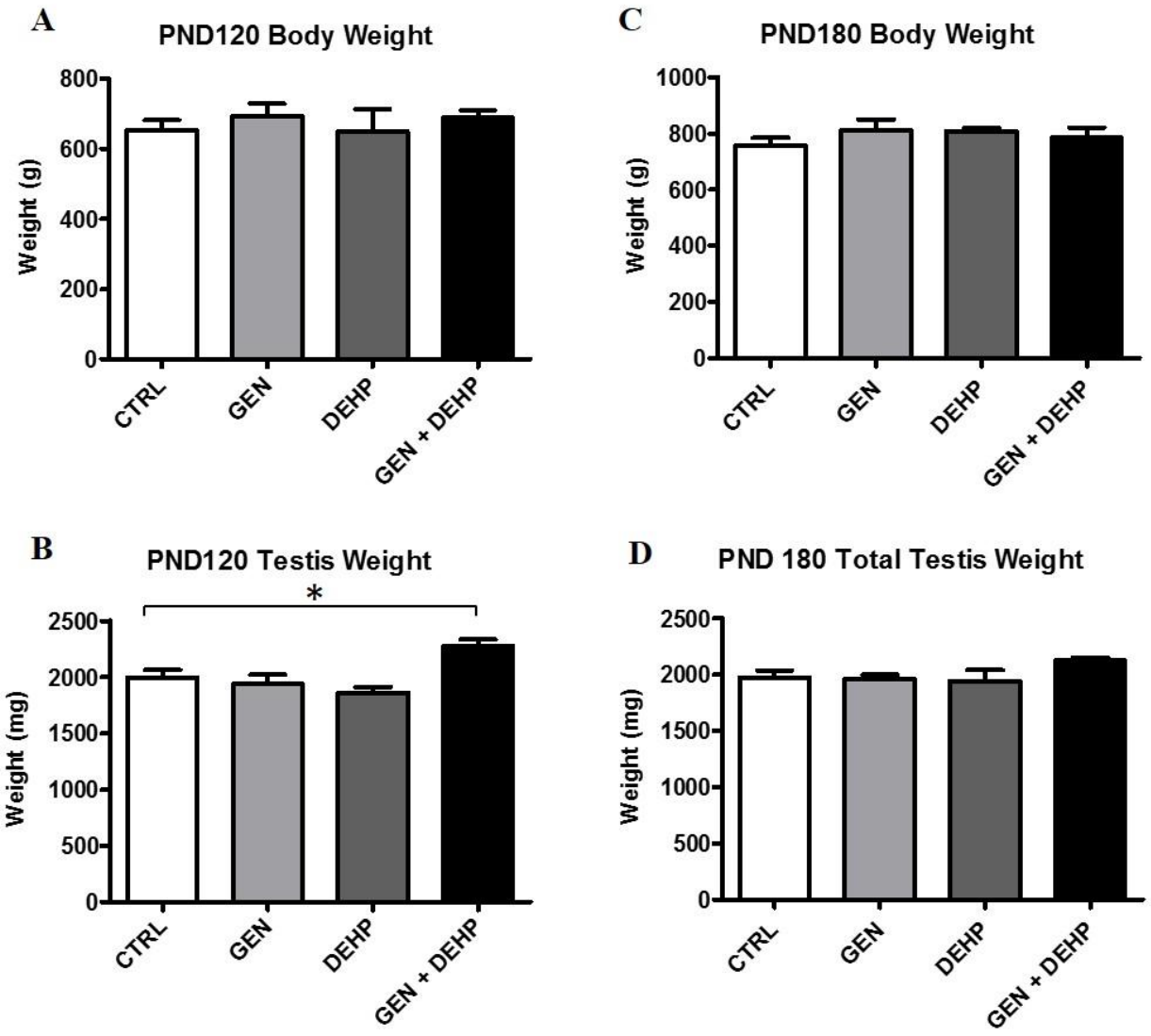

A
PND120 Body Weight
Weight (g)
800
600
400
200
0
CTRL
GEN
DEHP
GEN + DEHP
C
PND180 Body Weight
Weight (g)
1000
800
600
400
200
0
CTRL
GEN
DEHP
GEN + DEHP
B
PND120 Testis Weight
*
Weight (mg)
2500
2000
1500
1000
500
0
CTRL
GEN
DEHP
GEN + DEHP
D
PND 180 Total Testis Weight
Weight (mg)
2500
2000
1500
1000
500
0
CTRL
GEN
DEHP
GEN + DEHP

Figure 2.2. Effects of in utero exposure to genistein and DEHP on testicular mast cells and macrophages. Relative mRNA expression of correlated mast cell markers *Cd13* (A), *Cd33* (B) and *Cd38* (C) and macrophage markers *Cd68* (D) and *Cd163* (E) in PND 120 testis. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin (n = 3 to 6 per treatment). Asterisks indicate a significant difference relative to control ($p \leq 0.05$).

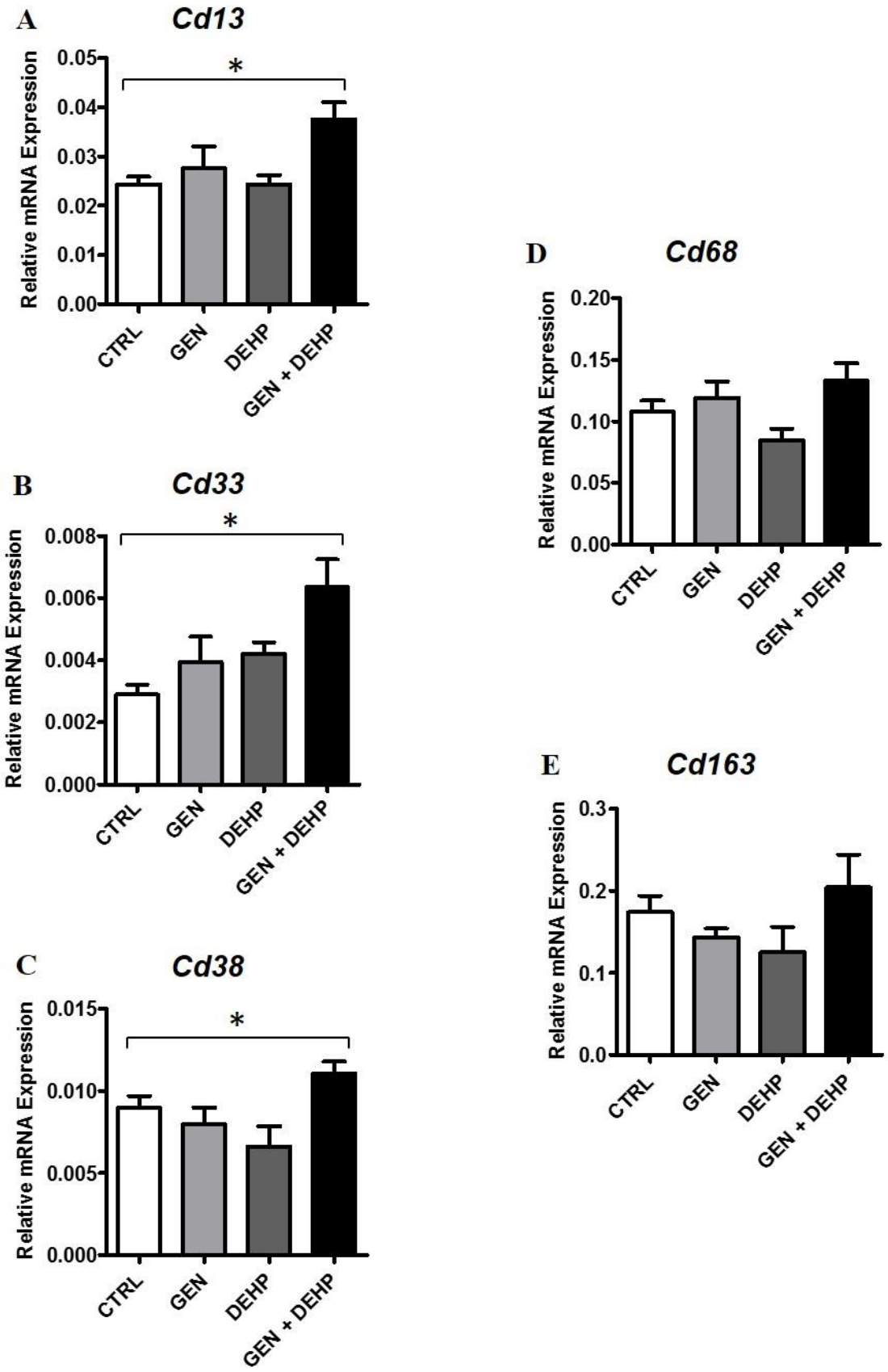

A
Cd13
Relative mRNA Expression
0.05
0.04
0.03
0.02
0.01
0.00
*
CTRL
GEN
DEHP
GEN + DEHP
B
Cd33
Relative mRNA Expression
0.008
0.006
0.004
0.002
0.000
*
CTRL
GEN
DEHP
GEN + DEHP
C
Cd38
Relative mRNA Expression
0.015
0.010
0.005
0.000
*
CTRL
GEN
DEHP
GEN + DEHP
D
Cd68
Relative mRNA Expression
0.20
0.15
0.10
0.05
0.00
CTRL
GEN
DEHP
GEN + DEHP
E
Cd163
Relative mRNA Expression
0.3
0.2
0.1
0.0
CTRL
GEN
DEHP
GEN + DEHP

Figure 2.3. Effects of in utero exposure to genistein and DEHP on Leydig, Sertoli and Myoid cells. Relative mRNA expression of Leydig cell markers, *Cyp11a1* (A) and *Tspo* (B), Sertoli cell markers, *Amh* (C) and *Wt1* (D) and Leydig/Sertoli/Myoid expressed *Ar* (E) and *Esr1* (primarily Leydig) (F) in PND 120 testis. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin (n = 3 to 6 per treatment). Asterisks indicate a significant difference relative to control ($p \leq 0.05$).

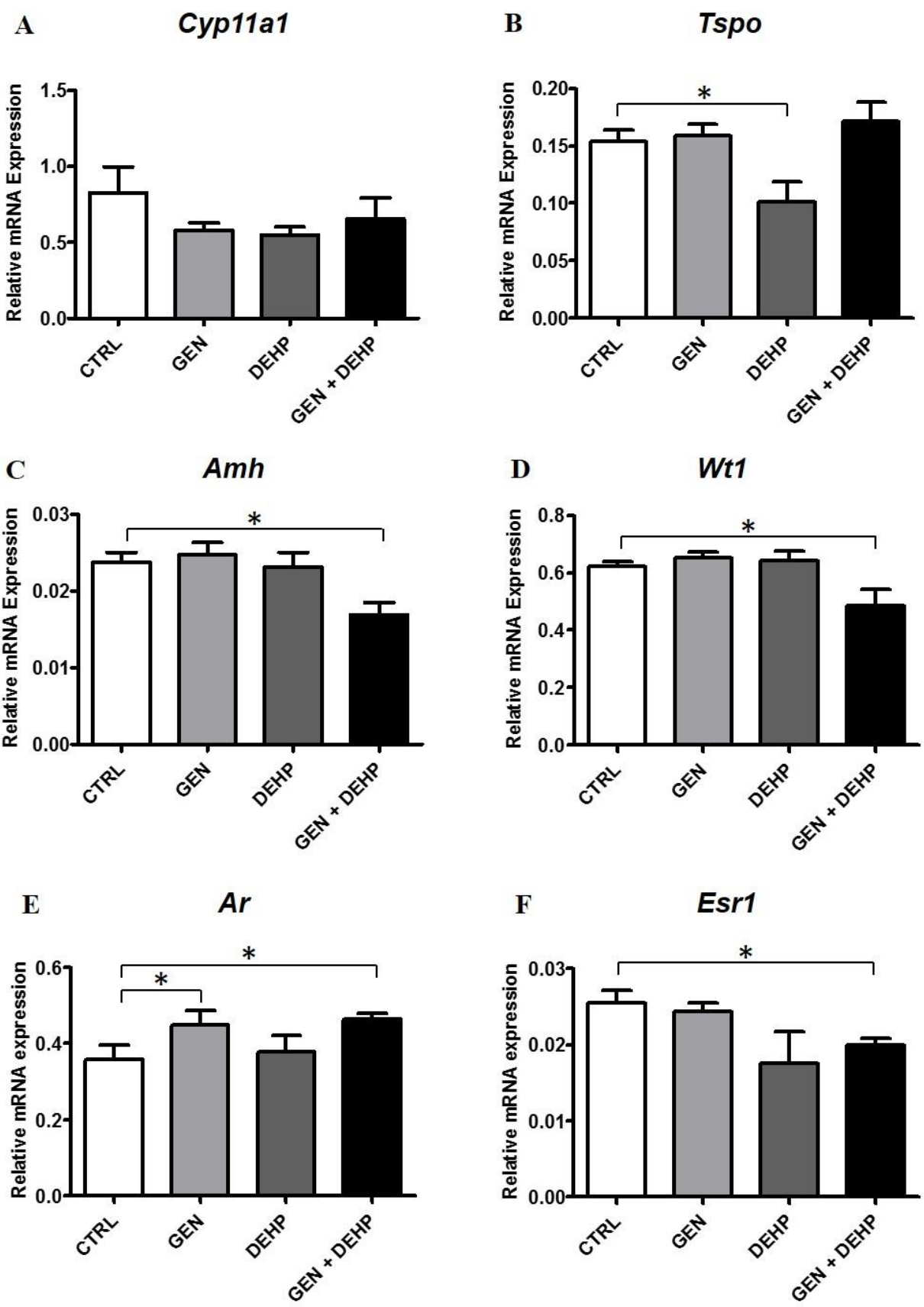

A
Cyp11a1
Relative mRNA Expression
1.5
1.0
0.5
0.0
CTRL
GEN
DEHP
GEN + DEHP
B
Tspo
Relative mRNA Expression
0.20
0.15
0.10
0.05
0.00
*
CTRL
GEN
DEHP
GEN + DEHP
C
Amh
Relative mRNA Expression
0.03
0.02
0.01
0.00
*
CTRL
GEN
DEHP
GEN + DEHP
D
Wt1
Relative mRNA Expression
0.8
0.6
0.4
0.2
0.0
*
CTRL
GEN
DEHP
GEN + DEHP
E
Ar
Relative mRNA expression
0.6
0.4
0.2
0.0
*
*
CTRL
GEN
DEHP
GEN + DEHP
F
Esr1
Relative mRNA expression
0.03
0.02
0.01
0.00
*
CTRL
GEN
DEHP
GEN + DEHP

Figure 2.4. Effects of in utero exposure to genistein and DEHP on adult germ cells. Relative mRNA expression of SSC/undifferentiated spermatogonia marker, *Thy1* (A), differentiating spermatogonial markers *cKit* (B) and *Sohlh2* (C), spermatogonia/pachytene marker, *Sox17* (D), zygotene marker *Scp1* (E) and round spermatid marker *Sp10* (F) in PND 120 testis. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin (n = 3 to 6 per treatment). Asterisks indicate a significant difference relative to control ($p \leq 0.05$).

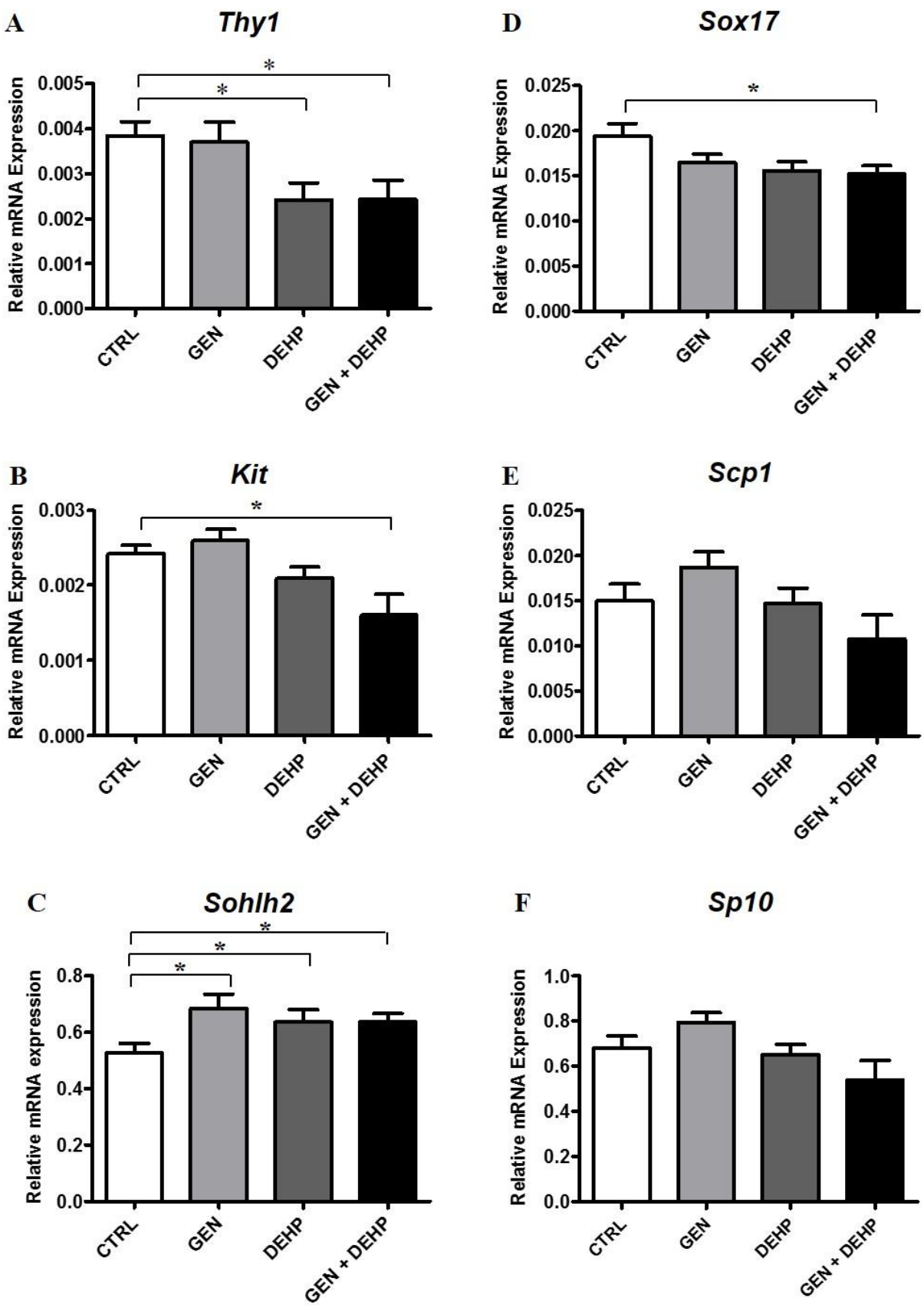
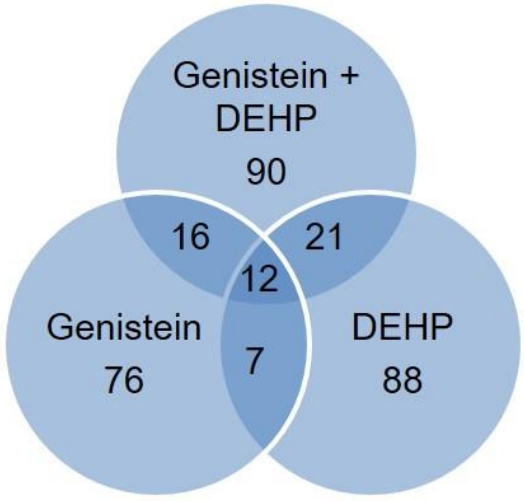
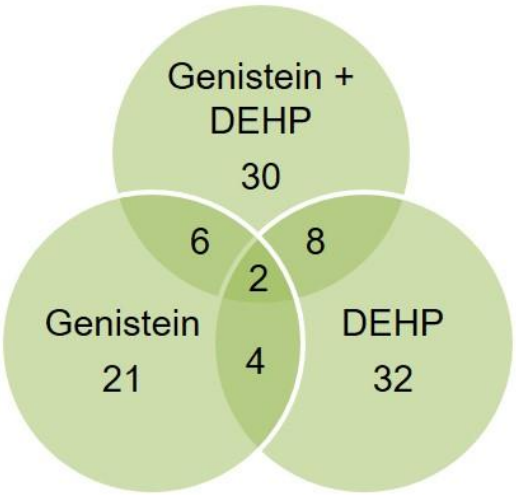

A
Thy1
Relative mRNA Expression
0.005
0.004
0.003
0.002
0.001
0.000
CTRL
GEN
DEHP
GEN + DEHP
D
Sox17
Relative mRNA Expression
0.025
0.020
0.015
0.010
0.005
0.000
CTRL
GEN
DEHP
GEN + DEHP
B
Kit
Relative mRNA Expression
0.003
0.002
0.001
0.000
CTRL
GEN
DEHP
GEN + DEHP
E
Scp1
Relative mRNA Expression
0.025
0.020
0.015
0.010
0.005
0.000
CTRL
GEN
DEHP
GEN + DEHP
C
Sohlh2
Relative mRNA expression
0.8
0.6
0.4
0.2
0.0
CTRL
GEN
DEHP
GEN + DEHP
F
Sp10
Relative mRNA Expression
1.0
0.8
0.6
0.4
0.2
0.0
CTRL
GEN
DEHP
GEN + DEHP

Figure 2.5. Effects of in utero exposure to genistein and DEHP on gene expression in adult testes. Affymetrix Rat Gene 2.0 ST MicroArray was performed on RNA extracts from the offspring of 3 dams per treatment, and further used to mine significantly altered genes in PND 120 testis. Data was normalized in Affymetrix Expression Console using PLIER and further sorted using FlexArray. Venn diagrams represent the total number of significantly (≥ 40% fold change; $p \leq 0.05$) altered genes, up regulated genes and down regulated genes relative to control. Values within individual circles represent the total number of gene changes within a particular treatment group relative to control. Numbers within shaded regions indicate the common gene changes between two specific treatment groups or all three (center).

