Investigating the role of environmental estrogens in lower urinary tract dysfunction

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Abstract

Benign prostate hyperplasia (BPH) is an age associated prostate gland enlargement that is closely associated with lower urinary tract symptoms (LUTS) which can cause problems with urine storage, voiding, or both. If left untreated, BPH/LUTS can cause urinary retention, renal impairment, and ultimately death. In humans, BPH/LUTS occurs in 50% of men over the age of 50 and 50% of men with histologic BPH demonstrate LUTS by age 90. While the molecular mechanisms associated with BPH/LUTS are poorly understood, steroid hormones such as Testosterone (T) and 17β-estradiol (E_2) have been implicated as key players. Human exposure to environmental estrogens, weak estrogenic compounds that are structurally similar to E_2, is widespread, but the urologic risks are largely unexplored. We found that the environmental estrogen, bisphenol-A (BPA), administered in combination with T can alter the voiding behavior of adult male mice. Additionally, by employing the use of a rodent model that recapitulates endpoints associated with BPH/LUTS, we determined that bisphenol analogs can also induce BPH/LUTS in adult male mice. Finally, we evaluated the contributions of the estrogen receptor subtypes alpha (ERα) and beta (ERβ) using *Esr1*$^{-/-}$ and *Esr2*$^{-/-}$ mice and we determined that ERα is a key mediator in BPA induced BPH/LUTS after two months of treatment. Moving forward, further characterization of rodent models associated with other aspects of the estrogen signaling pathway may provide insightful answers to explore the underlying factors that drive environmental estrogen induced lower urinary dysfunction and could lead to improved therapies for patients.

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Abbreviations

BPA, bisphenol-A

BPF, bisphenol-F

BPH, Benign Prostate Hyperplasia

BPS, bisphenol-S

CHO, Cholesterol

DHT, Dihydrotestosterone

E_2, 17β-estradiol

ER, Estrogen receptor

ERα, Estrogen receptor alpha

ERβ, Estrogen receptor beta

Esr1, Estrogen receptor 1 gene

Esr2, Estrogen receptor 2 gene

LUTD, Lower Urinary Tract Dysfunction

LUTS, Lower Urinary Tract Symptoms

SERMs, Selective estrogen receptor modulators

T, Testosterone

UNT, Untreated

Chapter 1: Introduction

Sections of this chapter have been adapted from the following publications:

Ricke WA, Williams K, **Wynder J**, Palapattu G, Wang Y, et al. The Molecular Basis of Human Cancer. 2 ed. Coleman WB, Tsongalis GJ, editors. New York: Springer; 2017. Chapter 20, Hormonal Carcinogenesis: The Role of Estrogens; p.307-322. 873p. ISBN 978-1-59745-458-2.

Wynder JL, Nicholson TM, DeFranco DB, Ricke WA. Estrogens and Male Lower Urinary Tract Dysfunction. Current urology reports. 2015;16(9):534. PMID: 26156791

1.1 Abstract

Benign prostate hyperplasia (BPH) and associated lower urinary tract symptoms (LUTS) are common clinical problems in urology and they affect the majority of men at some time during their lives. The development of BPH/LUTS is associated with increased ratio of estrogen to androgen levels, and this ratio, when mimicked in a variety of animals, induces BPH and lower urinary tract dysfunction (LUTD). While the precise molecular etiology remains unclear, estrogens have been implicated in the development and maintenance of BPH. Numerous endogenous and exogenous estrogens exist in humans. These estrogens act via multiple estrogen receptors to promote or inhibit prostatic hyperplasia and other BPH-associated processes. The prostate is an estrogen target tissue, and estrogens directly and indirectly affect growth and differentiation of the prostate. The precise role of estrogen action directly affecting prostate growth and differentiation in the context of BPH is an understudied area and remains to be elucidated. Estrogens and selective estrogen receptor modulators (SERMs) have been shown to promote or inhibit prostate proliferation illustrating their potential roles in the development of BPH as therapy. More work will be required to identify estrogen signaling pathways associated with LUTD in order to develop more efficacious drugs for BPH treatment and prevention.