Total Gene Changes



Up Regulated Genes



Down Regulated Genes

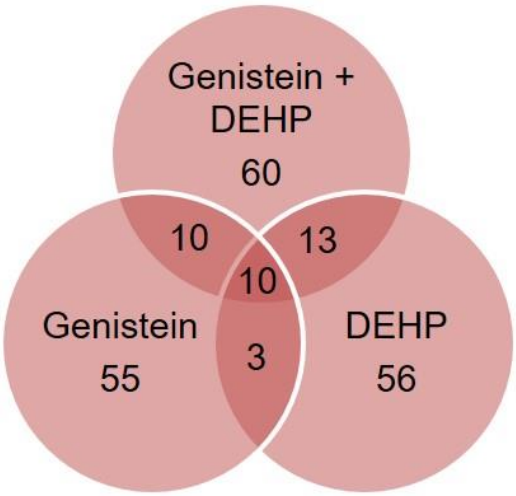


Figure 2.6. qPCR validation of gene expression changes identified in microarrays. The relative mRNA expressions of the candidate genes, *Pdgfra* (A), *Hsd3b* (B), *Foxa3* (C) and *Stc1* (D) were determined by qPCR analysis in RNA extracts from PND 120 testes. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin (n = 3 to 5 per treatment). Asterisks indicate a significant difference relative to control ($p \leq 0.05$).

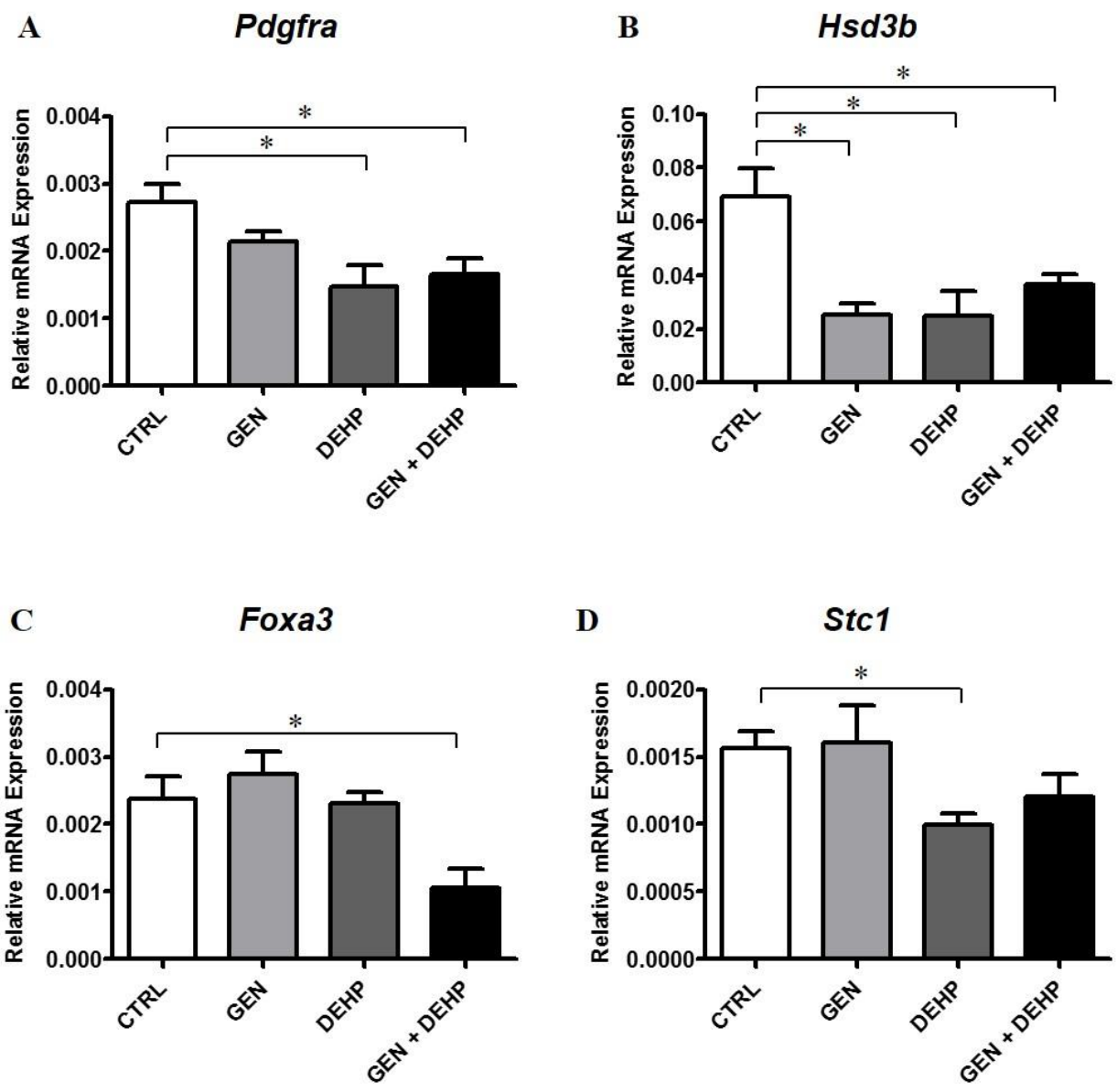

A
Pdgfra
Relative mRNA Expression
0.004
0.003
0.002
0.001
0.000
*
*
CTRL
GEN
DEHP
GEN + DEHP
B
Hsd3b
Relative mRNA Expression
0.10
0.08
0.06
0.04
0.02
0.00
*
*
*
CTRL
GEN
DEHP
GEN + DEHP
C
Foxa3
Relative mRNA Expression
0.004
0.003
0.002
0.001
0.000
*
CTRL
GEN
DEHP
GEN + DEHP
D
Stc1
Relative mRNA Expression
0.0020
0.0015
0.0010
0.0005
0.0000
*
CTRL
GEN
DEHP
GEN + DEHP

Figure 2.7. Effects of in utero exposure to genistein and DEHP on the expression levels of Leydig cell proteins. Immunohistochemical analysis of PDGFRa (A-D) and HSD3b (E-H) was performed on paraffin sections of PND 120 testes (photos taken at 20 and 40X magnification). Omission of the primary antibody was used as a negative control (data not shown). Representative pictures are presented.

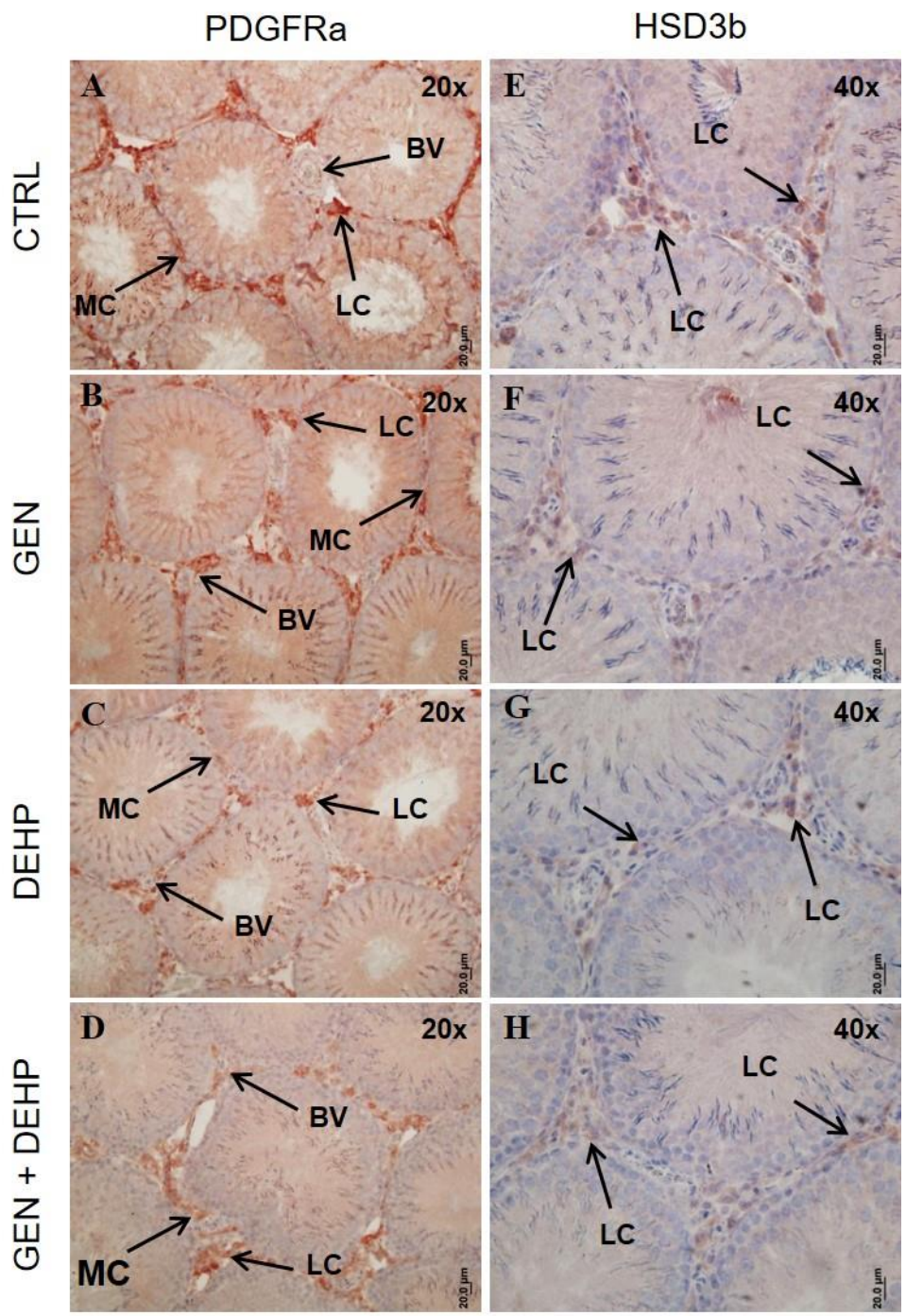

PDGFRa
HSD3b
CTRL
GEN
DEHP
GEN + DEHP
A
20x
BV
MC
LC
E
40x
LC
LC
B
20x
LC
MC
BV
F
40x
LC
LC
C
20x
MC
LC
BV
G
40x
LC
LC
D
20x
BV
MC
LC
H
40x
LC
LC
20.0 µm

Table 2.1. Primer sets used for quantitative real-time PCR

Gene symbol	Accession no.	Forward primer (5′–3′)	Reverse primer (3′–5′)
Amh	NM_012902.1	CGGGCTGTTTGGCTCTGATTCCCG	GTGGGTGGCAGCAGCACTAGG
Anxa1	NM_012904.2	TCTAACCAGCAAATCAGAGAGATTAC	CGAGAGCAAGCAAGGCATTA
Ar	NM_012502.1	CGGTCGAGTTGACATTAGTGAAGGACCG	ATTCCTGGATGGGACTGATGGT
Cd13	NM_013127	CACTCCGGCACCTAACATCG	CGCAACCACCAGGTACTCCGTGCG
Cd33	XM_002728749	CGGATACTGTGGAAAGAACCATCCG	GCCCAAGAATCAGGAGCTTGAC
Cd38	NM_031012	CACTCCGGCACCTAACATCG	CGCAACCACCAGGTACTCCGTGCG
Cd68	NM_001031638.1	CTCCTCACCCTGGTGCTCATT	CGACAGGCTGGTAGGTTGATTGTCG
Cd163	NM_001107887.1	CGGACCAATTTGGCTTGACAGT	CGGCCTTACACTCCCAAAGAGCCG
Ckit	NM_022264.1	ACGTGGTAAAAGGAAATGCACG	AAAATGCTCTCCGGTGCCA
Cyp11a1	NM_017286.2	CACGCACTTCCGGTACTTGG	CGGATATTTCCAGCTCTGCAATCCG
Esr1	NM_012689.1	TGCTGAACCACCTTTGATCTATT	TTCAAGGTGCTGGATAGAAATGT
Foxa3	NM_017077.2	ACTACCGGGAGAACCAGCA	TCATTGAAGGACAGCGAGTG
Hsd3b	NM_001007719.3	GACCAGAAACCAAGGAGGAA	CTGGCACGCTCTCCTCAG
Pdgfra	NM_012802.1	GCTACACGTTTGAGCTGTCAAC	ATGGTGGTCATCCACAAGC
Scp1	NM_012810	CGAAAGAAGTTCCTGAATGGCTTTTCG	GCATAGAGTCTGAACTGAAGCAGA
Sohlh2	NM_001034961.1	AGCCAGCTCCAGTTGTCTGT	GATGCTGGATGAGGCAGT
Sox17	NM_001107902	CGGTGCTGTCCGAGAGGTTCACACCG	TAAGCCGGAGATGGGTCTTCC
Sp10	NM_021747	GAAGGAATATGCACCACTCAAA	TGAACTGGAGCTTTCCACCT
Stc1	NM_031123.2	AACCCGGAAGCCATCACT	GGCTTCGGACAAGTCTGTTG
Thy1	NM_012673.2	CGGTCGTCAACCTTTTCAGTGACCG	CATGTAGTCGCCCTCATCCTTG
Tspo	NM_012515.1	CGCAATGGGAGCCTACTTTGTGCG	GCCAGGAGGGTTTCTGCAAG
Tuba1a	BC062238	CGGGGGAGAGTTCTCTGAGGCCCG	CAGAATCCACACCAACCTCCTC
Wt1	NM_031534	CGGTCGTCTTCAGGTGGTCGGACCG	GCACCAAAGGAGACACACAGGT

Table 2.2. Gene expression array analysis. Fold changes in significantly altered genes (≥ 40% fold change; $p \leq 0.05$) relevant to male reproductive function and development. Dark and light grey shades represent significant up or down regulated genes relative to control respectively.

Gene Symbol	Gene Accession Number	Gene Description	Treatments (fold change)		
			GEN	DEHP	GEN + DEHP
Adamts2	NM_001137622	ADAM metallopeptidase with thrombospondin type 1 motif, 2	-2.15	-1.79	-1.84
Anxa1	NM_012904	Annexin A1	-1.38	-1.64	-1.41
Cd96	NM_001025032	CD96 molecule	-	-	1.48
Cyp2a1	NM_012692	Cytochrome P450, family 2, subfamily a, polypeptide 1	-1.63	-1.44	-1.76
Dcn	NM_024129	decorin	-1.68	-1.96	-2.16
Epha3	ENSRNOT00000040065	Eph receptor A3	-	-	-1.44
Fn1	NM_019143	Fibronectin 1	-1.41	-1.17	-1.11
Foxa3	ENSRNOT00000019219	Forkhead box A3	1.217	-	-1.75
Gjb2	NM_001004099	Gap junction protein, beta 2	1.95	-	1.45
Gjb6	NM_053388	Gap junction protein, beta 6	1.35	1.41	1.414
Gsn	NM_001004080	Gelsolin	-1.33	-1.29	-1.51
Hsd3b	NM_001007719	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	-1.64	-1.74	-1.36
Lhcgr	NM_012978	Luteinizing hormone / choriogonadotropin receptor	-	-1.5	-1.29
Pdgfra	NM_012802	Platelet derived growth factor receptor, alpha polypeptide	-1.47	-1.4	-1.4
Prps1	NM_017243	Phosphoribosyl pyrophosphate synthetase 1	1.57	-	1.7
Rasgef1c	NM_001108273	RasGEF domain family, member 1C	-1.32	1.42	1.51
Scgb3a1	ENSRNOT00000051916	Secretoglobin, family 3A, member 1	-	2.44	2.134
Stc1	NM_031123	Stanniocalcin 1	-1.92	-1.55	-1.19

Preface to Chapter III

Results from chapter two demonstrated the ability of in-utero (GD14 to parturition) low dose (10mg/kg/day) mixture of Genistein and DEHP to induce long term alterations in testis function, gene and protein expression of critical germ and somatic cell markers, in a manner that is different from individual exposures. A microarray approach and subsequent qPCR and IHC validation, identified several novel candidate genes, in PND120 testis. Alterations in germ cell markers may reflect direct targeting of these cell types or indirect via targeting of somatic cells, as suggested by the significant number of altered Leydig cell - related genes (*Hsd3b*, *Anxa1*, *Foxa3* and *Pdgra*).

A follow up study was conducted to evaluate acute toxicity in early postnatal animals and gain insight into the early cellular and molecular events driving long term changes. Male offspring were euthanized at specific postnatal ages to evaluate testis function and development, including fetal Leydig cell activity and the initiation of gonocyte proliferation/migration (PND3) and differentiation to form a critical pool of SSCs (PND6) following in-utero exposure.

Chapter III

“Manuscript II”

IN UTERO EXPOSURE TO DI-(2-ETHYLHEXYL) PHTHALATE INDUCES TESTICULAR EFFECTS IN NEONATAL RATS THAT ARE ANTAGONIZED BY GENISTEIN CO-TREATMENT

Running Title: Neonatal effects of genistein and DEHP on testis

Summary Sentence: Fetal exposure to DEHP induces neonatal increases in Leydig, Sertoli and germ cell markers, as well as increased oxidative stress response genes that are normalized by co-treatment with Genistein, and distinct from their long-term effects.

Key Words: Endocrine Disruptor, genistein, Phthalate, mixture, Leydig, Sertoli, germ cells, Testis, ROS, Toxicology

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Affiliation: [1]*The Research Institute of the McGill University Health Centre,* [2]*division of Experimental Medicine, Departments of Medicine*[3] *and Pharmacology & Therapeutics*[4]*, McGill University, Montreal, Quebec, Canada,* [5]*Department of Urology, The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University*

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Tel: (514) 934-1934 x43752; FAX: (514) 937-6961; e-mail: martine.culty@mcgill.ca

ABSTRACT

Fetal exposure to endocrine disruptors (EDs) is believed to predispose males to reproductive abnormalities. Although males are exposed to combinations of chemicals, few studies have evaluated the effects of ED mixtures at environmentally relevant doses. Our previous work showed that fetal exposure to a mixture of the phytoestrogen genistein (GEN) and the plasticizer DEHP induced unique alterations in adult testis. In this follow−up study, we examined postnatal−day (PND) 3 and 6 male offspring exposed from gestational day 14 to parturition to corn oil, 10mg/kg GEN, DEHP or their combination, to gain insight into the early molecular events driving long term alterations. DEHP stimulated the mRNA and protein expression of the steroidogenic enzyme HSD3B, uniquely at PND 3. DEHP also increased the mRNA expression of Nestin, a Leydig progenitor/Sertoli cell marker, and markers of Sertoli cell (*Wt1)*, gonocyte (*Plzf, Foxo1*), and proliferation (*Pcna)* at PND 3, while these genes were unchanged by the mixture. Redox (*Nqo1, Sod2, Sod3, Trx, Gst, Cat*) and xenobiotic transporter (*Abcb1b, Abcg2*) gene expression was also increased by DEHP at PND 3, while attenuated when combined with GEN, suggesting the involvement of cellular stress in short-term DEHP effects and a protective effect of GEN. The direct effects of GEN and MEHP, principal bioactive metabolite of DEHP, on testis were investigated in PND 3 organ cultures, showing a stimulatory effect of 10 μM MEHP on basal testosterone production that was normalized by GEN. These effects contrasted with previous reports of androgen suppression and decreased gene expression in perinatal rat testis by high DEHP doses, implying that neonatal effects are not predictive of adult effects. We propose that GEN, through an antioxidant action, normalizes ROS-induced neonatal effects of DEHP. The notion that these EDs do not follow classical dose-response effects and involve different mechanisms of toxicity from perinatal ages to adulthood highlights the importance of assessing impacts across a range of doses and ages.

INTRODUCTION

The "developmental origins of disease hypothesis" states that adverse events or influences early in life can lead to permanent changes in physiology and predispose adults to disease [5, 6]. In that regard, fetal and neonatal exposure to endocrine disruptors (EDs) is thought to contribute to reported declines in male reproductive potential and increased incidence of developmental abnormalities such as testicular cancer and genital birth defects. A concerning 15% of couples have primary infertility, up to half of which can be attributed solely to male cause, and an estimated 40% of men are currently presenting sperm counts in the subfertile range [1, 248]. Evidence suggests semen quantity and quality have been decreasing steadily since early 19th century, alongside increasing incidence of cryptorchidism and hypospadias, now affecting 2-9% and 0.2-1% of male newborns, respectively [249]. These aberrations, collectively termed testicular dysgenesis syndrome (TDS), have shared risk factors and are thought to arise from alterations in fetal androgen levels, androgen/estrogen balance or hormone action and impaired development and reprogramming of progenitors during a developmentally sensitive fetal and neonatal window [1, 196-200, 249-251].

Following SRY induced sex determination of the fetus, somatic cells proliferate and differentiate to form supporting Sertoli and steroidogenic Leydig cells and an environment suitable for fetal primordial germ cells (PGCs). Fetal Sertoli and Leydig cells play a fundamental role in generating hormones required for differentiation of the urogenital tract and testis decent, including androgens, anti-mullerian hormone (AMH) and insulin-like growth factor 3 (INSL3) [17]. Once in the interstitium of the gonad primordia, these cells, now termed gonocytes, undergo active DNA remethylation and establishment of new paternal imprints, prior to forming a critical pool of renewable spermatogonial stem cells (SSCs) that will support

spermatogenesis throughout adulthood [27, 41, 202, 204]. Spermatogenesis is regulated by factors produced by Sertoli cells, themselves dependent on the gonadotropin FSH and on Leydig cell-produced testosterone (T). Interestingly, adult Leydig cells are not derived from fetal Leydig cells, but rather from neonatal mesenchymal precursors [59, 60]. It is therefore thought that affecting the pool of "stem" Leydig precursors, either directly or indirectly by altering the fetal testis environment, can ultimately impair adult Leydig development and function.

The default developmental program of the fetus is female and largely hormone independent, whereas hormone production is absolutely necessary for masculinization [7]. The "masculinization programming window" is driven principally by fetal Leydig T production, and in humans begins at 8 weeks gestation, peaks around 12-14 and steadily declines after week 20 [192]. Correspondingly, well established rat developmental models have an equivalent window with T production starting at gestational day (GD) 14.5-15-5, peaking around GD18-5-19.5 and declining shortly after parturition [192]. Thus, the hormonal dependence and developmental intricacy of the fetal period, in combination with a comparatively permeable fetal skin surface, minimal detoxifying capacity and lack of blood-testis barrier, make the developing male gonads particularly sensitive to EDs capable of crossing the placenta [7, 197, 198, 249]. Interestingly, several common environmental pollutants, including derivatives of paints, plastics and resins as well as natural soy derived phytoestrogens, have been detected in amniotic fluid, cord blood, breast milk and semen, and have also been shown to possess antiandrogenic or estrogenic properties [8-12].

Although EDs have been extensively studied in the context of single high dose chemical exposures, few studies have examined the additive or synergistic effects of ED co-exposure during critical periods of development. In reality, humans and animals are exposed not to one,

but a myriad of potentially harmful substances throughout their lifetimes. Previous work from our laboratory demonstrated that environmentally relevant in utero co-exposure to two common EDs, the plant derived phytoestrogen, Genistein (GEN), found in soy based infant formula and antiandrogenic plasticizer, Di-(2-ethylhexyl) phthalate (DEHP), primarily used in the production of polyvinyl chloride plastics (PVC), can induce alterations in testis development different or potentially more harmful than exposure to a single chemical at the same dose [252]. GEN is reported to have broad biochemical interactions, including estrogenicity, peroxisome proliferator-activated receptor (PPAR) activation, direct or indirect antioxidant action, and modulation of important signaling molecules and DNA methylation [175-177]. DEHP or its principal bioactive metabolite, MEHP, has been demonstrated to act as an anti-androgen via PPARs and alter DNA methylation status and estradiol-regulated proteins [145, 146, 205, 206].

The current follow up study was conducted to evaluate GEN and DEHP acute toxicity in early postnatal animals and gain insight into the early cellular and molecular events driving long term changes. We hypothesized that early postnatal hormonal alterations and reproductive toxicity of this ED mixture would correlate with or at least clarify the roots of the long term effects, and that the mixture would pose a greater risk than individual compounds. A combination of in-vivo, ex-vivo and in-vitro approaches in rat however, demonstrated that these EDs do not follow classical dose-response effects and involve different mechanisms of toxicity from perinatal ages to adulthood.

MATERIAL AND METHODS

Treatments and Tissue Collection

Pregnant Sprague Dawley rats were purchased from Charles River Laboratories, and kept on a 12L:12D photoperiod with ad libitum access to food and water. To address early life alterations in testis development and function, pregnant Sprague Dawley rats were treated from GD14 to parturition by gavage with either control corn oil or corn oil + GEN, DEHP or GEN + DEHP, both used at a dose of 10 mg/kg, a dose within the range of human exposure. This dose was selected from previous dose-response studies in which we had examined the short and long term effects of in utero exposure to GEN or DEHP used separately [32, 132, 133, 174, 195]. These studies had shown that a fetal exposure to 10 mg/kg/day GEN did not induce changes in circulating T nor germ cell numbers in adult rats [195], while long term effects of

DEHP were seen for much higher doses [132, 133].

Gestationally treated male offspring were raised on a normal diet to defined ages, sacrificed and analyzed. Treatment doses were adjusted according to changes in dam weights. Male offspring were euthanized at specific postnatal ages to evaluate testis function and development, including fetal Leydig activity and the initiation of gonocyte proliferation/migration (PND3) and differentiation to form a critical pool of SSCs (PND6) following in-utero exposure. Testes were collected, weighed and either snap frozen, fixed in paraformaldehyde or dissected for organotypic cultures (each end-point was determined using randomly selected offspring of 4 dams per treatment, where N is defined as the number of offspring from independent litters). Animals were handled according to protocols approved by the McGill University Animal Care and Use Committee.

RNA Extraction and Quantitative Real-Time PCR

Testis RNA was extracted using QIAGEN RNeasy Plus Mini kit (Qiagen, Santa Clarita, CA). cDNA was synthesized from isolated RNA using the single-strand cDNA transcriptor synthesis kit (Roche Diagnostics, Laval, QC). Quantitative real-time PCR (qPCR) was performed as previously described using the LightCycler 480 Real-Time PCR System (LC480, Roche Diagnostics) [209]. Alpha tubulin was used as an endogenous control and for normalization of gene targets. A minimum of three male offspring from different litters were evaluated in triplicate. The comparative Ct method was used to calculate relative expression. Data are represented as mean relative mRNA expression in arbitrary units. Complete primer sequences for all gene targets can be found in Table 3.1.

Histology, Hematoxylin and Eosin Staining and Immunohistochemistry

Testis from male offspring were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin or used for immunohistochemical analysis as previously described [195]. Briefly, tissue sections were dewaxed and rehydrated, followed by antigen retrieval using a DAKO solution. Primary antibodies, HSD3B (Abcam, ab65156) and NQO1 (Abcam, ab28947) were incubated at 1/300 and 1/100 dilutions respectively overnight at 4^{o}C and revealed using species appropriate secondary antibodies (mouse or rabbit), HRP/colorimetric HRP chromogen (AEC) and hematoxylin counterstaining. Representative images are shown. For quantification, digital images of stained sections were loaded into ImageJ analysis software and RGB stacks/montages were generated ($n \geq 3$). Uniquely for HSD3B quantification, seminiferous tubules were manually removed using the "cut" feature, leaving on the interstitium. The threshold feature was adjusted to specifically identify positive red/brown immunostaining. Once set, identical settings were used for all images quantified. Percent of

positive staining relative to total area was calculated using the measure feature and subsequently expressed in term of fold change over control.

Ex-Vivo and In-Vitro Testis Organ Culture

Ex-vivo organ culture of PND 3 testis was performed to evaluate basal and hormone stimulated androgen production following in-utero exposure, as reported previously [132]. Briefly, PND 3 testes were excised and cut into 8-10 small fragments, placed on filter papers and cultured on trans-well inserts in normal DMEM or DMEM + hCG for three days. Supernatant containing steroids was collected daily and replenished with new medium. T levels in supernatant were measured by radioimmunoassay (RIA), expressed in ng/testis/3 days or fold change relative to control. In-vitro organ culture of testes from PND 3 untreated rats was performed in the presence of either control DMSO, GEN, MEHP or GEN + MEHP at 10 μM. A paradigm similar to that described above was followed, with T measured by RIA. After organ cultures, testis fragments were collected, fixed and sent for embedding and cutting. Histological and morphological alterations were evaluated following hematoxylin and eosin counter staining.

Statistical Analysis

All statistical analysis was performed using unpaired two-tailed t-test with statistical analysis functions in GraphPad Prism version 5.0 software (GraphPad Inc., San Diego, CA). Asterisks indicate a significant change relative to control ($P \leq 0.05$).

RESULTS

In Utero Genistein Exposure Antagonizes Pro Androgenic Effects of DEHP In Young Male Offspring

Litters of treated dams did not display any abnormalities in pup number or sex ratio (data not shown). Examination of PND 3 and PND 6 body weights (FIG. 3.1A) and Testis Weights (data not shown) revealed no significant differences between control and treated animals. Anogenital distances (AGD normalized to body weight) (FIG. 3.1B) were significantly reduced uniquely in PND 3 GEN, but not DEHP or combined GEN and DEHP offspring, suggesting early feminizing effects. Initial alterations in AGD were resolved with age however, as no significant alterations were observed at PND 6 in any treatment group. The overall tissue morphology appeared normal (data not shown).

The qPCR analysis of PND 3 and PND 6 testes revealed a significant decrease in *Cyp11a1* (FIG. 3.2A) only in combined GEN and DEHP-treated PND 3 testes. Two other steroigenenic mediators, *Hsd3b* (FIG. 3.2B) and *Ar* (FIG. 3.2C), were increased uniquely in PND 3 DEHP treated animals, suggesting an early pro androgenic effect of DEHP that is resolved by co-treatment with GEN. Similar trends were not observed in PND 6 testes, with *Ar* (FIG. 3.2C) being the only significantly increased gene, unique to GEN treated offspring. Alterations in PND 3 *Hsd3b* mRNA were further validated at the protein level by immunohistochemistry and subsequent quantitative image analysis (FIG. 3.2D-E). Consistent with gene expression data, HSD3B stained strongest in the interstitial Leydig cells of DEHP-treated PND 3 testis (FIG. 3.2D). Quantitative image analysis of interstitial HSD3B also revealed a significant increase only in PND3 DEHP treated offspring relative to control (FIG. 3.2E). .

PND 3 testes basal and hCG stimulated androgen production were evaluated using ex-vivo organ culture (FIG.3.3). T measurement demonstrated no significant alterations in any treatment group compared to control (FIG. 3.3A). Testes from all treatment groups responded well to hCG stimulation, with no significant alterations or obvious trends (FIG 3.3B).

To decipher between direct effects on testis, and effects involving the hypothalamus-pituitary-testis axis, untreated PND 3 testes were exposed in vitro over three days to medium, DMSO (0.2%), GEN, MEHP (the principal bioactive metabolite of DEHP) or combined GEN and MEHP, both at 10 μM, a concentration within the range of reported human blood levels [42,44,45] (FIG. 3.3C) under basal conditions. Consistent with the observed in vivo pro-androgenic gene expression data, MEHP significantly stimulated PND 3 testes T production in vitro, an effect that was attenuated by combination treatment with GEN. Interestingly, histological analysis of paraffin sections from the cultured testes fragments also revealed the presence of large multinucleated germ cells (MGC) uniquely in the seminiferous tubules treated with 10 μM MEHP (FIG. 3.3D).

Further qPCR analysis of PND 3 and PND 6 testes was conducted to evaluate impacts on Sertoli cell, proliferation and early germ cell markers (FIG. 3.4). The Sertoli cell marker *Wt1* and the Sertoli/Leydig progenitor marker *Nestin* (FIG. 3.4A-C) were significantly increased uniquely in PND 3 DEHP treated offspring. By contrast, the marker of Sertoli cell differentiation *Abp* was reduced in DEHP-exposed PND 6 pups, an effect partially corrected in pups exposed to GEN + DEHP mixture. Early germ cell markers *Plzf* and *Foxo1* (FIG. 3.4D-E) displayed a similar trend, being significantly increased in PND 3 DEHP, but not GEN or combined GEN and DEHP. Interestingly, the marker of differentiating spermatogonia Sohlh2 was significantly decreased in DEHP-exposed pups at PND 6, an effect alleviated by combination with GEN (FIG 3.4F). The cell proliferation marker *Pcna*, was significantly increased uniquely in PND 3 DEHP treated offspring (FIG. 3.4G).

In Utero Exposure to Genistein and DEHP Up Regulates Cellular Junction Markers in Early Postnatal Testes

The qPCR analysis of PND 3 and PND 6 testes revealed a stimulatory effect of the ED mixture on several gap, tight and adherens junction markers (FIG. 3.5). Gap junction marker *Gja1* (Connexin 43) was significantly upregulated in GEN and combined GEN + DEHP treated offspring at PND 6, but not in animals treated with DEHP alone (FIG. 3.5A). Similarly, tight junction maker *Tjp1* (FIG. 3.5B) and several adherens junctions *Cdh1*, *Cdh2* and *Cdh3* (FIG. 3.5C-E) were increased uniquely by combined GEN and DEHP at PND 6. Excluding a consistent upregulation of *Cdh3* in PND 3 animals exposed to combined GEN and DEHP, no significant alterations or trends were observed for cellular junction markers immediately following exposure at PND 3.

In Utero Genistein Co Exposure Antagonizes Pro Oxidant Cellular Stress of DEHP in Early Postnatal Testes

The qPCR analysis of PND 3 and PND 6 testes revealed a significant (2-3 fold) up-regulation of numerous downstream NRF2 antioxidant gene targets, including *Nqo1*, *Sod2*, *Sod3*, *Trx1*, *Gsta1* and *Cat* (FIG. 3.6A-F) specifically in PND 3 testes of DEHP treated animals. This up-regulation was attenuated in PND 3 testes of animals treated with combined GEN and DEHP and was not observed in GEN treated animals. No obvious trends for any anti-oxidant makers evaluated were observed in PND 6 testes. *Nqo1* gene alterations were further validated at the protein level by immunohistochemistry and subsequent quantitative image analysis (FIG. 3.7A-B): NQO1 protein stained strongest in the PND 3 interstitial Leydig cells of DEHP treated animals, an effect that was not present in animals receiving combined GEN and DEHP or GEN alone (FIG 3.7A). Quantitative image analysis of NQO1 also revealed a consistent, significant increase only in PND3 DEHP treated offspring relative to control (FIG. 3.7B). Lastly, consistent with anti-oxidant expression data, qPCR analysis of PND 3 and PND 6 xenobiotic transporters *Abcb1b* and *Abcg2* (FIG. 3.7C-D) revealed a significant upregulation uniquely in PND 3 DEHP treated testes, suggesting a

concerted effort to detoxify cells by physically eliminating chemical stressors. The effects of DEHP on the transporters were normalized in pups exposed to the GEN and DEHP combination.

DISCUSSION

Previous work from our laboratory demonstrated that gestational exposure (GD14-parturition) to combined 10mg/kg/day GEN and DEHP induces long term alterations in testis function, gene and protein expression of critical germ and somatic cell markers [252]. This dose corresponds to 1.6-2.0 mg/kg/day in human equivalents, after conversion using the body surface area normalization method [208]. Under normal conditions, human exposure to GEN and DEHP ranges from 0.01 to 0.2 mg/kg/day and 0.003-0.03 mg/kg/day, respectively. Neonates fed soy-based infant formula or undergoing medical intervention however, can experience levels that are 10 to 100 fold higher than the general population [9, 11, 119-123]. In search of mechanistic clues for the previously observed long term effects in testis, we hypothesized that GEN and DEHP would target the early postnatal precursors of adult somatic and germ cells by altering similar genes/pathways in these cells, and that the mixture of the two EDs would have stronger effects than single compounds. The present results showed that it is not the case.

Unexpected gene profile alterations in response to DEHP, but not GEN or their combination.

PND 3 and PND 6 gross measurements revealed a significant decrease in PND 3 AGD of GEN-exposed animals, suggesting possible feminizing effects in response to this soy derived phytoestrogen. An intriguing effect of GEN was its consistent induction of Ar expression between PND 6 and adulthood. Interestingly, GEN has been reported to exert a biphasic effect on Ar expression in prostate cancer cells, inducing its expression at 0.5-5 μM, and inhibiting it at 25-50 μM [253]. Thus, it is possible that a similar process takes place in testis. No other significant gene

expression or protein alterations were found in GEN treated offspring for the endpoints evaluated. Paradoxically, the effects of GEN on AGD were normalized in rats treated in-utero with combined GEN and DEHP, a previously reported anti-androgen [254]. The expression of genes altered in adult testes by fetal exposure to the mixture revealed different gene profiles in neonatal testes. Noticeably, exposure to DEHP, but not GEN and DEHP mixture, induced a significant up-regulation of *Hsd3b* and *Ar*, whose expression was previously shown to be induced by T [255]. Concomitantly, DEHP up-regulated the expression of the Sertoli cell marker *Wt1*, early germ cell markers (*Plzf*, *Foxo1*), and proliferation marker *Pcna*, which at these early ages reflects the behavior of Sertoli, germ and myoid cells [256], suggesting a general stimulatory effect of DEHP on neonatal testis development. Our finding that the transcript of *Nestin*, an intermediate filament protein found in Sertoli and progenitor Leydig cells [257, 258], was increased in DEHP-exposed rats is reminiscent of an earlier study where we found that in utero exposure to higher DEHP doses (≥ 250 mg/kg/day) increased nestin expression in fetal Leydig cells right before birth [144]. Interestingly, these DEHP-induced effects were normalized by adding GEN, implying that GEN-activated pathways opposed those triggered by DEHP in neonatal testes.

At PND 6, the decreased expression of the Sertoli cell differentiation marker *Abp* [259] and the differentiating spermatogonia marker *Sohlh2* [260] in DEHP-exposed rats only, suggested that DEHP had altered the differentiation program of juvenile Sertoli and germ cells. Moreover, *Cyp11a1*, the enzyme responsible for cholesterol conversion to pregnenolone, was decreased uniquely by combined GEN and DEHP, indicating a selective targeting of specific steroidogenic mediators by the ED mixture.

Despite the significant up-regulation of androgen related genes by DEHP at PND 3, ex-vivo T production was not altered, potentially reflecting the functional recovery of testis fragments after

three days in culture without DEHP, similarly to the normalization of gene expression observed in PND 6 rats. By contrast, direct treatment of unexposed PND 3 testis fragments over three days with 10 uM MEHP, a concentration in the range of those measured in human blood under specific conditions [123, 261], revealed a pro-androgenic effect of MEHP, which was attenuated by combining GEN with MEHP, matching in-vivo PND3 expression data. These results contrast with previous studies where the same window of DEHP exposure using higher doses showed reduced basal and hormone-induced T production for ≥ 100 and ≥ 900 mg/kg/day DEHP respectively, suggesting differential DEHP sensitivity of basal and hormone-induced steroidogenesis mechanisms [132]. Moreover, studies using testis organ cultures fromGD14-16 fetuses reported the suppression of basal T production by high doses DBP; and an inhibitory effect of MEHP but stimulatory effect of DEHP on basal and LH stimulated T production, implying that MEHP and DEHP direct modes of action on fetal Leydig cells [136, 262].

Occurrence of Multinucleated Germ Cells (MNGs) In-Vitro

A recent study using organ cultures of human fetal testes incubated with 100 μM ^{14}C-MEHP determined that similar concentrations were present in the medium and testis fragments after 24 hours [263], suggesting that intra-testicular MEHP levels were close to the 10 μM added in PND 3 testis cultures, where abnormal MNGs were observed in vitro in the present study. Considering these data, together with the facts that in vivo MNGs were observed in rats treated in utero with ≥ 500 mg/kg/day DEHP, and that 10 mg/kg/day DEHP would elicit blood levels ~ 1 μM [264], the absence of MNGs in neonatal testes from rats treated in utero with 10 mg/kg/day DEHP is not surprising. Nonetheless, MNG formation is a phthalate-induced in-vitro phenotype consistent across rat, mouse and human fetal testis, and independent of hormone alterations [137, 262, 265]. Although this phenotype still remains poorly understood, selective targeting of critical cellular

junctions has been proposed as a mechanism contributing to endocrine - dependent or independent phthalate reproductive toxicity [266, 267].

In the adult, dynamically regulated tight junctions between Sertoli cells form a functional blood-testis barrier (BTB) that physically divides basal and apical compartments of the seminiferous epithelium, creating a microenvironment that allows the maturation and movement of dividing germ cells [268-273]. Although no MNG was observed at the dose used in the current in-vivo study, combined GEN and DEHP significantly up-regulated PND 6 testis mRNA levels of several cellular junction-related genes, including cadherins (*Cdh1* and *Cdh2*), tight junction protein 1 (*Tjp1*) and gap junction gene *Gja1*. Interestingly, an up-regulation of N-cadherin and catenins was also reported in rats displaying testicular atrophy and degeneration of the seminiferous epithelium following high dose DEHP treatment [274]. Inhibition or up-regulation of adhesion proteins by combined GEN and DEHP during early testis development may be related to long term reproductive toxicity and be a contributing factor to the observed MNG phenotype in-vitro. Further research is necessary to determine the cell specificity of junctional changes and their implications, and potential relationship with phthalate-induced MNGs, in which organ culture may serve as a valuable toxicological tool.

Are ROS Involved in DEHP Biphasic Effect?

The in-vitro data suggest that MEHP stimulates T production, a process abrogated by GEN co-treatment. Other studies have reported “non-monotonic” or “biphasic” effects of EDs, including phthalates. Rats treated gestationally (GD2-20) with DEHP had increased T at 10mg/kg/day and decreased T at higher doses [275]. Non-monotonic effects of DEHP on fetal T were also observed at GD20 and in-vitro following MEHP treatment [276-278]. Several studies point to the

involvement of ROS in modifying Leydig mitochondrial function: low concentrations of H_2O_2 and MEHP increased ROS, T production and STAR/CYP11A1 activities in isolated rat Leydig cells, an effect that was attenuated by co-treatment with the antioxidant Vitamin C [279]. Interestingly, high concentrations of H_2O_2 and MEHP had a suppressive effect on T production and steroidogenic enzyme activity, suggesting a biphasic effect of ROS on androgen production. Although not fully understood, research using MA-10 Leydig cells demonstrated a potential role for ROS in cAMP-mediated modulation of cellular signalling pathways, an effect that was attenuated by an uncoupler of oxidative phosphorylation [280]. Alternatively, or perhaps in parallel, evidence suggests that oxysterols generated in part by an oxidative cellular environment, can regulate STAR expression and also serve as ligands for liver x nuclear receptors (LXR), critical mediators of steroid and lipid metabolism and male reproductive function [281, 282]

Does GEN Protect Against DEHP Mediated Cellular Stress?

Although we have been investigating GEN in the context of a phytoestrogen with potential endocrine disrupting properties, GEN is also a relatively promiscuous compound with broad biological interactions, including PPAR activation, modulation of signalling molecules, as well as direct or indirect antioxidant action [175, 177]. It is therefore plausible that GEN may attenuate DEHP-mediated oxidative stress during the fetal period, independently of the long term effects of GEN and DEHP on male reproductive development.

To test this ROS hypothesis in PND 3 and PND 6 animals, gene and protein expression profiles of several downstream targets of the KEAP1/NRF2 mediated antioxidant response element (ARE) were examined [283]. In the event of oxidative environment changes, stress sensing cysteine in cytoplasmic KEAP1 results in a conformational change and subsequent dissociation and migration

of NRF2 to the nucleus, activating a battery of antioxidant and detoxifying gene targets intended to restore cell homeostasis.

qPCR analysis revealed a significant up-regulation of downstream *Nrf2* antioxidant and detoxifying gene targets, including *Nqo1*, *Sod2*, *Sod3*, *Gst*, *Trx* and *Ca*t specifically in PND 3 testes of DEHP-treated rats. Co-treatment with GEN attenuated these effects, suggesting a protective effect of GEN in neonatal testes. The DEHP-induced oxidative stress responses were no longer visible at PND 6, implying that the pro-oxidant effects of DEHP occurred only in the presence of the chemical or its metabolite MEHP in the tissues, in agreement with pharmacokinetic studies reporting that the total elimination of DEHP/MEHP and genistein can take 5 to 7 days [155]. Our data concur with previous studies reporting changes in oxidative stress genes in fetal testes exposed to various phthalates [284]. Immunohistochemical and quantitative image analysis indicated that DEHP-induced NQO1 upregulation occurred primarily in interstitial Leydig cells, fitting with the observed phenotype and ROS hypothesis, given the close proximity of these cells to interstitial macrophages and endothelial cells of blood vessels trafficking toxicants.