1.2 Benign prostate hyperplasia and lower urinary tract symptoms

Benign prostatic hyperplasia (BPH) and the bothersome lower urinary tract symptoms (LUTS) that accompany it are some of the oldest clinical entities in urology. With advancing age, virtually all men will develop histologic evidence of BPH. A meta-analysis of autopsy studies demonstrated the ubiquity of BPH histology: while the prevalence in men 51-60 years old is 42+/-9.7%, this increases steadily with advancing decades to 88 +/- 10.9% in men 81-90 years (8). LUTS are also common in men and typically worsen with age. Among men older than 65 years, one third with no or mild symptoms will develop clinical significant LUTS within 2 years (97). For those men who already have moderate to severe symptoms, one quarter will experience symptom progression (97). BPH-LUTS have a major impact on the lives of men and their families and represent a significant financial burden on the healthcare system, with direct costs estimated at 3.9 billion dollars annually (115).

Classically, BPH is thought of as new glandular and stromal growth in the transition zone, which leads to benign enlargement of the prostate and causes bladder outlet obstruction. As such, LUTS result due to obstruction from the enlarged prostate. There is increasing recognition that age-related declines in detrusor function, neurologic control of micturition and other unknown factors also contribute to bladder outlet obstruction (BOO) and LUTS, perhaps independently of the prostatic hyperplasia (56). Moreover, common comorbidities of BPH, including obesity, hypertension, diabetes and the metabolic syndrome, may also adversely affect voiding function (42, 116). Adding layers of complexity, there is likely a spectrum of BPH-LUTS disease, and possibly distinct phenotypic and/or molecular subtypes of BPH. While not all male LUTS occur in the setting of BPH, benign enlargement of the prostate remains the most common cause of bladder outlet obstruction in men.

1.3 Clinical manifestations and current standards of care for BPH/LUTS

For most of the 20th century the common treatments for LUTS associated with BPH was surgical intervention through resection or enucleation of the prostate (69). In the 1980s the development of therapies to shrink the prostate, relax the prostate, and/or relax the bladder became the standard of care.

These drugs include α-blockers(95), 5-α reductase inhibitors (5-ARIs)(11, 43), and anticholinergics respectively. New age therapies that are currently in use or being proposed include combination therapy, antimuscarinics such as phosphodiesterase-5-inhibitors (PDE5)(95),and selective estrogen receptor modulators (140).

Diagnosis of BPH/LUTS include evaluation of medical history to gauge the symptom severity using a questionnaire referred to as the International Prostate Symptom Score index (IPSS). Watchful waiting is the standard of care for patients who received a score of 0-7 which is indicative of mild symptoms. Urologic evaluation and ultimately medical intervention is sought for patients who demonstrate moderate, IPSS scores 8-19, to severe symptoms, IPSS scores 20-35. Physical examinations include measuring the size of the prostate and checking for prostate cancer by performing a digital rectal exam while other examinations include urinalysis to screen for urinary tract infections and bladder cancer. Urodynamic functional tests are used to evaluate bladder capacity by measuring bladder function through the use of noninvasive urine flow rates(uroflowmetry), post void residual measurement (PVR) measurements, pressure-flow studies, and filling Cystometry (69). In extreme cases renal or transrectal ultrasound (TRUS) are performed by the urologist (128). Many of these functional tests provide valuable information in regard to urine storage and voiding events.