In addition to detoxifying enzymes, the BTB and Blood-Epidymal Barrier created by tight junction proteins at later ages, specialized xenobiotic efflux transporters actively regulate the type and quantity of compounds entering the male reproductive tract. Several members of the ATP-binding cassette (ABC) family of energy dependent efflux transporters, including ABCB1A/B (P-GP), ABCBG2 (BCRP) and ABCC1 (MRP1), are expressed in testicular germ, Sertoli, Leydig and endothelial cells, epididymal principal cells and maturing spermatozoa [285-289]. In our study, the xenobiotic transporters *Abcb1b* and *Abcg2* were significantly upregulated in PND 3, but not PND 6 testes of DEHP-exposed rats, suggesting a concerted effort to detoxify the cells by physically eliminating chemical stressors. Interestingly, DEHP and MEHP are not known

substrates for these xenobiotic transporters. However, oxidative stress can upregulate ABCB1, ABCG2 and ABCC1 expression via NRF2, P53 and NFKB pathways [290-292]. DEHP has also been proposed as a potential chemosensitizer, capable of modifying drug entry in multi-drug resistant tumours [293]. BRCP (-/-) mice displayed increased bioavailability and plasma levels of GEN metabolites and accumulation of several phytoestrogens in testis and epididymis, suggesting an important role in GEN toxicokinetics [287, 294]. Our results therefore reaffirm an important role for xenobiotic transporters in mediating testicular bioavailability and detoxification, endpoints that are often overlooked and warrant more investigation.

Conclusion

The results of the present study were somewhat unexpected, as they identified DEHP as the main inducer of changes in neonatal testis, and GEN as a protective agent. This contrasts with the effects observed in adult testis, where fetal exposure to the mixture of DEHP and GEN induced deleterious long-term effects in various testicular cell types, and GEN acted with DEHP to induce effects unique to their mixture. Although much of the literature has focused on EDs in relation to androgen suppression, research suggests that any deviation from normal hormonal programming, including androgen up-regulation or altering the balance of androgens and estrogens, can have profound consequences on male reproductive development [7, 295, 296]. To our knowledge, this is the first in-vivo study to provide mechanistic insight into low dose DEHP-induced early responses and the differential effects of GEN and DEHP mixture at doses below their known thresholds of toxicity.

We propose a mechanism by which GEN, through its antioxidant action, normalizes the ROS-mediated and pro-androgenic effects of DEHP in early postnatal testes. Thus, co-treatment of antioxidants devoid of endocrine disrupting side effects may help attenuate DEHP mediated

alterations during the fetal and neonatal periods. Paralleling our findings, low dose GEN antagonized the neonatal germ cell toxicity of vinclozolin, an anti-androgenic food contaminant, while conversely exacerbating adult toxicity in a synergistic manner [207, 297]. It is likely that GEN long term effects are not related to its antioxidant properties, but rather to its estrogenic properties, since protection against PND3 oxidative stress did not prevent long term effects.

Discrepancies between acute and delayed long term effects may be related to the non-monotonic nature of the effects. The latent effects of our mixture may be independent of hormonal or testicular environment aberrations triggered at much higher doses. Longitudinal effects likely reflect permanent alterations in early versions or progenitors of the testicular cells affected in adulthood. For example, adult Leydig cells are derived not from fetal Leydig cells but rather from neonatal mesenchymal precursors [59, 60]. Similarly, damages of primordial germ cells and gonocytes in-utero can lead to disrupted spermatogenesis in adulthood [41, 202, 203]. GEN and DEHP may have epigenotoxic effects in early fetal development that affect the developmental progression of key testicular cell types. Thus, future work will be focus at identifying correlative epigenetic alterations, such as DNA methylation and histone modifications, between fetal precursors and adult cell types that may be driving long-term toxicity.

Finally, this study highlights how short term studies of GEN and DEHP testicular effects cannot predict their long term effects. The notion that these EDs do not follow classical dose-response effects with consistent mechanisms of toxicity from perinatal ages to adulthood further stresses the importance of assessing impacts across a range of doses during appropriate windows of exposure and at different ages. We feel that these findings raise pertinent questions for regulatory agencies performing chemical risk assessments and determining acceptable exposure levels.

FIGURES AND LEGENDS

Figure 3.1. Effects of in utero exposure to genistein (GEN) and DEHP on general and reproductive health parameters. PND 3 and PND 6 average body weight (A) and anogenital distance (AGD) normalized to body weight (B). Data are represented as mean measurements (SEM) of parameters measured in the offspring of four dams per treatment. Asterisks indicate a significant difference relative to control in respective age groups ($p \leq 0.05$).

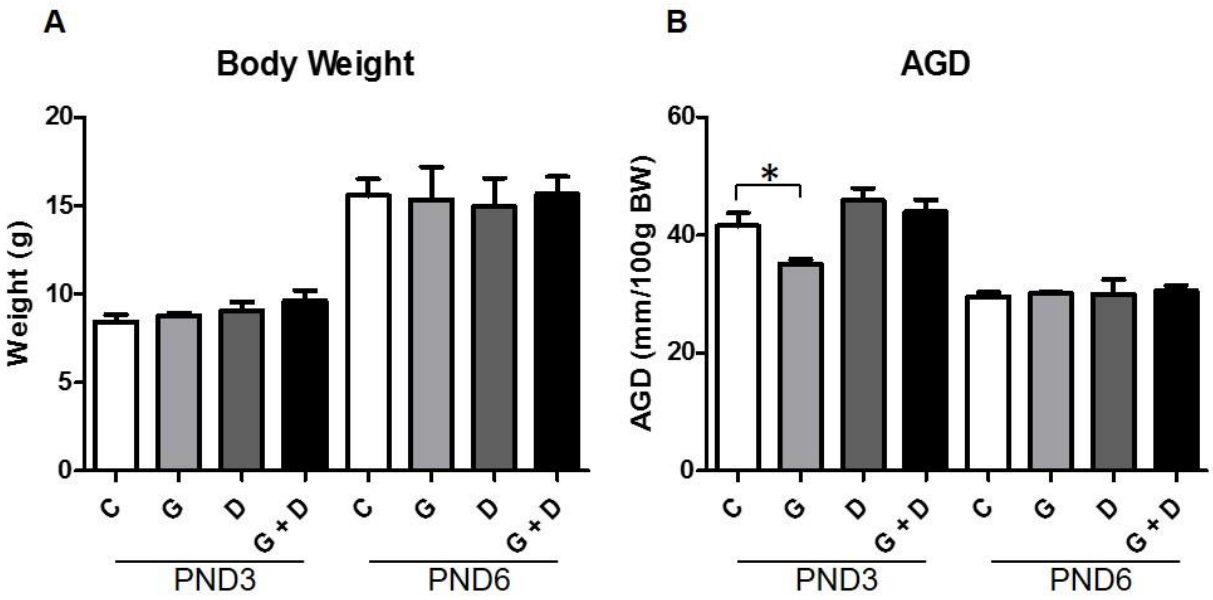
A
Body Weight
Weight (g)
20
15
10
5
0
C
G
D
G+D
C
G
D
G+D
PND3
PND6
B
AGD
AGD (mm/100g BW)
60
40
20
0
*
C
G
D
G+D
C
G
D
G+D
PND3
PND6

Figure 3.2. Effects of in utero exposure to genistein (GEN) and DEHP on steroidogenic mediators. Relative mRNA expression of steroidogenic enzymes *Cyp11a1* (A) and *Hsd3b* (B), and androgen receptor (*Ar*) (C) in PND 3 and PND 6 testes. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin (n = 4 per treatment). To further validate mRNA alterations, immunohistochemical analysis of HSD3B (D) was performed on PND 3 testes and positive interstitial staining was quantified by image analysis (E) (photos taken at 40x magnification. Immunohistochemical quantification is represented by mean fold change ± SEM of HSD3B staining relative to control ($n \geq 3$). Omission of the primary antibody was used as a negative control (data not shown). Representative pictures are presented. Asterisks in both mRNA and protein analyses indicate a significant difference relative to control ($p \leq 0.05$).

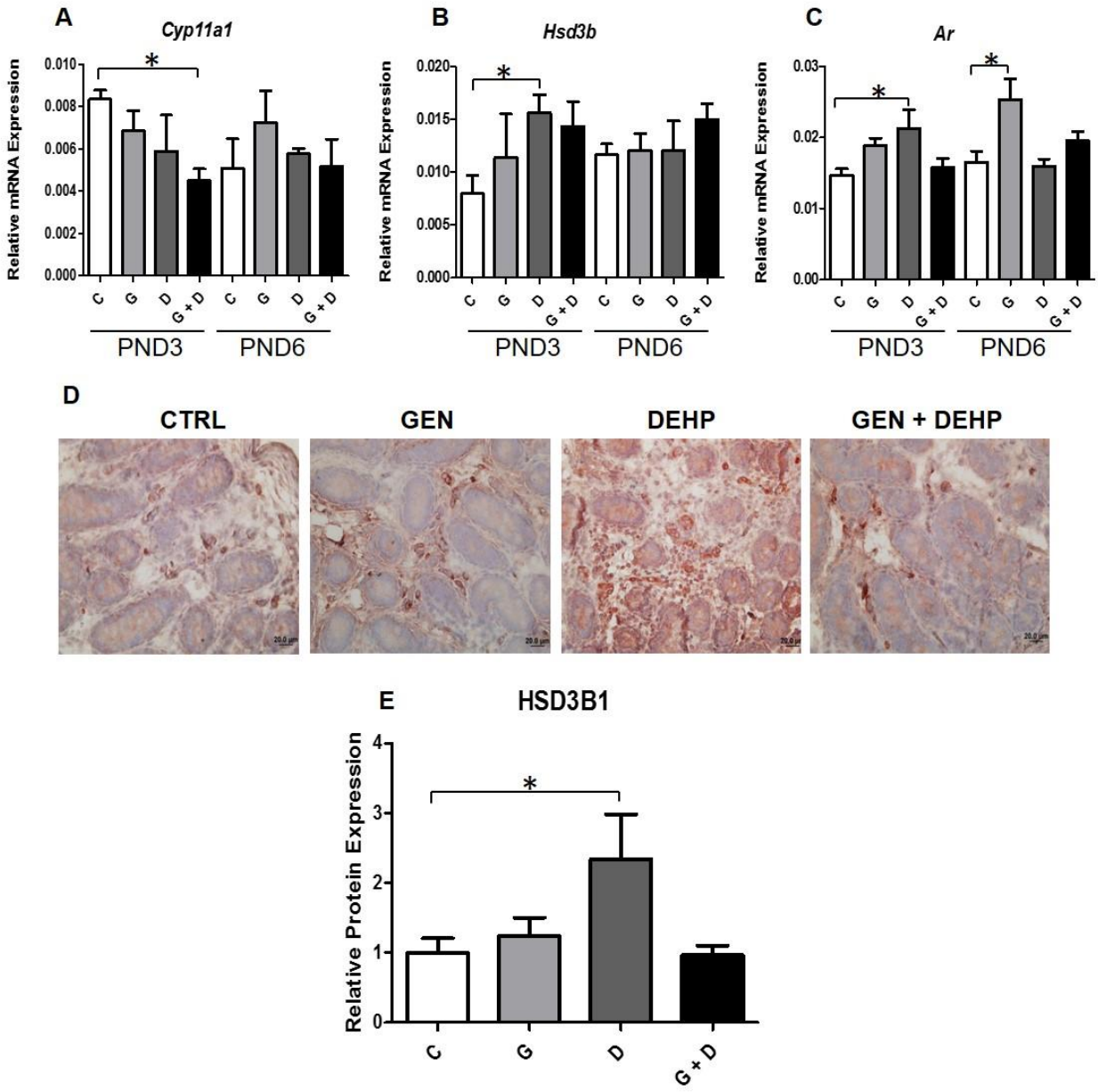
A
Cyp11a1
Relative mRNA Expression
0.010
0.008
0.006
0.004
0.002
0.000
*
C G D G+D C G D G+D
PND3 PND6
B
Hsd3b
Relative mRNA Expression
0.020
0.015
0.010
0.005
0.000
*
C G D G+D C G D G+D
PND3 PND6
C
Ar
Relative mRNA Expression
0.03
0.02
0.01
0.00
* *
C G D G+D C G D G+D
PND3 PND6
D
CTRL
GEN
DEHP
GEN + DEHP
E
HSD3B1
Relative Protein Expression
4
3
2
1
0
*
C G D G+D

Figure 3.3. Effects of in utero and in vitro exposure to genistein (GEN) and DEHP on PND 3 testes testosterone production. Ex-vivo testis organ culture was performed over three days in basal (A) or hCG (B) containing medium using PND 3 testes from in utero treated offspring. Control (untreated) PND 3 testes were also treated in vitro over three days with either plain medium (control), vehicle (DMSO), 10µM GEN, 10µM MEHP or combined 10µM GEN + MEHP. Testosterone levels in supernatant medium for both ex vivo and in-vitro organ cultures were determined by radioimmunoassay (RIA) and expressed in ng/testes. Graphs represent the sum of testosterone produced over three days (supernatant collected once daily). Asterisks indicate a significant difference relative to control ($p \leq 0.05$, n=4). To assess histological alterations, testes from ex vivo (data not shown) and in vitro organ cultures (D) were collected for processing and hematoxylin and eosin staining (photos taken at 100x magnification). Representative pictures are presented.

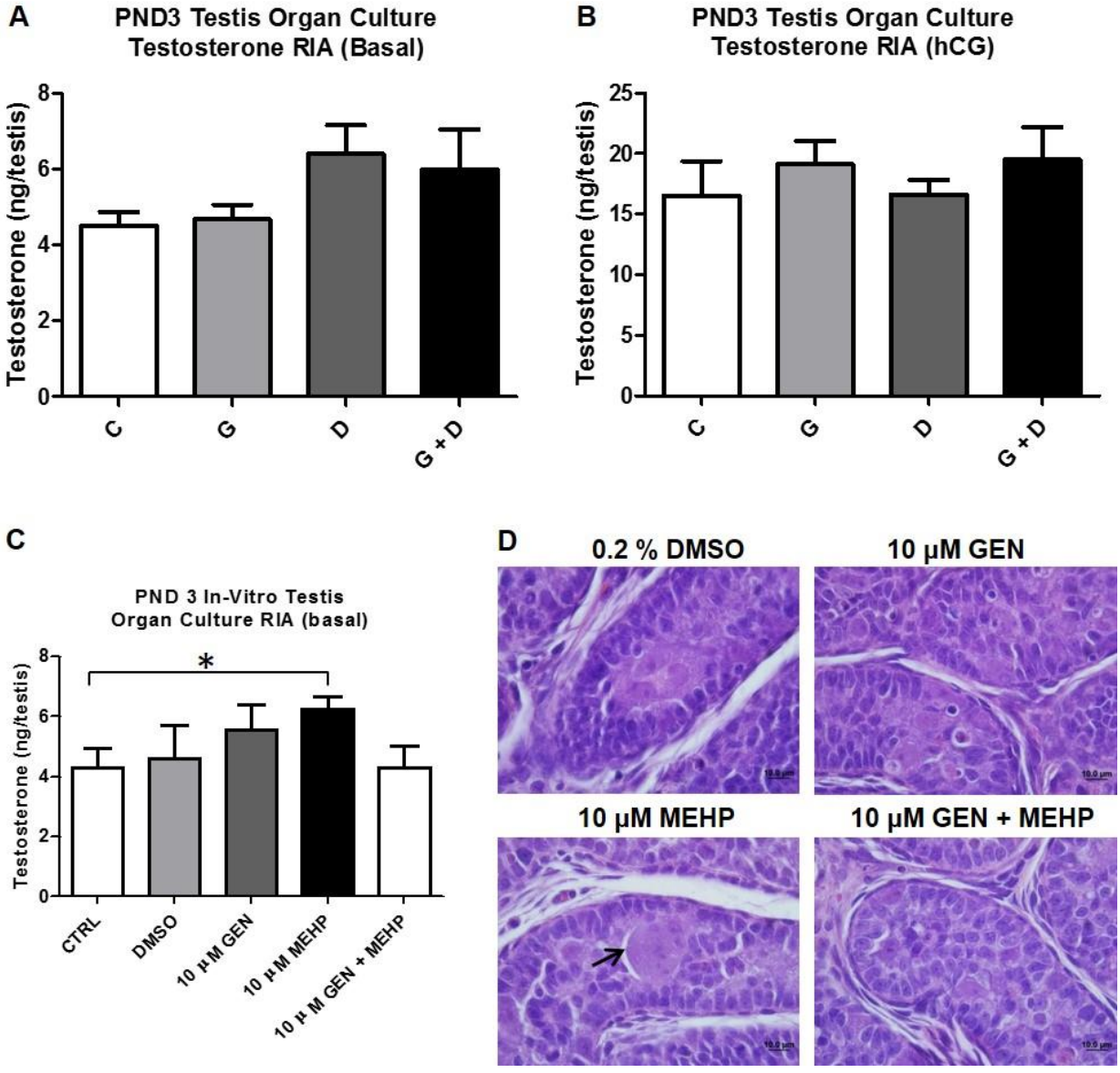
A
PND3 Testis Organ Culture
Testosterone RIA (Basal)
Testosterone (ng/testis)
0
2
4
6
8
C
G
D
G+D
B
PND3 Testis Organ Culture
Testosterone RIA (hCG)
Testosterone (ng/testis)
0
5
10
15
20
25
C
G
D
G+D
C
PND 3 In-Vitro Testis
Organ Culture RIA (basal)
Testosterone (ng/testis)
*
0
2
4
6
8
CTRL
DMSO
10 µM GEN
10 µM MEHP
10 µM GEN + MEHP
D
0.2 % DMSO
10 µM GEN
10 µM MEHP
10 µM GEN + MEHP

Figure 3.4. Effects of in utero exposure to genistein (GEN) and DEHP on Sertoli, proliferation and early germ cell markers. Relative mRNA expression of Sertoli cell markers *Wt1* (A) and *Abp* (B), Sertoli cell and Leydig marker *Nestin* (C), early germ cell markers *Plzf* (D), *Foxo1* (E) and *Sohlh2* (F), and proliferation marker *Pcna* (G), in PND 3 and PND 6 testes. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin (n = 4 per treatment). Asterisks indicate a significant difference relative to control ($p \leq 0.05$).

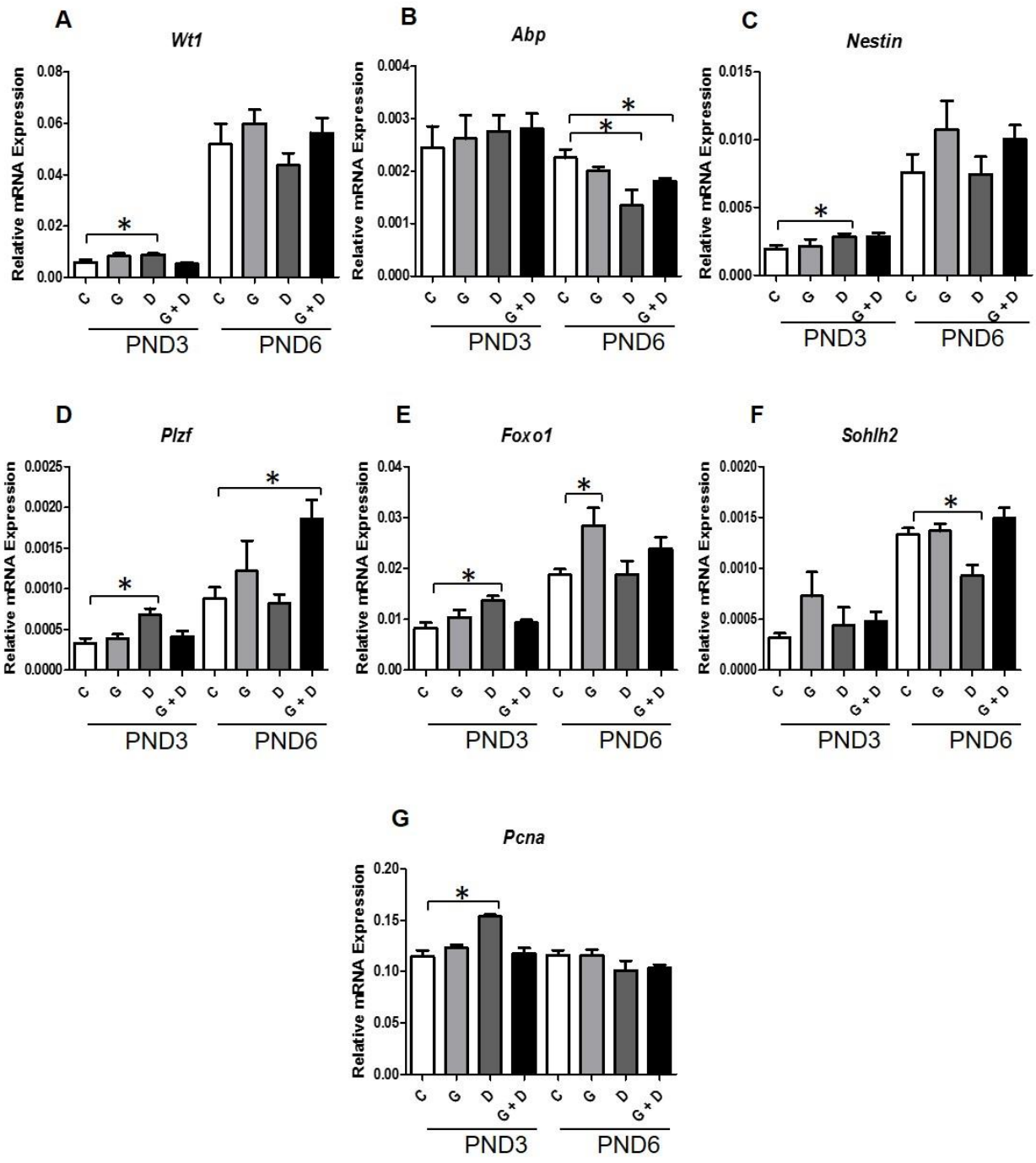
A
Wt1
Relative mRNA Expression
0.08
0.06
0.04
0.02
0.00
C G D G+D C G D G+D
PND3 PND6
B
Abp
Relative mRNA Expression
0.004
0.003
0.002
0.001
0.000
C G D G+D C G D G+D
PND3 PND6
C
Nestin
Relative mRNA Expression
0.015
0.010
0.005
0.000
C G D G+D C G D G+D
PND3 PND6
D
Plzf
Relative mRNA Expression
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
C G D G+D C G D G+D
PND3 PND6
E
Foxo1
Relative mRNA Expression
0.04
0.03
0.02
0.01
0.00
C G D G+D C G D G+D
PND3 PND6
F
Sohlh2
Relative mRNA Expression
0.0020
0.0015
0.0010
0.0005
0.0000
C G D G+D C G D G+D
PND3 PND6
G
Pcna
Relative mRNA Expression
0.20
0.15
0.10
0.05
0.00
C G D G+D C G D G+D
PND3 PND6

Figure 3.5. Effects of in utero exposure to genistein (GEN) and DEHP on cellular junctions. Relative mRNA expression of gap junction *Gja1* (A), tight junction protein *Tjp1* (B) and adherens junctions *Cdh1* (C), *Cdh2* (D) and *Cdh3* (E) in PND 3 and PND 6 testes. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin (n = 4 per treatment). Asterisks indicate a significant difference relative to control ($p \leq 0.05$).