1.4 Implication of steroid hormones in BPH/LUTS

The importance of androgens, which act via androgen receptors, in prostate development and growth are well established (27). Additionally, 5α-reductase inhibitors have been used as a therapy to decrease prostate volume, improve symptoms, and increase urinary flow rates in men with LUTS (59, 80). However, complete androgen deprivation does not result in symptomatic improvement for all men (80). In fact, men castrated before puberty and men with low serum androgen driven by hypopituitarism do not develop BPH (14, 31). Furthermore, as men age serum testosterone levels decrease steadily after age 39 (13) while estrogen levels remain steady resulting in a change in the androgen: estrogen ratio (131). The androgen/estrogen ratio is well established in prostate development and disease, but the molecular

mechanisms are poorly understood (6, 7, 89). Estrogens are critical steroid hormones that target a variety of reproductive organs including the prostate. However, the role of estrogen/estrogen receptor (ER) signaling in the prostate is poorly understood. Steroid hormones including estrogen are derived from cholesterol. Cholesterol is subsequently converted to pregnenolone, progesterone, and then to testosterone (T) via several different enzymes. Testosterone is converted to estradiol by the P450 aromatase/CYP19 gene product. Therefore, aromatase is a key controller of the ratio of androgen to estradiol. This could be a critical key in BPH because as men age the ratio of serum E_2:T increases. Given that aromatase is expressed in the prostate, it is highly possible that local estradiol production could play an important role in prostatic function (37, 38). Another source of estrogen in men is the adrenal glands which produce androgen that can be converted to estrogens via the action of aromatase.

1.5 Modeling BPH

There has been substantial progress in the medical and surgical management of BPH in the past century, but the underlying molecular etiology of this disease remains elusive. Our understanding of the critical and likely permissive role of androgens in the prostate has revolutionized the clinical approach to BPH. With contemporary medical management, including the use of 5-α reductase inhibitors (5ARI) [to block the production of the potent androgen receptor ligand dihydrotestosterone (DHT) from testosterone (T)], for most men, BPH-LUTS is a chronic disease that can be managed medically. However, not all men with LUTS tolerate or improve with medical management, and some will progress despite treatment. In pioneering experiments, Walsh and Wilson demonstrated that estradiol (E_2) acts in synergy with the DHT metabolite androstanediol to induce BPH in dogs (135). Forty years of subsequent studies have confirmed the importance of estrogens in the development of BPH-LUTS, but the molecular basis for estrogen action remains unexplained. In part, detailed mechanistic studies for estrogen targets in BPH-LUTS have been limited by a lack of models that are both suitable for interrogating mechanisms and recreating the clinical aspects of BPH-LUTS. We recently developed a mouse model induced with a combination of testosterone (T) and E_2 to mimic the increased estrogen to androgen ratio that develops with age in men.

This model exhibits many clinical features of BPH, including urinary voiding dysfunction, bladder enlargement, new glandular prostatic growth, and BOO (86). While this model is a logical extension of past BPH models, it utilizes a genetically tractable organism and offers a useful tool for investigating the underlying biology in BPH and is useful for testing novel treatment strategies. Since the publication of this work, several groups have presented different models for BPH. Cadmium, which may act via steroid hormone receptor, induces a BPH-like state in the rat prostate (103). Vignozzi et all. demonstrated that rabbits fed a high-fat diet to induce metabolic syndrome developed an increased E_2:T ratio and lower urinary tract fibrosis, which is improved by exogenous T therapy (132). Another mouse model of lower urinary tract dysfunction (LUTD) is the accelerated aging SAMP6 mouse fed a high-fat diet. These mice are diabetic and obese and, with time, developed LUTD (42). Along with the male mouse treated with T+E_2, these models are important tools for understanding the underlying biology of BPH, and for developing future treatment strategies.