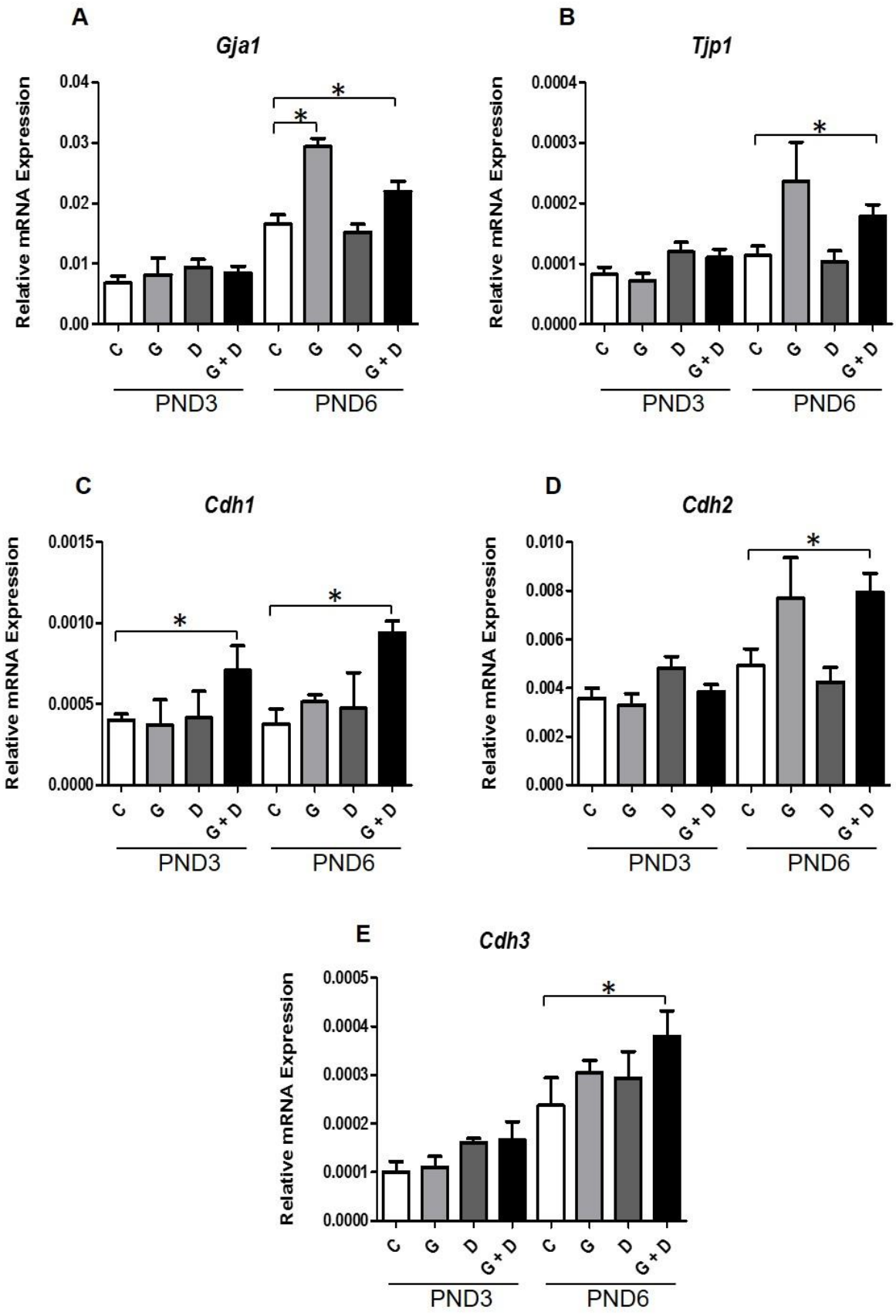
A
Gja1
Relative mRNA Expression
0.04
0.03
0.02
0.01
0.00
C G D G+D C G D G+D
PND3 PND6
B
Tjp1
Relative mRNA Expression
0.0004
0.0003
0.0002
0.0001
0.0000
C G D G+D C G D G+D
PND3 PND6
C
Cdh1
Relative mRNA Expression
0.0015
0.0010
0.0005
0.0000
C G D G+D C G D G+D
PND3 PND6
D
Cdh2
Relative mRNA Expression
0.010
0.008
0.006
0.004
0.002
0.000
C G D G+D C G D G+D
PND3 PND6
E
Cdh3
Relative mRNA Expression
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
C G D G+D C G D G+D
PND3 PND6

Figure 3.6. Effects of in utero exposure to genistein (GEN) and DEHP on cellular defence mediators. Relative mRNA of downstream NRF2 antioxidant and detoxifying enzymes *Nqo1* (A), *Sod2* (B), *Sod3* (C), *Trx1* (D), *Gsta* (E) and *Cat* (F) in PND 3 and PND 6 testes. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin (n = 4 per treatment). Asterisks indicate a significant difference relative to control ($p \leq 0.05$).

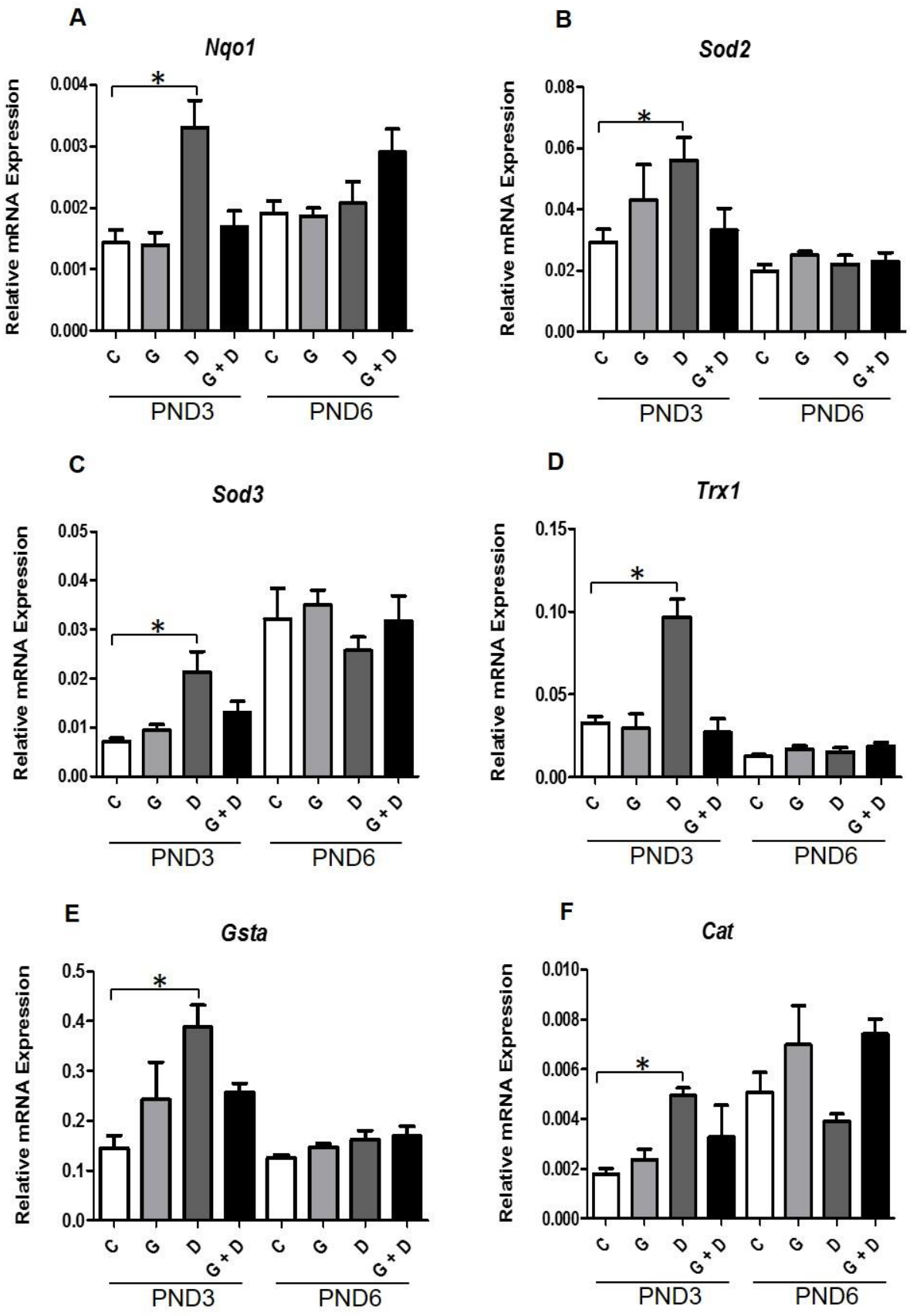
A
Nqo1
Relative mRNA Expression
0.004
0.003
0.002
0.001
0.000
*
C G D G+D C G D G+D
PND3 PND6
B
Sod2
Relative mRNA Expression
0.08
0.06
0.04
0.02
0.00
*
C G D G+D C G D G+D
PND3 PND6
C
Sod3
Relative mRNA Expression
0.05
0.04
0.03
0.02
0.01
0.00
*
C G D G+D C G D G+D
PND3 PND6
D
Trx1
Relative mRNA Expression
0.15
0.10
0.05
0.00
*
C G D G+D C G D G+D
PND3 PND6
E
Gsta
Relative mRNA Expression
0.5
0.4
0.3
0.2
0.1
0.0
*
C G D G+D C G D G+D
PND3 PND6
F
Cat
Relative mRNA Expression
0.010
0.008
0.006
0.004
0.002
0.000
*
C G D G+D C G D G+D
PND3 PND6

Figure 3.7. Effects of in utero exposure to genistein (GEN) and DEHP on anti-oxidant protein and xenobiotic transporter gene expression. Immunohistochemical analysis of NQO1 (A) was performed on PND 3 testes (A) and positive staining was quantified by image analysis (B)(photos taken at 40x magnification). Immunohistochemical quantification is represented by mean fold change ± SEM of NQO1 staining relative to control ($n \geq 3$). Omission of the primary antibody was used as a negative control (data not shown). Representative pictures are presented. Relative mRNA expression of xenobiotic transporters *Abcb1b* (C) and *Abcg2* (D) in PND 3 and PND 6 testes. Data are expressed as mean relative mRNA levels ± SEM normalized to alpha-tubulin ($n = 4$ per treatment). Asterisks in both mRNA and protein analyses indicate a significant difference relative to control ($p \leq 0.05$).

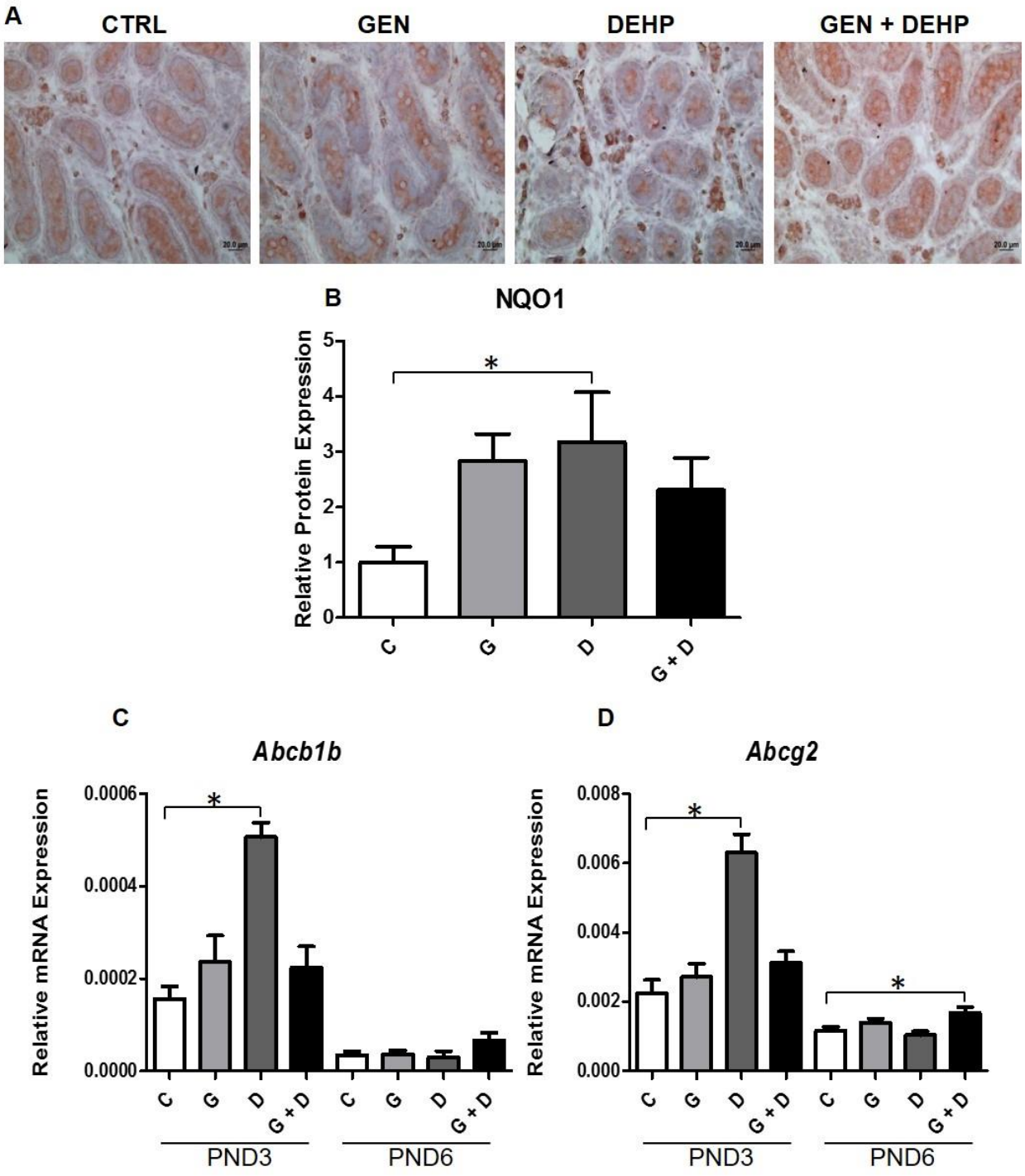
A
CTRL
GEN
DEHP
GEN + DEHP
B
NQO1
Relative Protein Expression
0
1
2
3
4
5
*
C
G
D
G+D
C
Abcb1b
Relative mRNA Expression
0.0000
0.0002
0.0004
0.0006
*
C
G
D
G+D
C
G
D
G+D
PND3
PND6
D
Abcg2
Relative mRNA Expression
0.000
0.002
0.004
0.006
0.008
*
*
C
G
D
G+D
C
G
D
G+D
PND3
PND6

Table 3.1. Primer sets used for quantitative real-time PCR

Gene name and symbol	Accession no.	Forward primer (5′–3′)	Reverse primer (3′–5′)
ATP-binding cassette, subfamily b member 1 (*Abcb1b*)	NM_012623.2	CAAATCCATGCTGCGACA	GCTACATTCTGGGTAACTACAGCA
ATP-binding cassette, subfamily g member 2 (*Abcg2*)	NM_181381.2	GGCCTGGACAAAGTAGCAGA	CTCCATTCCTATGCTTGTCCTT
Androgen binding protein *(Abp)*	NM_012650.1	TGAACGGGGATTCACTGCTAT	GGTCACTCAGGCAAAAGGAAGCAG
Androgen receptor *(Ar)*	NM_012502.1	CGGTCGAGTTGACATTAGTGAAGGACCG	ATTCCTGGATGGGACTGATGGT
Catalase (*Cat*)	NM_012520.1	TTCATCAGGGATGCCATGT	GGGTCCTTCAGGTGAGTTTG
Cadherin-1 (*Cdh1*)	NM_031334.1	GATCCTGGCCCTCCTGAT	TCTTTGACCACCGTTCTCCT
Cadherin-2 (*Cdh2*)	NM_031333.1	CCATCATCGCGATACTTCTG	CCATACCACGAACATGAGGA
Cadherin-3 (*Cdh3*)	NM_053938.2	CTTGGAGGTGGGAGGAACT	TGTCCAGCCAATGCCTCT
Cytochrome P450, family 11, subfamily A, polypeptide 1 (*Cyp11a1*)	NM_017286.2	CGGATATTTCCAGCTCTGCAATCCG	CACGCACTTCCGGTACTTGG
Forkhead box protein o1 (*Foxo1*)	NM_001191846.2	TCAGGCTAGGAGTTAGTGAGCA	GGGGTGAAGGGCATCTTT
Gap junction alpha-1 (*Gja1*)	NM_012567.2	AGCCTGAACTCTCATTTTTCCTT	CCATGTCTGGGCACCTCT
Glutathione s-transferase alpha-1 (*Gsta1*)	NM_031509.2	GGCCAGGCTAAGGAATGAT	AGAATGGCTCTGGTCTGCAC
Hydroxy-delta-5-steroid dehydrogenase, 3 beta (*Hsd3b*)	NM_001007719.3	GACCAGAAACCAAGGAGGAA	CTGGCACGCTCTCCTCAG
Heat shock protein 90, alpha (*Hsp90a*)	NM_175761.2	TTTCGTGCGTGCTCATTCT	AAGGCAAAGGTTTCGACCTC
Nestin (*Nestin*)	NM_012987.1	CCCTTAGTCTGGAGGTGGCTA	GGTGTCTGCAACCGAGAGTT
NAD(P)H dehydrogenase, quinone 1 (*Nqo1*)	NM_017000.3	GCCTACACGTATGCCACCA	CCCTGCAGAGAGTACATGGAG
Proliferating cell nuclear antigen (*Pcna*)	NM_022381.3	GAACTTTTTCACAAAAGCCACTC	GTGTCCCATGTCAGCAATTTT
Zinc finger and BTB domain containing 16 (*Plzf*)[a]	NM_001013181	AGCCCAGTTCTCAAAGGA	AGACAGAAGACAGCCATGCC
Superoxide dismutase 2 (*Sod2*)	NM_017051.2	TGGACAAACCTGAGCCCTAA	GACCCAAAGTCACGCTTGATA
Superoxide dismutase 3 (*Sod3*)	NM_012880.1	CTTGGGAGAGCTTGTCAGGT	CACCAGTAGCAGGTTGCAGA
Spermatogenesis and oogenesis specific basic helix-loop-helix 2 (*Sohlh2*)	NM_001034961.1	AGCCAGCTCCAGTTGTCTGT	GATGCTGGATGAGGCAGT
Tight junction protein 1 (*Tjp1*)	NM_001106266.1	GCAAGACCCAGCAAAGG	GGTTTTGTCTCATTTCCTCA
Tubulin alpha-1a (*Tuba1a*)	BC062238	CGGGGGAGAGTTCTCTGAGGCCCG	CAGAATCCACACCAACCTCCTC
Thioredoxin reductase 1 (*Trx1*)	NM_031614.2	TGGCCTCTCTGAAGAAAAAGC	TGGCCAGAAGAAACTGTGGT
Wilms tumor 1 (*Wt1*)	NM_031534	CGGTCGTCTTCAGGTGGTCGGACCG	GCACCAAAGGAGACACACAGGT

[a] BTB, BR-C, ttk and bab domain (found in Zinc finger proteins).

Preface to Chapter IV

Findings from chapters 2 and 3 identified fetal and adult Leydig cells, as well as their progenitors, as potential targets of GEN and DEHP. To further investigate direct effects of GEN and DEHP independent of systemic endocrine effects and interactions with other testicular cell types, MA-10 murine Leydig cells were exposed in-vitro to varying concentrations of GEN and MEHP, the principle bioactive metabolite of DEHP. MA-10 Leydig cells are a clonal strain of gonadotropin (hCG) and EGF responsive tumour cells. They are a useful and convenient model for Leydig cell function, steroidogenesis, regulation and response to external stimuli. Using this model, we were able to evaluate a full dose response of GEN and MEHP effects on Leydig cell viability, steroid production, gene expression and lipid homeostasis, an endpoint logistically challenging to isolate in complex tissue and cell based animal models.

Chapter IV

“Manuscript III”

STIMULATORY EFFECTS OF COMBINED ENDOCRINE DISRUPTORS ON MA-10 LEYDIG CELL STEROID PRODUCTION AND LIPID HOMEOSTASIS

Key Words: Endocrine Disruptor, Genistein, Phthalate, Leydig, MA-10, In-Vitro, Phthalate, Testis, Lipids, Steroidogenesis, Toxicology

Running Title: Distinct effects of combined genistein and DEHP on Leydig cell function

Summary Sentence: A mixture of genistein and DEHP exerts stimulatory effects on basal steroid production, lipid formation and associated gene expression at a concentration relevant to human exposure.

Authors: Steven Jones[1,2], Annie Boisvert[1,3], Andrada Naghi[4], Françoise Hullin−Matsuda [5,6], Peter Greimel[5], Toshihide Kobayashi[5], Vassilios Papadopoulos[1,4] and Martine Culty[1,2,3,4]

Affiliation: [1]*The Research Institute of the McGill University Health Centre, [2]division of Experimental Medicine, Departments of Medicine[3] and Pharmacology & Therapeutics[4], McGill University, Montreal, Quebec, Canada, [5]Lipid Biology Laboratory, RIKEN Institute, Wakoshi, Saitama, Japan, [6]INSERM UMR1060, University Lyon 1, Villeurbanne, France*

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ABSTRACT

Previous work in our laboratory demonstrated the ability of in utero exposure to a mixture of the phytoestrogen, Genistein (GEN), and plasticizer, DEHP, to induce long term alterations in gene and protein expression that are substantially different from individual exposures. Recent data identified fetal-type and adult Leydig cells, as well as their progenitors, as sensitive targets for low dose endocrine disruptor (ED) mixtures. To further investigate the direct effects of GEN and DEHP and elucidate specific mechanisms of toxicity, MA-10 Leydig cells were exposed in-vitro to varying concentrations of GEN and MEHP, the principle bioactive metabolite of DEHP.

10 μM combined GEN + MEHP had a stimulatory effect on basal MA-10 progesterone production. Consistent with increased androgenicity, the mRNA of steroidogenic and cholesterol mediators *Star, Cyp11a*, *Srb1* and *Hsl*, as well as upstream orphan nuclear receptors *Nr2f2* and *Sf1* were all significantly increased uniquely in the mixture treatment group. *Insl3*, a sensitive marker of Leydig endocrine disruption and cell function, was significantly decreased by combined GEN + MEHP. Lipid analysis by high-performance thin layer chromatography demonstrated the ability of combined 10 μM combined GEN + MEHP, but not individual exposures, to increase levels of several neutral lipids and phospholipid classes, indicating a generalized deregulation of lipid homeostasis. Further investigation by qPCR analysis revealed a concomitant increase in cholesterol (*Hmgcoa*) and phospholipid (*Srebp1c*, *Fasn*) mediator mRNAs, suggesting the possible involvement of upstream LXRα agonism.

These results suggest a deregulation of MA-10 Leydig function in response to a combination of GEN + MEHP, suggesting synergism and in some cases bi-phasic effects. We propose a working model for low doses GEN + MEHP exposure that likely involves LXR agonism and direct or

indirect activation of other transcription factors. Taken more broadly, this research highlights the importance of assessing the impact of ED mixtures in multiple toxicological models across a range of environmentally relevant doses.

INTRODUCTION

Early life exposure to endocrine disrupting (ED) chemicals is thought to contribute to reported declines in male reproductive potential and increased incidence of congenital reproductive tract malformation. Whereas the default reproductive development of the fetus is female and largely hormone independent, the proper masculinization, development and maintenance of male reproductive tract is highly dependent on androgens. Exposure to compounds capable of mimicking, antagonizing or impairing the normal functioning of endogenous hormones can alter the developmental program of the testis and have long-term consequences.

Two common early life exposures are those of genistein (GEN), a natural phytoestrogen found at high levels in soy-based infant formula and other soy-derived products, and di-(2-ethylhexyl) phthalate (DEHP), a ubiquitous plasticizer used in the manufacturing of polyvinyl chloride (PVC) plastics. Babies fed soy-based infant formula receive up to 9mg/kg/day of GEN and are reported to experience blood levels in micromolar (μM) ranges [119, 123, 151, 157]. Despite GEN being substantially less estrogenic than endogenous estradiol, circulating levels can be orders of magnitude higher than endogenous hormones and within the range of biological activity. Under normal circumstances, exposure to DEHP, and its principal bioactive metabolite, MEHP, is in the range of 0.003-0.03 mg/kg/day [10, 119, 121, 298, 299]. Specific populations however, such as neonates undergoing medical interventions using PVC medical tubing, blood bags and medical devices, can experience exposures up to 100 times the general population, while DEHP and MEHP levels up to 5 μM have been found in human cord blood [9, 300].

Early life individual exposures to GEN and DEHP have been associated with impaired male reproductive development, in high dose in-vivo and in-vitro models. GEN has been demonstrated to impair fetal testosterone production via ERα [297], inducing alterations in prepubertal spermatogenesis, Leydig and Sertoli cell function and development. DEHP has been shown to suppress fetal and adult testosterone biosynthesis [132, 144, 254], resulting in alterations in Leydig, Sertoli and germ cells, anogenital distance and external genitalia dysgenesis [134, 135]. Mechanistic evidence suggests that DEHP is an androgen receptor-independent anti-androgen [138], proposed to act via peroxisome proliferator-activated receptors (PPAR α/γ), LXR, or by inducing epigenetic alterations [145, 146, 205, 301].

Despite extensive efforts to establish toxicological evidence for regulatory interventions, criticism has been placed on the reliability of doses used and the appropriateness of animal and cell based models in the context of human exposure. Compounding the skepticism are an increasing number of reports highlighting bi-phasic or non-monotonic effects of low dose EDs, including phytoestrogens and phthalates [190]. An additional challenge for regulators is that studies have largely been mechanism-focused, using individual compounds rather than chemical mixtures more representative of life-long human exposure, and levels well exceeding reported human levels. Recent studies from our laboratory have demonstrated the ability of in-utero low dose combined GEN + DEHP to induce long term alterations in rat testicular function, involving different mechanisms of toxicity from perinatal ages to adulthood and from individual exposures [252, 302].

To further investigate the effects of GEN and DEHP and elucidate specific mechanisms of toxicity independent of systemic endocrine effects and indirect responses from other testicular cell types, MA−10 murine Leydig were exposed in−vitro to varying concentrations of GEN and MEHP, the principal bioactive metabolite of DEHP. MA-10 cells are a well characterized clonal strain of

mouse Leydig tumour cells, responsive to human chorionic gonadotrophin (hCG) and epidermal growth factor (EGF) that produces progesterone as major steroid [303]. Analysis of progesterone levels, gene expression and endpoints logistically challenging to isolate in complex tissue and cell-based animal models, such as lipid homeostasis, revealed specific effects of the ED mixture on basal steroid production and lipid homeostasis.

MATERIALS AND METHODS

Cell Culture and Treatment

MA-10 cells were grown in DMEM/nutrient mixture F-12 Media, supplemented with 2.5% horse and 5% fetal bovine serum, in 96-well (MTT and steroid measurements) or 6 cm culture dishes (RNA and lipid analyses) as previously described [304]. 24 h prior to treatment, medium was switched for serum-free medium containing 1% Nutridoma SP (Sigma-Aldrich, St Louis, MO), a biochemically defined supplement composed of albumin, insulin, transferrin and other defined organic and inorganic compounds, but no other growth factors, mitogens, hormones or steroids. Cell were grown to subconfluence, and incubated with either medium alone, vehicle (0.2 % DMSO), GEN, MEHP or GEN + MEHP at concentrations up to 100μM for 48 h. After treatment, cells and media were collected for subsequent MTT, Progesterone radioimmunoassay (RIA) for basal and hCG-stimulated cells, RNA, protein or lipid analyses. To determine steroid production, cells were washed with serum-free medium, and incubated for 2 h in the presence or absence of a saturating concentration of hCG (CR-125, 50 ng/ml; National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Control basal levels of progesterone varied between 4 and 12 ng/mg protein. Control hCG stimulated cells displayed robust increases in progesterone production, with levels between 62 and 148 ng/mg protein.

Analysis of Cell Viability

The effect of GEN and MEHP on cell viability was assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell viability/cytotoxicity assay as previously described [305] .

Steroid Quantification

Progesterone production by MA-10 cells was measured by radioimmunoassay (RIA) [304].

RNA Extraction and Quantitative Real-Time PCR

RNA was extracted using QIAGEN RNeasy Plus Mini kit (Qiagen, Santa Clarita, CA). cDNA was synthesized from isolated RNA using the single-strand cDNA transcriptor synthesis kit (Roche Diagnostics, Laval, QC). Quantitative real-time PCR (qPCR) was performed as previously described [252] using the LightCycler 480 Real-Time PCR System (LC480, Roche Diagnostics). Alpha tubulin was used as an endogenous control and for normalization of gene targets. A minimum of three male offspring from different litters were evaluated in triplicate. The comparative Ct method was used to calculate relative expression. Data are represented as mean relative mRNA expression in arbitrary units. Complete primer sequences for all gene targets can be found in Table 4.1.

Lipid Extraction and Analysis

Alterations in neutral lipid and phospholipid content were determined after separation by High Performance Thin Layer Chromatography (HPTLC). Total lipid was extracted from MA-10 cells following in vitro treatment using the Bligh-Dyer Method [306]. Chloroformic lipid extracts were blown off in pure nitrogen and stored at -20^{o}C until further use. Dry extracts were re-suspended in

a chloroform/methanol (2/1) mixture and spotted, along with appropriate commercial lipid standards, on silica HPTLC plates. Lipid migration was done in glass chambers with either chloroform/methanol/acetic acid/water (65/43/1/3) (to separate phospholipid classes) or hexane/diethyl ether/acetic acid (80/20/2) (to separate neutral lipids). Plates were dried and visualized using Primuline dye (phospholipids)(Sigma-Aldrich) or cupric acetate (neutral lipids). Lipid bands were measured by image analysis (ImageQuant software) using LAS 4000 image analyzer (Fujifilm) and normalized to total protein content (Bradford assay).

Statistical Analyses

Multiple means were compared using one-way ANOVA (Prism version 3.0; GraphPad Software, San Diego, CA).

RESULTS & DISCUSSION

The present study demonstrates a deregulation of Leydig cell function in response to concentrations of combined GEN and MEHP relevant for human exposure. Interestingly, synergistic effects on steroid production and lipid homeostasis and correlated gene expression were seen at doses below those previously reported to exert anti-androgenic effects for individual compounds [237, 307-309]. The novelty of the current findings is a demonstrated ability of two common ED to activate Leydig cell functions in the absence of hCG, behaving as hormonally active chemicals.

3.1. GEN at high concentration affects cell metabolic activity

At high concentration (100 µM), GEN induced a small but significantly increased MTT reduction in MA-10 cells, suggesting increased cellular metabolic activity or proliferation (FIG 4.1). This

effect was also found with the mixture, but not with MEHP, implying that GEN alone was exerting the observed effect on MTT reduction, independent of MEHP. A previous study had reported that 50 µM GEN had no effect on MTS tetrazolium reaction, an assay similar to MTT, at 48 h, the same time as in the present study, while it exerted an inhibitory effect after 72 h treatment [307]. These findings suggest different effects of GEN in function of the length of exposure.

3.2. Low dose of combined GEN and MEHP stimulates basal steroidogenesis

As previously reported, high dose MEHP exposure to MA-10 and primary rat Leydig cell had a suppressive effect on hCG-stimulated progesterone production (FIG 4.2B). Noticeably, co-treatment with GEN did not prevent the inhibitory effect of MEHP on hCG-induced steroid production, despite the reported anti-oxidant effect of genistein [177, 302], suggesting different mechanisms of action on basal and hormone-induced steroidogenesis.

Interestingly, 10 µM combined GEN and MEHP, but not individual exposures, resulted in a 2-fold increase in basal progesterone production, suggesting androgenic activity of the ED mixture (FIG 4.2A). A correlated increase in steroidogenic mediators (*Star, Cyp11a1*) and cholesterol mediators (*Srb1*, *Hsl* and *Hmgcoa*) (FIG 4.3 and 4.7), and upstream transcription factors *Sf1* and *Nr2f2* [96] (FIG 4.4) gene expression further confirmed an androgenic effect. 10 µM MEHP alone also had a stimulatory effect on *Star* and *Cyp11a1* gene expression, while inducing an increasing trend on *Srb1* (FIG 4.3), but no changes in progesterone levels (FIG 4.2A). These data suggest that GEN may be potentiating MEHP-induced transcriptional changes, permitting to reach expression levels in steroidogenesis-related genes required for optimal progesterone production in basal conditions. Interestingly, low concentration (≤ 1 µM) mono-butyl phthalate (MBP), a metabolite of di-butyl phthalate commonly used as a plasticizer in PVC plastics, was shown to increase hCG-stimulated

progesterone production, as well as *Star* mRNA and protein in a MLTC-1 Leydig tumour cell line [310]. Low concentrations of MEHP were also found to stimulate hCG regulated genes in MA-10 cells [237], suggesting multiple mechanisms of androgenic activity.

100µM combined GEN and MEHP had a suppressive effect on *Nr2f2* gene expression (FIG 4.4), highlighting a bi-phasic effect of the mixture specifically on this gene, but not other steroidogenesis-related genes (FIG 4.3 and 4.7). The inhibitory effect of the mixture on Nr2f2 expression paralleled the inhibition of hormone-induced, but not basal, progesterone production (FIG 4.2), suggesting a critical role of this gene in hormone-regulated steroidogenesis. Such central role of NR2F2 is in agreement with a study reporting that NR2F2 activates the expression of StAR, a key element of hormone-regulated steroidogenesis, in MA-10 cells [96].

Insl3, a gene encoding for a Leydig-derived hormone involved in fetal testicular descent was decreased by combined 10µM GEN and MEHP, suggesting a deregulation of cell function (FIG 4.4). Paradoxically, *Sf1* and *Nr2f2*, both increased in our study (FIG 4.4), have been demonstrated to directly regulate *Insl3* gene expression in MA-10 cells [95]. Nonetheless, *Insl3* has been proposed as a biomarker of Leydig function [311], sensitive to phthalates and estrogens, presenting level changes often paralleling steroid production changes observed *in-vitro* [312-314].

Characterization of MA-10 lipid composition

Given the deregulation of basal steroidogenesis and the previously reported ability of MEHP to interact with testis PPARs and LXR, we hypothesized that lipid homeostasis of MA-10 cells would also be altered. The lipid profile of this commonly utilized Leydig model, proposed as an ED screening tool [315], was evaluated under normal and ED treated conditions. Under control conditions, MA-10 cell neutral lipids displayed a high-percentage of cholesterol esters, followed

by free cholesterol, triglycerides (TG) and free fatty acids (FFA) (FIG 4.5A). A similar ratio of cholesterol ester to free cholesterol was reported in primary adult Leydig cells isolated from Wistar rats [316]. Primary cultures in comparison however, had substantially more glycerides (mono, di and tri-acyl) than MA-10 cells. This is likely due to a reduced number of TG-rich lipid droplets in MA-10 cells [308, 317]. Lipid composition changes between primary cultures and cultured cells or cell lines are also often reported due to difference in lipid composition of foetal bovine serum or new calf serum used in cell culture versus blood in vivo. Although proportions varied, phospholipid composition in MA-10 cells and adult rat Leydig cells was also similar, with phosphatidylcholine (PC) being the dominant phospholipid, followed by phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyeline (SM) (FIG 4.5B). Other specific cellular compartment phospholipids such as mitochondrial Cardiolipin (CL) and lysosomal Bis (Monoacylglycero) Phosphate (BMP) were not considered since they represent a minor portion of the total phospholipid content and were not separated in this TLC system [318-320].

Low dose of combined GEN and MEHP increases MA-10 lipid contents

Treatment of MA-10 cells with the same 10 µM GEN and MEHP combination that upregulated steroid synthesis, also significantly increased neutral lipid and phospholipid levels in these cells (FIG 4.6B-D). The amount of cholesterol ester did not increase at the same dose (FIG 4.6B), presumably because of the substantial free cholesterol required for progesterone synthesis. Indeed, mRNA expression of *Hsl*, a gene coding for the hormone-sensitive lipase responsible for the liberation of free cholesterol from cholesterol esters in lipid droplets of steroid cells, was also shown to be upregulated (FIG 4.3E). Additionally, nearly all phospholipid classes, including PC, PI, PE and PS were significantly increased relative to control (FIG 4.6D). Consistent with these

findings, a phospholipid modifying transcription factor downstream of LXRα, sterol regulatory element-binding protein-1c (*Srebp1c*) was upregulated along with downstream gene target *fatty acid synthase (Fasn)* (FIG 4.7C-D). *Fasn* is coding for a key enzyme involved in the *de novo* synthesis of fatty acids incorporated into various phospholipids and TG. *Hmgcoa* gene encoding HMG-CoA reductase, the rate-limiting enzyme for cholesterol synthesis, downstream of another LXRα regulated transcription factor sterol regulatory-element binding protein 2 (*Srebp2*), was also increased (FIG 4.7A-B). This gene expression change was consistent with the demonstrated increase in free cholesterol and demand for steroid precursors.

Low concentration MEHP was previously reported to increase lipid droplet size while decreasing mitochondrial volume fraction in MA-10 cells [308]. Similar effects were observed in human testis explants treated with MEHP in-vitro: Interstitial cells from treated explants displayed increased lipid droplets and consistent upregulation of downstream LXRα lipid mediators, suggesting the involvement of LXR agonism by MEHP in Leydig cells [143]. MEHP-induced increase in all neutral lipids and phospholipid classes were also reported in steroidogenic trophoblasts [321]. Interestingly, in addition to modifying transcription factors implicated in cholesterol and phospholipid homeostasis, LXRα ligands have been demonstrated to directly activate *Star* gene expression. Alternatively, or perhaps in parallel, HSL expression has been demonstrated to increase the effectiveness of LXRα ligand-mediated upregulation of StAR, SREBP1C and ATP-binding cassette (ABC) transporter ABCA1 [322]. Thus, increased HSL expression, resulting from the heightened cellular cholesterol pool, or by virtue of GEN or MEHP – mediated transcriptional regulation, may further propagate LXR-mediated changes. Similarly, oxysterols generated as a bi-product of steroidogenesis are endogenous LXRα ligands and may also be contributing to the observed phenotype [281, 282].

Among GEN plethora of reported biological effects are direct activation of estrogen receptors, PPARs and LXRs, demonstrating a potential for steroid and lipid modifying ability [123, 175, 180, 323]. Much of the focus of GEN as a potential lipid modifier however, has been in systemic dietary studies: soy and low GEN increased LXRα, PPAR and HSL in adipocytes [29, 180]. Soy isoflavones have also been demonstrated to increase circulating adiponectin, a protein hormone involved in glucose and fatty acid metabolism [324]. Interestingly, developmental expression of adiponectin and adiponectin receptors has been reported in rat Leydig cells and also shown to be regulated by gonadotropins and PPAR [325]. In MA-10 cells, adiponectin increased progesterone, StAR and Cyp11a1 dose dependently [326], highlighting an additional avenue for androgenic activity and also a complex interplay between steroid and lipid metabolism.

CONCLUSION

The current study demonstrates the ability of two common ED at early life exposures to activate Leydig cells, resulting in significant changes in endogenous steroid production, correlated with gene expression changes as well as an upregulation of lipid levels, including cholesterol and several lipids known for their involvement as the source of diverse cell signaling processes [327-329]. Effects unique to the combination of GEN and MEHP were observed at 10 μM, suggesting synergism at a concentration within the range of levels measured in human blood. We propose a working model that likely involves LXR agonism and direct or indirect activation of other transcription factors, including SF1, NR2F2 and SREBP1C (FIG 4.8).

Our results parallel a proposed adrenal cortex model, in which LXRα agonism drives StAR expression, the conversion of cholesterol into steroid hormones and a crosstalk with fatty acid synthesis [330]. GEN may either potentiate the effects of MEHP, acting on similar receptors or

co-factors, or perhaps impacts its toxicokinetics. Although much emphasis has been placed on the potential anti-androgenic effects of EDs, equally important is maintaining a hormonal balance between androgens and estrogens [7, 295, 296]. Any deviation from normal hormonal programming, including increased androgenicity, can have profound effects on male reproductive function and development. A distinction between acute and long-term toxicity must also be made; we recently demonstrated in-vivo that while GEN attenuated early life androgenic effects of low dose DEHP, combined in-utero GEN + DEHP posed a greater reproductive risk in adulthood compared to individual exposures [252, 302]. Inconsistencies between in-vivo and in-vitro may be related to species differences, the tumorigenicity of cell lines, or the absence of other testicular cell types and systemic effects. Still, MA-10 Leydig cells are a useful and convenient toxicological tool for ED evaluation.

Although initially debated, non-monotonic effects are now being acknowledged by regulatory agencies and are the subject of recent expert review panel discussions. There is a push to incorporate endocrine principles in chemical risk assessment, rather than traditional toxicology and pharmacology based dose-response curves [190, 191, 331]. It is well established that endogenous hormones follow biological principles in which activity is dependent on numerous factors, including substrate concentration, receptor density and presence of co-factors. Hormones also act at very low doses and have a non-linear relationship between concentration and receptor occupancy, often leading to non-monotonic response curves well below toxic levels. It is logical to assume that exogenous substances capable of mimicking, blocking or potentiating the effects of endogenous hormones would act in a similar fashion.

The current study emphasizes how EDs and particularly ED mixtures do not follow a classic dose response. For hormonally active toxicants, it is no longer correct to assume that dose escalation

will linearly push the frequency and magnitude of effects observed at low doses. Although challenging, discussions on bi-phasic effects and the possible additive or synergistic effect of ED mixtures should be incorporated into chemical risk assessment. The current findings and also our previous in-vivo animal models for GEN and DEHP, highlight a need for a more comprehensive evaluation of EDs, individually or in combination, across a range of doses and different toxicological model. A culmination of evidence and scientific exchanges will ultimately lead to interventions aimed at improving male reproductive and developmental health.

FIGURES AND LEGENDS

Figure 4.1. Effect of genistein and MEHP on MA-10 cell viability. Cells were treated for 48 h with either medium (CTRL), vehicle (0.2% DMSO), 0.01, 1, 10 or 100 µM genistein (GEN), MEHP or combined genistein and MEHP (GEN + MEHP). Data are represented as mean ±SEM fold change relative to control (n=3). Asterisks indicate a significant difference relative to control in respective treatment groups (ANOVA, $p \leq 0.05$).