1.6 A contemporary perspective on BPH/LUTS

While estrogens may be important in BPH-LUTS, there is growing evidence that BPH-LUTS is a lower urinary tract manifestation of systemic metabolic derangement, which includes obesity, metabolic syndrome, and diabetes. In a recent epidemiologic study of 780 men, plasma E_2 predicted progression of both storage and voiding symptoms as assessed by the American Urological Association Symptom Index (78). A recent BPH case-control study showed that a gene polymorphism in the steroidogenic enzyme CYP17, which metabolizes and inactivates an estrogenic compound (3β-adiol), was associated with BPH (66). Greater abdominal fat and sleep apnea risk predicted progression of storage of LUTS, underscoring the interaction of obesity with BPH-LUTS (78). Obesity is a common comorbidity of BPH and is also an independent predictor of progression of LUTS (79). Recent evidence shows that leptin, a hormone produced by adipocytes, stimulates proliferation of human BPH cells, and that this effect may be partially mediated by the direct effect of leptin on estrogen metabolism, as leptin induces aromatase expression (45). This provides yet another link of estrogens to BPH, suggesting that leptin might also be

therapeutically targeted, perhaps with beneficial effects on obesity as well. Greater physical activity at baseline predicts improvement in storage and voiding symptoms in men, suggesting that lifestyle interventions might be a promising future strategy for BPH-LUTS and obesity (78). The interaction of obesity, estrogens, and lower urinary tract dysfunction is an important area for future study.

More broadly, estrogens are important in several normal and pathologic processes in men. Estrogen deficiency, in addition to androgen deficiency, is an important contributor to age-related hypogonadism (declining T with age). Androgens regulate lean mass, muscle size, and strength; estrogen deficiency leads to fat accumulation and sexual function is regulated by both androgens and estrogens (76). An important question for future research is which estrogen receptor (ER) mediates estrogen action in the maintenance of sexual function or adiposity.

1.7 Estrogens and Estrogen receptors

Estrogens are a broad term for compounds that activate receptors (i.e., the estrogen receptors [ERs]) for the major physiologic estrogen in higher organisms, 17β-estradiol (E_2). However, in addition to E_2 there are numerous estrogens found in the body (**Fig. 1-1**) including those that are endogenously produced, as well as those acquired from external sources. Estrogens from outside the body (xenoestrogens) can be derived from plant sources (e.g., phytoestrogens) or synthetic such as many endocrine disruptors or environmental estrogens whose effects on human health have been widely debated (see below). Furthermore, a number of compounds can act either as agonists or antagonists for ERs depending upon the cell and tissue context. These selective ER modulators (SERMs) function by triggering unique conformations of ERs and thereby influence their interactions with specific partners including transcriptional coregulator proteins. Estrogens and SERMs have been shown to enhance or inhibit prostate proliferation and therefore could contribute to the pathogenesis of BPH (87).

1.8 Environmental Estrogens

Estrogens can exhibit tumor promoter and/or suppressor activities, but their actions vary with the dose and the type of estrogenic agent used (138). ERs have a reasonable high affinity for environmental

estrogens such as bisphenol A (BPA), phthalates, pesticides, and polycyclic aromatic hydrocarbons (48). These environmental estrogens are also known as endocrine disruptors because they can mimic and/or interfere with estrogen signaling in ER responsive tissues and organs. Bisphenol-A (BPA) has emerged as one of the most important and controversial environmental chemicals. It is well recognized as an endocrine disruptor, which is a substance with hormone-like action, or that affects the activity of naturally occurring hormones in the body. In 2010, Canada was the first country to formally designate BPA as a toxin. The European Union prohibited the use of BPA in baby bottles in 2011, and the U.S. Food and Drug Administration (FDA) followed suit in July 201. Despite these restrictions, BPA continues to be used in food packaging (71), certain plastics (134), and thermal ink paper products(39, 51). The urinary and reproductive tract, and particularly the prostate, is acutely sensitive to BPA exposure during development (104). Due to its estrogenic activity and ubiquitous exposure, BPA is likely an etiologic factor in BPH-LUTS, but more robust clinical and epidemiological studies are needed to establish this link (51, 102). Understanding the role of BPA, as well as other estrogens, in the role of prostate diseases, may lead to regulatory changes regarding endocrine disruptor exposure and/or new treatment strategies targeting estrogen action.