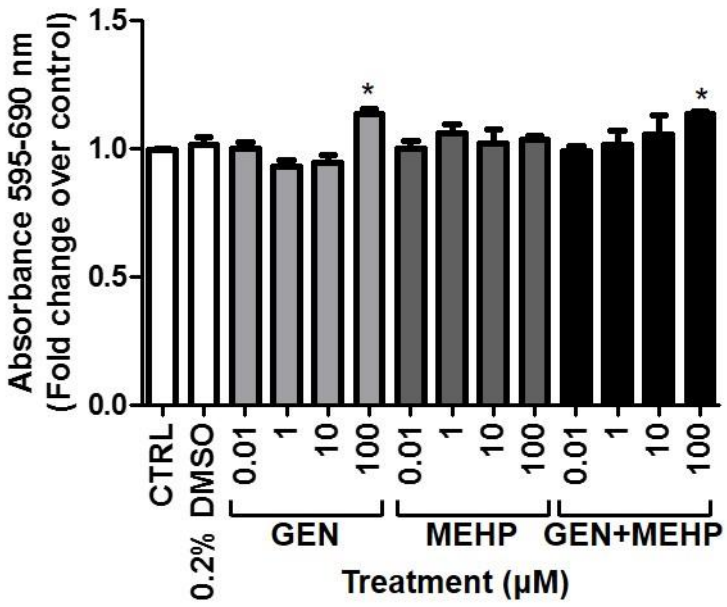
Absorbance 595-690 nm
(Fold change over control)
1.5
1.0
0.5
0.0
*
*
CTRL
0.2% DMSO
0.01
1
10
100
0.01
1
10
100
0.01
1
10
100
GEN
MEHP
GEN+MEHP
Treatment (μM)

Figure 4.2. Effect of genistein and MEHP on MA-10 basal (A) and hCG-stimulated (B) progesterone production. Cells were treated for 48 h with either vehicle containing 0.2% DMSO (CTRL), 0.1, 10 or 100 µM genistein (G), MEHP (M) or combined genistein and MEHP (G + M). hCG was then added in half of the samples for 2 hours. Basal or hCG-stimulated progesterone levels were determined by radioimmunoassay (RIA). Data are represented as mean ±SEM fold change relative to control (n=3). Asterisks indicate a significant difference relative to control in respective treatment groups (ANOVA, $p \leq 0.05$).

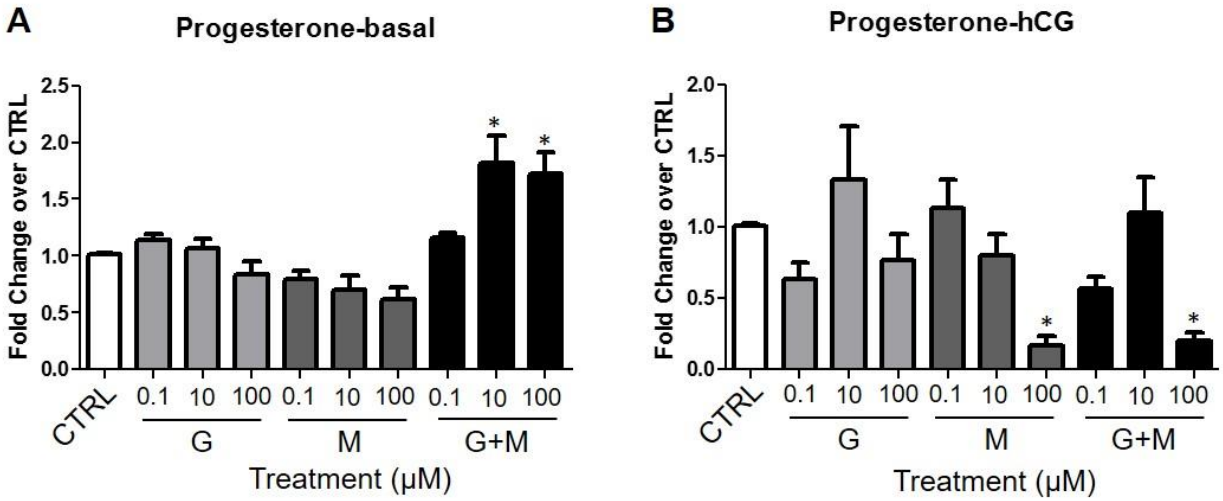
A
Progesterone-basal
Fold Change over CTRL
2.5
2.0
1.5
1.0
0.5
0.0
*
*
CTRL
0.1
10
100
0.1
10
100
0.1
10
100
G
M
G+M
Treatment (μM)
B
Progesterone-hCG
Fold Change over CTRL
2.0
1.5
1.0
0.5
0.0
*
*
CTRL
0.1
10
100
0.1
10
100
0.1
10
100
G
M
G+M
Treatment (μM)

Figure 4.3. Effect of genistein and MEHP on MA-10 steroidogenic gene expression. Cells were treated for 48 h with either vehicle containing 0.2% DMSO (CTRL), 0.1, 10 or 100 µM genistein (G), MEHP (M) or combined genistein and MEHP (G + M). *Star* (A), *Tspo* (B), *Cyp11a1* (C), *Hsd3b* (D), *Hsl* (E) and *Srb1* (F) mRNA levels were determined by real-time PCR and normalized to alpha-tubulin (n=3). Data are expressed as the mean ±SEM. Asterisks indicate a significant difference from control treatments groups (ANOVA, $p \leq 0.05$).

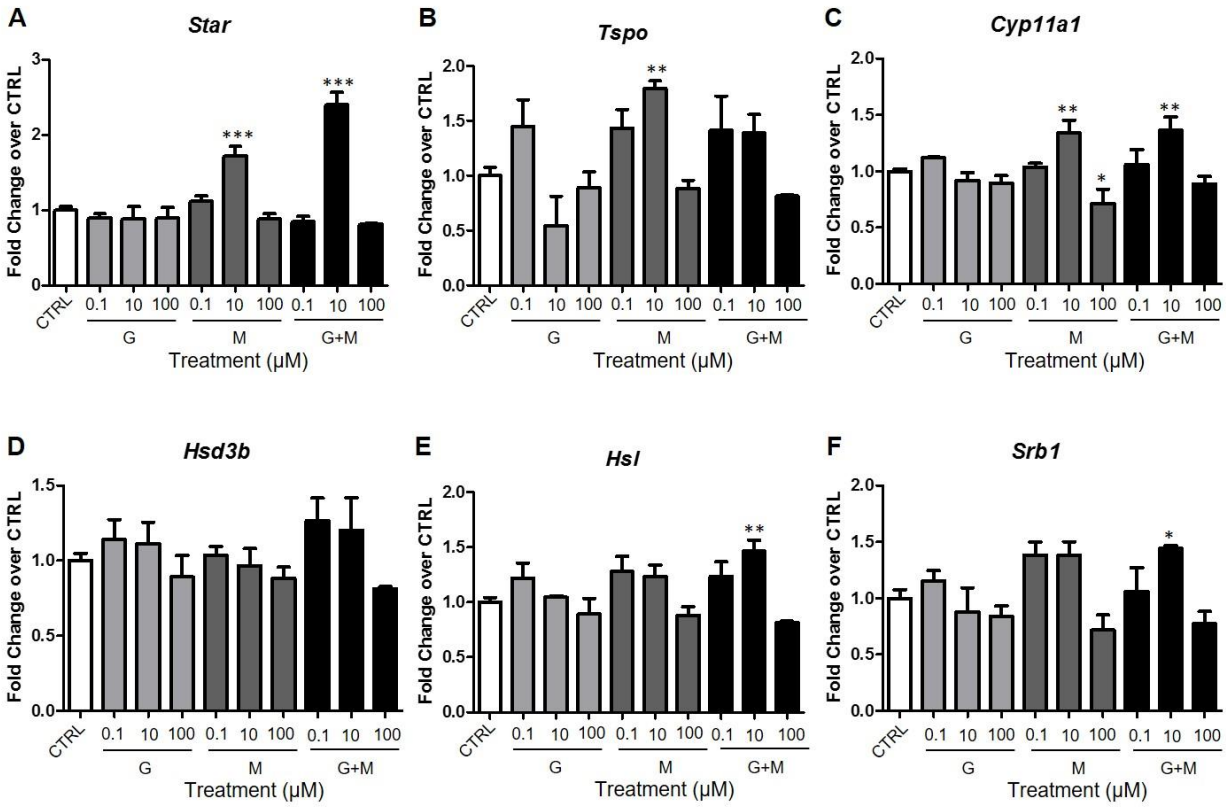
A
Star
Fold Change over CTRL
CTRL
0.1 10 100 0.1 10 100 0.1 10 100
G
M
G+M
Treatment (μM)
B
Tspo
C
Cyp11a1
D
Hsd3b
E
Hsl
F
Srb1

Figure 4.4. Effect of genistein and MEHP on the expression of key Leydig cell genes in MA-10 cells. Cells were treated for 48 h with either 0.2% DMSO (CTRL), 0.1, 10 or 100 µM genistein (G), MEHP (M) or combined genistein and MEHP (G + M). *Nr2f2* (A), *Sf1* (B) and *Insl3* (C) mRNA levels were determined by real-time PCR and normalized to alpha-tubulin (n=3). Data are expressed as the mean ±SEM. Asterisks indicate a significant difference from control treatments groups (ANOVA, $p \leq 0.05$).

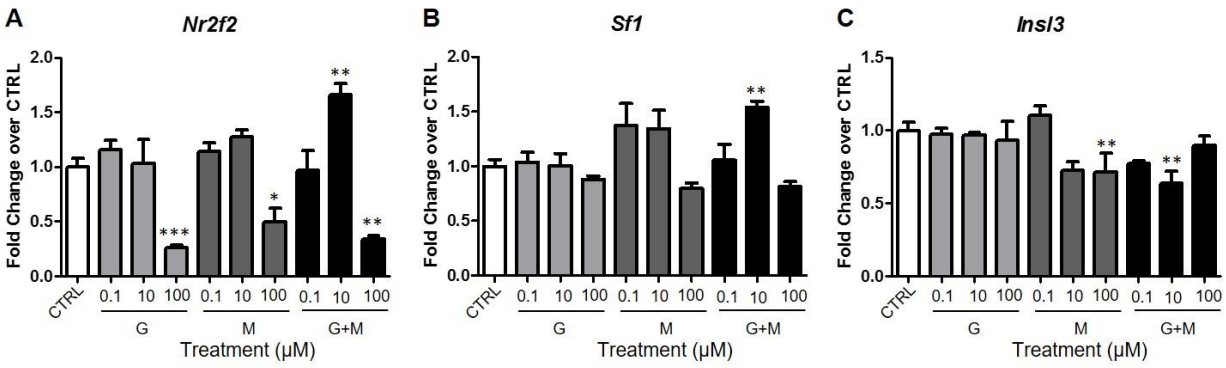
A
Nr2f2
Fold Change over CTRL
2.0
1.5
1.0
0.5
0.0
***
*
**
**
CTRL
0.1
10
100
0.1
10
100
0.1
10
100
G
M
G+M
Treatment (μM)
B
Sf1
Fold Change over CTRL
2.0
1.5
1.0
0.5
0.0
**
CTRL
0.1
10
100
0.1
10
100
0.1
10
100
G
M
G+M
Treatment (μM)
C
Insl3
Fold Change over CTRL
1.5
1.0
0.5
0.0
**
**
CTRL
0.1
10
100
0.1
10
100
0.1
10
100
G
M
G+M
Treatment (μM)

Figure 4.5. Relative neutral lipid (A) and phospholipid (B) levels in untreated MA-10 Leydig cells. Representative images of lipids after separation by high performance thin layer chromatography (HPTLC) are shown in left panels. Data in right panels are represented as percentage (%) of total lipid content within their respective lipid class as determined by quantitative image analysis (n=3). EChol, Esterified Cholesterol; TG, Triglycerides; FFA, Free Fatty Acid; Chol, Cholesterol; PL, Phospholipid; NL, Neutral Lipids; BMP, Bis (Monoacylglycero) Phosphate; CL, cardiolipin; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PS, Phosphatidylserine; PC Phosphatidylcholine; SM Sphingomyelin.

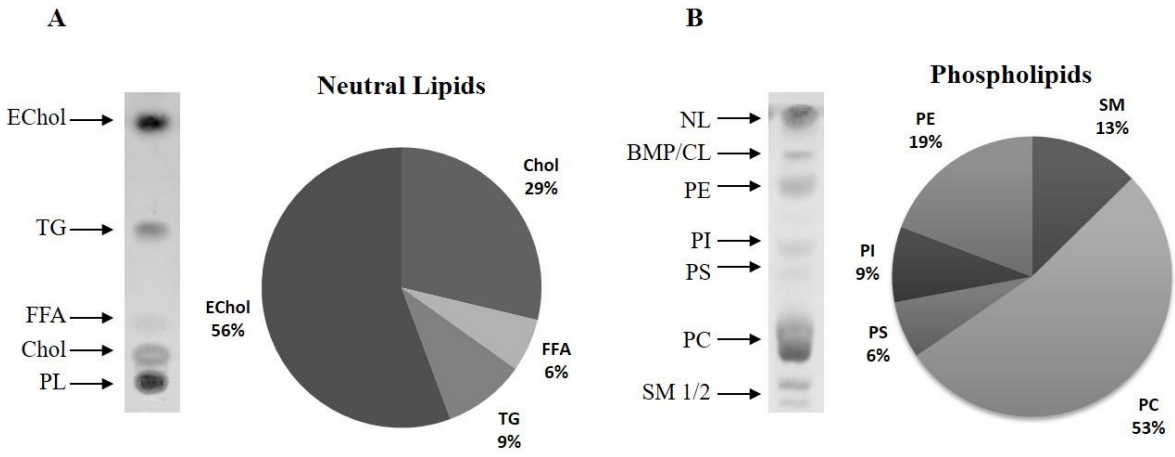
A
EChol
TG
FFA
Chol
PL
Neutral Lipids
Chol
29%
FFA
6%
TG
9%
EChol
56%
B
NL
BMP/CL
PE
PI
PS
PC
SM 1/2
Phospholipids
PE
19%
SM
13%
PI
9%
PS
6%
PC
53%

Figure 4.6. Effect of genistein and MEHP on relative neutral lipid and phospholipid levels in MA-10 Leydig cells. Cells were treated for 48 h with either 0.2% DMSO (CTRL), 0.1 μM (A,C) or 10 μM (B, D) Genistein (GEN) , MEHP or combined Genistein and MEHP (GEN + MEHP). Data are represented as mean (SEM) fold change relative to control as determined by quantitative thin layer chromatography (HPTLC) after normalization by protein content (n=3). Asterisks indicate a significant difference relative to control in respective treatment groups (ANOVA, $p \leq 0.05$). EChol, Esterified Cholesterol; TG, Triglycerides; FFA, Free Fatty Acid; Chol, Cholesterol; PE, Phosphatiylethanolamine; PI, Phosphatidylinositol; PS, Phosphatidylserine; PC Phosphatidylcholine; SM Sphingomyelin.

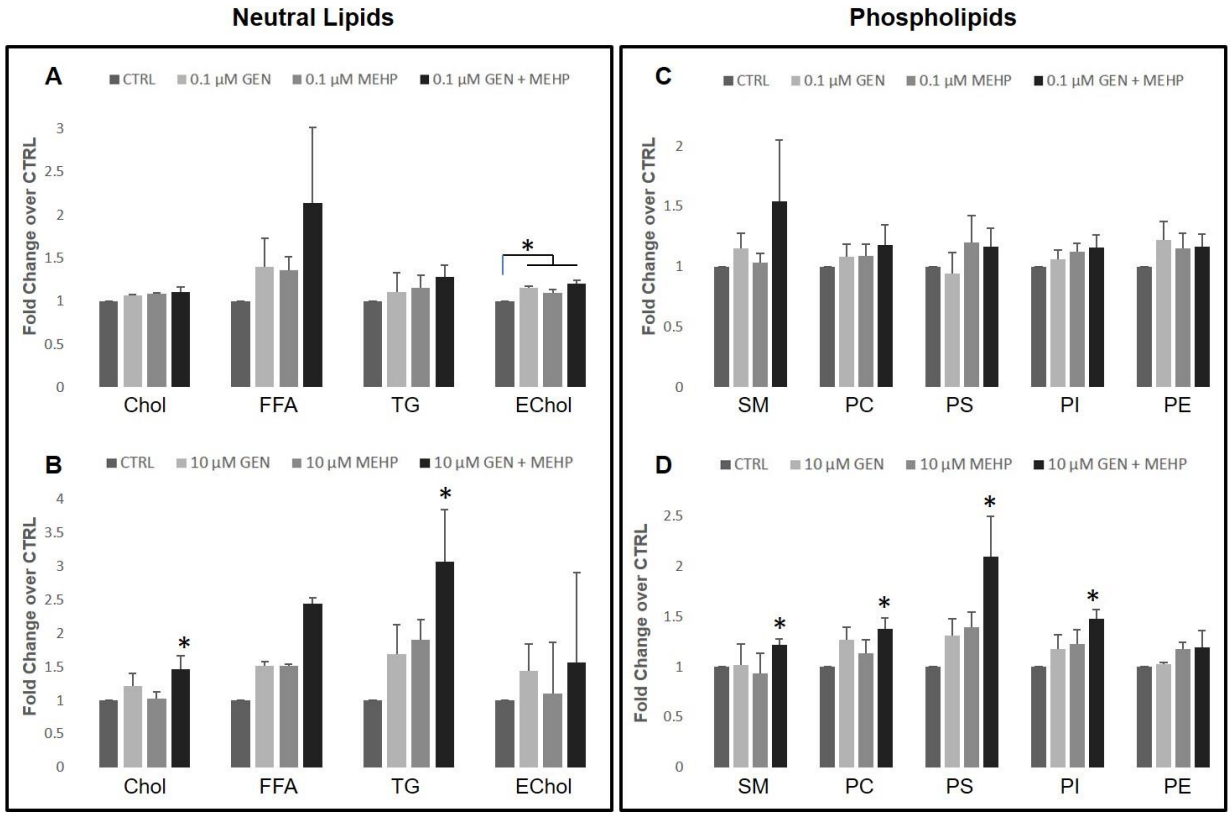
Neutral Lipids
Phospholipids
A
CTRL
0.1 µM GEN
0.1 µM MEHP
0.1 µM GEN + MEHP
Fold Change over CTRL
Chol
FFA
TG
EChol
B
10 µM GEN
10 µM MEHP
10 µM GEN + MEHP
C
D
SM
PC
PS
PI
PE

Figure 4.7. Effect of genistein and MEHP on MA-10 cholesterol and phospholipid mediator gene expression. Cells were treated for 48 h with either 0.2% DMSO (CTRL), 0.1, 10 or 100 µM genistein (G), MEHP (M) or combined genistein and MEHP (G + M). *Srebp2* (A), *Hmgcoa* (B), *Srebp1c* (C) and *Fasn* (D) mRNA levels were determined by real-time PCR and normalized to alpha-tubulin (n=3). Data are expressed as the mean ±SEM. Asterisks indicate a significant difference from control treatments groups (ANOVA, $p \leq 0.05$).

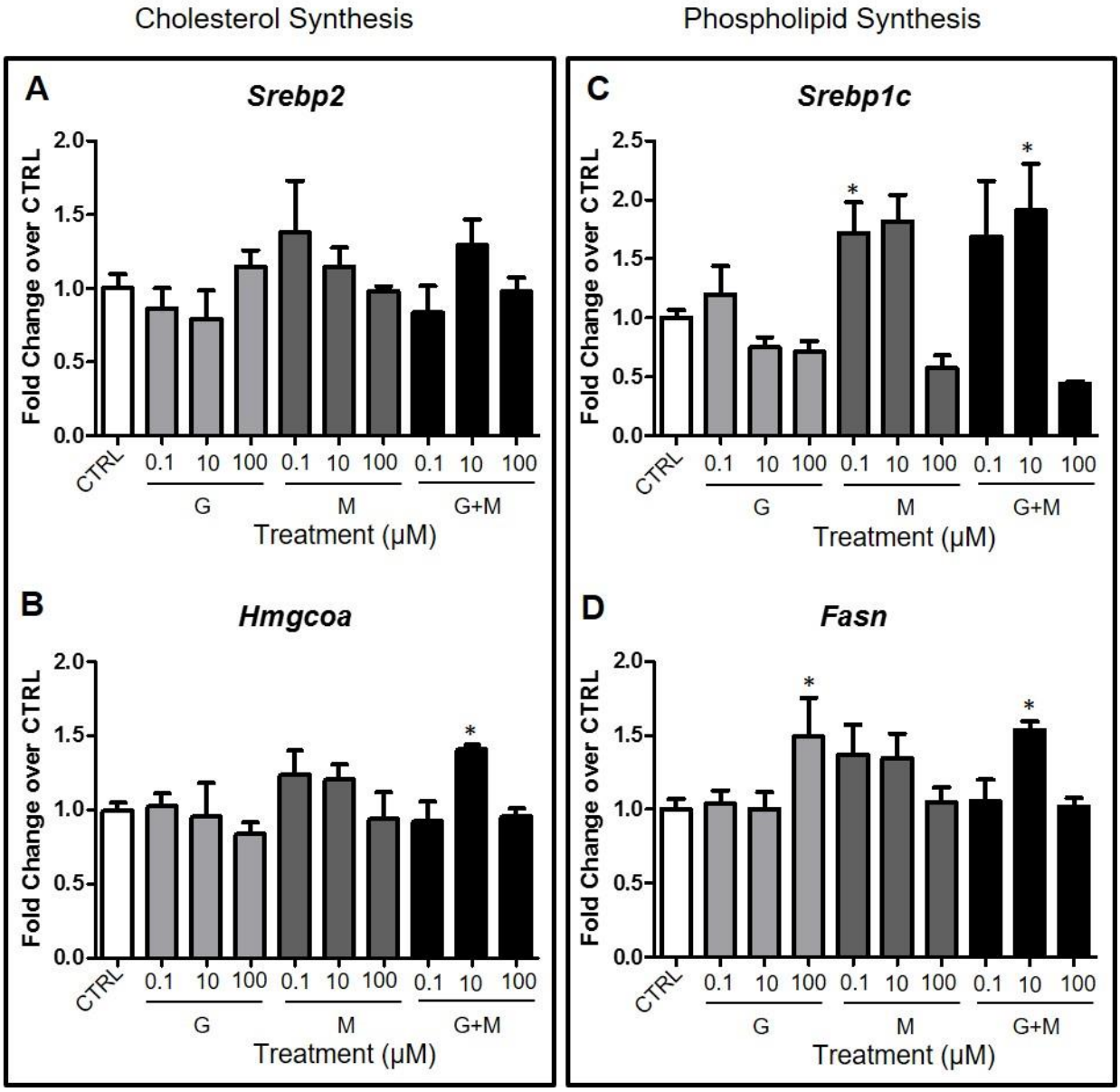
Cholesterol Synthesis
Phospholipid Synthesis
A
Srebp2
B
Hmgcoa
C
Srebp1c
D
Fasn
Fold Change over CTRL
CTRL
0.1
10
100
G
M
G+M
Treatment (μM)

Figure 4.8. Working model for combined 10µM GEN + MEHP induced steroid and lipid metabolism up-regulation

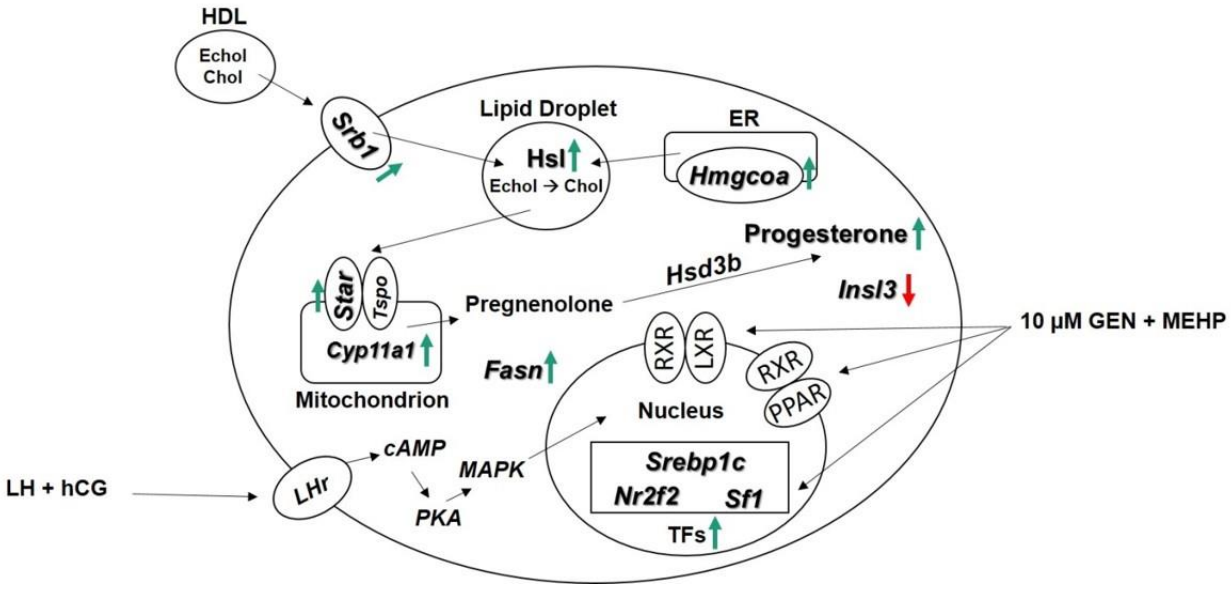

Table 4.1. Primer sets used for quantitative real-time PCR

GENE SYMBOL	ACCESSION NO.	FORWARD PRIMER (5' - 3')	REVERSE PRIMER (3' - 5')
3-hydroxy-3-methylglutaryl-Coenzyme A reductase (***Hmgcoa***)	NM_008255.2	tgccacactctgcactaaaga	ctgagaggtccgacttgctt
Cytochrome P450, family 11, subfamily a, polypeptide 1 (***Cyp11a1***)	NM_019779.3	cggagcagaattgaagttcaaaatctcc	ggttctcaggcatcaggatgag
Fatty acid synthase (***Fasn***)	NM_007988.3	caacatgggacaccctgag	gttgtggaagtgcaggttagg
Hormone senstive lipase (***Hsl***)	NM_010719.5	cagaaggcactaggcgtgatg	gcttgcgtccacttagttcca
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (***Hsd3b***)	NM_008293	tgttggtgcaggagaaagaactg	cgacctcctccttggtttctggtc
Insulin-like 3 (***Insl3***)	NM_013564.6	aagaagccccatcatgacct	tttatttagacttttgggacacagg
Mus musculus ovalbumin upstream promoter transcription factor II COUP-TFII (***Nr2f2***)	U07635.1	cctcaaagtgggcatgagac	tgggtaggctgggtaggag
Nuclear receptor subfamily 5, group A, member 1 (***Sf1***)	NM_139051.3	ctgggatatggggactagca	caccccgactcttggaaa
Scavenger receptor class B, member 1 (***Srb1***)	NM_016741.2	catggctcagagagtgactaca	gcacgaagggatcgtcatagcc
Steroidogenic acute regulatory protein (***Star***)	NM_011485.4	atctccttgacatttgggttcca	cggtctctatgaagaacttgtggac
Sterol regulatory element binding protein 2 (***Srebp2***)	NM_033218.1	acctagacctcgccaaaggt	gcacggataagcaggtttgt
Sterol regulatory element-bindingprotein-1c (***Srebp1c***)	AB046200.1	ttcctcagactgtaggcaaatct	agcctcagtttacccactcct
Translocator protein (***Tspo***)	NM_009775.4	cccgcttgctgtacccttacc	caccgcatacatagttgacgact
Tubulin alpha-1a (***Tuba1a***)	NM_011653	cggaaccagcttggacttctttccg	ggaactggctctggcttcacc

Chapter V

Summary and Discussion

The present thesis challenges the long running doctrine that assessing chemical safety in the context of high dose, single exposure animal and cell based models is an appropriate mean of determining environmental exposure risks. In contrast to specific accidental occupational or residential exposures to a well-defined single toxicant (e.g. drugs, lead, mercury, solvents, industrial intermediates in chemical reactions), environmental exposures usually involve mixtures of chemicals, which could potentially influence each other's toxicokinetics and effects. Because testing complex mixtures presents challenges in the interpretation of the results, especially in tissues comprising a variety of cell types with multiple functions, our approach was to use a simplified model, in which only two chemicals were used, alone or in combination, limiting exposure to a critical period of sensitivity for the male reproductive system, and doses for which we had previously found that each chemical used alone did not exert long term deleterious effects in testis. Although this is still a much simpler model than real human exposure, the study allowed us to reach important conclusions and identify sensitive markers of exposure in different testicular cell types. Results also highlight how short term studies may not be predictive of long term effects. The notion that these EDs do not follow classical dose-response effects with consistent mechanisms of toxicity from perinatal ages to adulthood further stresses the importance of assessing impacts across a range of doses during appropriate windows of exposure and at different ages. These are very pertinent concepts in the context of chemical risk assessment and the ability of regulatory agencies to determine safe exposure thresholds.

An established rodent model for in-utero exposure to EDs was employed [132, 144, 254]; pregnant dams were treated with environmentally relevant doses of GEN and DEHP, individually or in combination, from GD14 to parturition, encompassing the peak fetal androgen production period and paralleling the human masculinization window beginning at 8 weeks gestation. Given the critical importance of androgens in male reproductive development, we hypothesized that combined low dose exposure to GEN, a phytoestrogen with estrogenic activity, and DEHP, a previously reported anti-androgen, would pose a greater risk to male reproductive health than individual compounds.

Indeed, a seven day gestational treatment of GEN and DEHP alone was sufficient to induce significant reproductive abnormalities in adulthood at PND120. Testicular effects unique to our ED mixture included an increase in testicular weight, qualitative increase in interstitial fibrosis and correlated increase in mast cell and inflammatory gene markers (Fig. 5.1). Early spermatogonial germ cell markers and Sertoli cells markers were also suppressed, suggesting a potentially compromised pool of germ cell precursors and supportive somatic cells. These changes may reflect direct targeting of these cell types in early testis development, or result from the large number of Leydig cell - related genes compromised in adulthood.

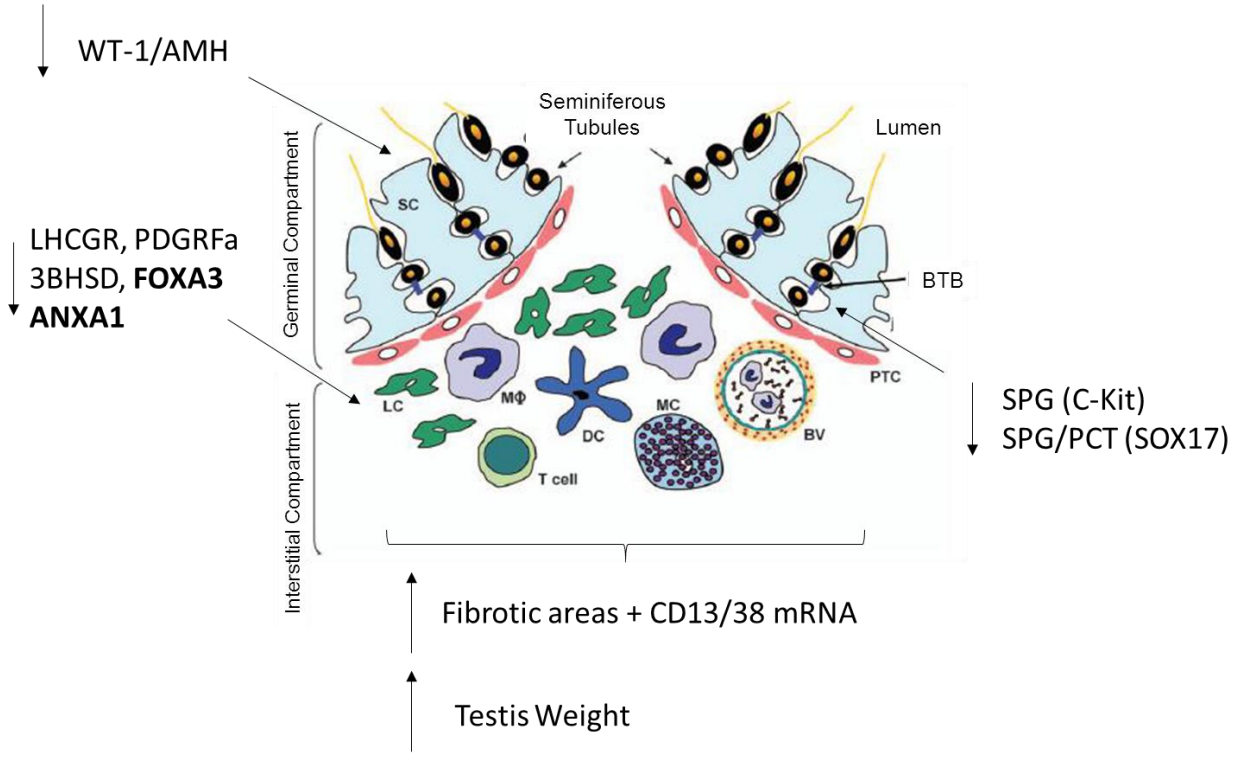

Figure 5.1. Effects of fetal exposure to combined GEN and DEHP on adult rat testis

Despite significant alterations in key somatic and germ cell markers and testis physiology, treated offspring, absolute sperm number and fertility were unaffected. We hypothesize that these changes may still sensitize the testis to subsequent environmental insult. A partially compromised system may jeopardize the ability to maintain homeostasis in response to stressors. This concept could be further evaluated in future experiments employing a "multiple hit" model, in which in-utero exposed male offspring would be re-exposed in adulthood to similar compounds or other established male reproductive toxicants. Although complex, such a model may more accurately mimic continuous or intermittent ED exposure from the time of conception through adulthood.

Interestingly, these alterations were found 120 days post treatment, reflecting permanent alterations in testis function and targeting of cell types not yet present during the exposure period. Our results suggest that either direct contact with GEN and DEHP or an altered testicular

environment may affect developmental pattern of somatic and germ cell precursors or progenitors. This notion was reinforced by subsequent analysis of newly identified adult Leydig target genes, *Anxa1* and *Foxa3* that were found to not be yet expressed in PND3 and PND6 testis (data not presented). Long term alterations in genes not expressed during the time of exposure may involve epigenetic mechanisms. Elucidation of these phenomena will be a goal of our laboratory going forward.

As a starting point, two critical early postnatal periods of testis development were characterized immediately following in-utero exposure, to evaluate fetal Leydig activity as well as the initiation of gonocyte proliferation/migration (PND3) and differentiation to form a critical pool of SSCs (PND6). We hypothesized that early alterations in the testicular environment and early cell types would correlate with long term toxicity. A series in-vivo, ex-vivo and in-vitro analyses however, demonstrated GEN and DEHP do not follow a classical dose response and involved different mechanisms of toxicity from perinatal ages to adulthood.

Paradoxically, low dose of DEHP alone had a stimulatory effect on PND3 androgen related processes, Sertoli cells, germ cells, and proliferation markers, an effect that was attenuated when combined with GEN. A mechanism was proposed by which GEN, through non-endocrine activity as an antioxidant, normalizes ROS-mediated and pro-androgenic effects of DEHP in early postnatal testis. These results suggest that an antioxidant devoid of endocrine disrupting properties may be beneficial in mitigating DEHP toxicity in fetal and neonatal periods. An interesting follow-up experiment would therefore be to add additional treatment groups containing an antioxidant (ex. Vitamin E) or free radical scavenger alone or in combination with DEHP.

These results allowed for an important distinction between early acute effects and late onset toxicity mediated by GEN, DEHP or combined GEN and DEHP. Despite GEN being protecting against DEHP - mediated alterations in early fetal and postnatal periods, combined GEN and DEHP was associated with the most significant testicular aberrations in adulthood. It is likely that GEN long-term reproductive toxicity may be related to its ability to act as an ED via mechanisms unrelated to its anti-oxidant properties.

Although there is extensive literature on individual exposure of both GEN and DEHP, to our knowledge this is the first study assessing their in utero effects in combination. Future studies could also include a full dose response of GEN and DEHP, individually or in combination. This would allow full toxicity evaluation across the range of human exposure. Evaluation of ultra-low GEN exposure would however require consideration of additional challenges; conventional rat chow, optimized for general health and reproductive function, is soy-based, providing several mg/kg day of phytoestrogens, including the glucuronidated form of genistein. True evaluation of GEN doses below 1 mg/kg bw would require a switching of maternal diet to casein - based rat chow, which in itself has been associated changes in male reproductive development and response to phytoestrogen supplementation [332, 333].

In both studies, fetal and adult Leydig cells, as well as their progenitors, were identified as potential targets of GEN and DEHP. Exemplified in the schematic of effects in adult testis (Fig.1) however, the ultimate phenotype is the result of complex interplay between multiple cell types, intra-testicular and circulating hormonal signals. To further investigate direct effects of GEN and DEHP independent of systemic endocrine effects and interactions with other testicular cell types, MA-10 murine Leydig cells were exposed in-vitro to varying concentrations of GEN and MEHP, the principle bioactive metabolite of DEHP. MA-10 Leydig cells are a clonal strain of hCG and EGF

responsive tumour cells. These cells, albeit with clear differences from normal rodent and human Leydig cells, are a useful and convenient model for Leydig cell function, steroidogenesis, regulation and response to external stimuli.

Using this model, we were able to evaluate a full dose response of GEN and MEHP effects on Leydig cell viability, steroid production, gene expression and lipid homeostasis, an endpoint logistically challenging to isolate in complex tissue and cell - based animal models. Paralleling short term in-vivo and organ culture models, low doses of EDs had a stimulatory effect on MA-10 basal Leydig steroid production and lipid homeostasis, an effect that was not observable with very low or high concentrations of GEN and MEHP.

Although 10 µM MEHP alone had a stimulatory effect on steroid mediator mRNA (*Star, Tspo, Cyp11a1 and Srb1*), it was only combined 10 µM GEN and MEHP that demonstrated a true increase in steroid production, correlated with gene expression changes as well as an upregulation of lipid levels, including cholesterol and several lipids known for their involvement as the source of diverse cell signaling processes [327-329]. MEHP effects were not attenuated by GEN as was observed in in-vivo and organ culture models. It is likely MEHP induced ROS - mediated upregulation of androgenic responses and interplay with GEN are dependent on the presence of multiple testicular cell types, including interstitial immune cells, endothelial and myoid cells and germ cells. Speaking to the complexity of ED effects on testicular cell types, Leydig cells alone have at least 26 identified nuclear receptors and transcription factors, many of which are "orphaned" with no known endogenous ligands [86]. Even with MA-10 results being derived from a closed in-vitro system, we can only suggest that the mechanism likely involves LXR agonism and upregulation of other transcription factors implicated in steroidogenesis and lipid homeostasis.

Still, we have demonstrated the ability of two common EDs to activate Leydig cells in-vitro, resulting in significant physiological and functional changes. Consistent across both in-vivo and in-vitro acute exposure models, low doses of EDs had a stimulatory effect below doses previously reported to suppress steroid production. There is likely merit in pursuing "in between" primary culture models that may better reflect in-vivo physiology. Crude preparations of interstitial Leydig and germ cells would allow for maintenance of interactions with neighboring cell types during toxicological assessments.

General Discussion

Taken together these studies warrant further investigation of EDs across a range of environmentally relevant doses, in different toxicological systems and across various windows of exposure. Historically, an emphasis has been placed not only on establishing a relationship between exposure and outcome, but also cause and effect, through accurate characterization of specific mechanisms of toxicity. The assumption has been that dose escalations will increase the frequency of effects observed at low doses and that biological mechanisms will be conserved.

The present study reinforces a growing body of evidence that hormonally active compounds do not follow a linear dose response for many toxicological endpoints. Effects at low doses often vary in magnitude or even oppose those observed at high doses. These non-monotonic or hormetic effects have been well documented in the context of classical endogenous endocrine systems. Physiological circulating levels of endogenous hormones are in parts per billion to parts per trillion ranges, often below environmental chemical exposures and certainly orders of magnitude below high dose ED toxicity models; the most significant anti-androgenic phthalate phenotypes have been reported in-vivo and in-vitro, at doses exceeding 300mg/kg/day and 100μM, respectively.

Endogenous hormones rely on high receptor specificity, rather than concentrations, to elicit their biological effects. It has been reported that, at any given time, fewer than 5% of hormone receptors are occupied. This system allows for relatively small increases in hormone levels to produce significant activity. Considering that dose response is not linear, and that a small increase in hormone levels can greatly increase the relative proportion of occupied receptors, flooding an already saturated system with high hormone levels has little effect on receptor occupancy. This kinetic contributes to non-linear and non-monotonic dose response curves for endocrine endpoints. Based on these principles, exogenous compounds capable mimicking, antagonizing or altering the effects of endogenous hormones likely follow similar rules and should be evaluated in the context of their endocrine activity.

The relatability of higher dose animal toxicity data has been a great challenge for regulatory agencies performing human health risk assessments for potentially harmful environmental exposures. Criticism has been placed on both the relevance of animal physiology and the doses used in the context human risk. At the same time, low dose effects have been heavily debated as they do not fit within classical pharmacological and toxicological frameworks. More recently, a growing body of evidence has sparked international workshops on the state of science of low dose effects and non-monotonic dose-response curves [331]. There is discussion about the incorporation of aforementioned endocrine principles into ED risk assessment [188, 189].

Equally important are context and windows of exposure with regards to ED toxicity. The endocrine properties of EDs are dependent on the presence of the receptors they're acting on. Dynamic expression of hormone receptors throughout different developmental windows is an additional critical regulator of hormone action. A relevant example is that while adult Leydig testosterone production is highly LH dependent, production of testosterone from fetal Leydig cells precedes

the expression of the LH receptor and is mediated by unknown mechanisms. Thus the action of EDs is also very much dependent on the periods of exposure and the target organs/systems examined.

In the context of male reproductive development and function, it is generally accepted that the fetal and neonatal period corresponds to a critical hormone dependent "masculinization" window. Whereas the development of the female fetus is largely hormone independent, fetal testosterone production is absolutely necessary for masculinization. The hormonal dependence and developmental intricacy of the fetal period in combination with immature defence mechanisms creates a vulnerability to EDs crossing the placenta. Paired with the developmental origins of disease hypothesis, early life exposure to EDs is thought to predispose males to reproductive abnormalities observable later in life.

An additional layer of complexity is that while humans are exposed to a myriad of EDs, other environmental chemicals and pharmaceuticals from the time of conception through adulthood, risks are generally assessed in the context of single chemical exposures. Relatively few studies have attempted to discern the potential additive, synergistic or antagonistic effects of chemical mixtures. A major deterrent of mixture studies has been that precise characterization of toxicological mechanisms is often difficult or perhaps impossible. Perhaps in that regard, toxicological research should be phenotype and endpoint driven instead of being focused on elegant characterization of cellular and molecular pathways, as is often required by peer reviewed journals. Independent of knowing how a group of compounds act, it may be sufficient to flag a group of compounds as risky for human health.

From a regulatory perspective, it is also challenging to implement interventions and new legislations when the true culprits in a complex mixture may be unknown. A hierarchical approach may be more appropriate, conducting initial toxicological assessments with environmentally relevant ED mixtures, and sequentially designing follow-up experiments to zero in on specific classes of pollutants. This approach was successfully applied in the laboratory of Dr Daniel Cyr at INRS Institut Armand-Frappier, which employs a diverse array of toxicological models spanning multiple species; environmental xenoestrogens were thought to be implicated the occurrence of sexually ambiguous fish in the St Lawrence river [334, 335]. An approach was taken to first reproduce the effect using different percentages of crude sewage effluent, followed by repeat experiments with column separated and fractionated effluent. Although initially a daunting task, these experiments, along with translational rodent fish feeding models [334, 335], ultimately led to the identification of an independently risky class of EDs, industrial alkylphenols that were the subject of subsequent individual toxicity evaluation [288, 336, 337].

It is not possible for all classes of EDs to be assessed in a similar ecological context. There is likely merit however in employing a similar hierarchical approach to toxicity testing, even if beginning with a simple mixture of related or unrelated compounds more accurately reflecting human exposure. We have taken a more holistic approach to identifying risks to early life male reproductive development and long-term reproductive function. As a proof of concept, we employed multiple toxicological models to demonstrate the ability of two purported male reproductive toxicants to act in an additive or synergistic manner, below previously reported thresholds of toxicity, and involving different mechanisms of toxicity from perinatal ages through adulthood. Our design had the additional benefits of differentiating individual effects from combination, acute vs chronic toxicity and directs effects from systemic or multi-cell mechanisms.

Several vulnerable cell types were identified in perinatal and adult periods, along with potential mechanisms of toxicity. This work has generated numerous hypotheses that will be the subject of future experiments.

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