While BPA is perhaps the most visible and well documented environmental estrogen, other endocrine disruptors may influence BPH-LUTS given their widespread use in consumer products and reported estrogenic activity. Bittner and colleagues recently reported estrogenic activity in various products marketed to consumers as BPA-free (9). A number of these "BPA-free" products contain compounds that have tested positive for estrus response (28, 112), share structural similarities (29, 30), and physiochemical properties (18, 39) with the endocrine disruptor BPA (**Table 1-1**). Perhaps, the most significant structural component conserved across many bisphenol analogues is the location of the two hydroxyl groups in the *para* position on both aromatic rings which allows for hydrogen bonding and causes the aromatic ring to be more reactive. The spacing of the phenolic rings along with the presence of an aliphatic spacer has been proposed as a structural feature that may enable environmental estrogens to mimic the actions of E_2, an endogenous hormone, by binding to ligand regulated transcription factors such

as estrogen receptors and interfering with normal estrogen signaling (4).While the usage for many of these BPA analogues are largely undocumented, the production of these compounds are on the rise with approximately 16 BPA analogues having documented industrial application (18). Many of these BPA analogues, bisphenol-S and bisphenol-F, have a variety of reported toxic effects (3, 20, 44, 63, 93, 112, 141) that are comparable to BPA. Despite these well documented adverse effects the environmental fate of many of these bisphenol analogues remain unclear. One study demonstrated that BPF and BPS displayed estrogenic activity and altered Testosterone (T) secretion by human fetal testes similar to BPA (36). Equally important, patients with BPH have higher levels of several endocrine disrupting organochloride pesticides, including pp-DDE and endosulfan-α compared to health age-matched controls (66). The potential risks to human health from BPA analogues, and many other environmental endocrine disruptors, are largely unknown. BPA can bind to several kinds of receptors including the ER, AR, and aryl hydrocarbon receptor (82). BPA has also been identified as a G protein-coupled estrogen (GPER) agonist which suggests that BPA can signal through a non-genomic pathway to mobilize cellular responses to estrogen without initially affecting transcriptional responses of ERs (105). BPA exposure has also been found to lower serum PSA levels in prostate cancer patients based on an assessment of urinary BPA levels. Bisphenol-S, Tritan, polyethersulfone (PES), and polyethylene terephthalate (PET) were proposed as safer BPA alternatives (58). Recent studies have stressed the importance of further analysis of these BPA analogues, as well as their metabolites, based on the estrogenic potency associated with exposure to these compounds and the fact that they are ubiquitous in our environment (36, 47, 58, 113). The physiochemical properties presented in **Table 1-1**, calculated using the U.S. Environmental Protection Agency's (EPA) Estimation Program Interface (EPI) Suite Version 4.11, indicate that these BPA analogues are water soluble (BPF>BPS>BPA) given their low octanol water partition coefficient (K_{ow}) values they have low bioaccumulation potential (18, 39, 46). However, BPS is considered by some an unacceptable BPA replacement because of its persistence in the environment compared to BPA (51). In rodent studies, BPA-treated mice had a higher plasma E_2/T ratio which could be correlated with prostate disease (15). Equally important BPA has been shown to increase aromatase mRNA and protein

levels in rodent prostates (15). Phthalates have also been shown to have weak estrogenic activity and urinary excretions of metabolites are associated with adverse effects in humans. For example, phytoestrogens derived from grains are presumed to have beneficial effects based on epidemiological studies. Phytoestrogens commonly act via the beta isoform of ERβ to elicit their effects (114). Hence, ERβ-agonists may prove useful in the treatment of prostatic diseases.

1.9 Estrogen Receptors

For decades, ERα, a 66-kDa member of the nuclear receptor superfamily, was assumed to be the only ER (116) until 1995 when a second receptor for estrogen (i.e., ERβ) was discovered (89). ERs (ERα and ERβ) have highly conserved sequence homology in their central DNA-binding domains (DBD) with less sequence conservation in their C-terminal ligand-binding domain (LBD). The NH_2-terminal domains between the two receptors are variable. ERα and ERβ have similar affinities for E_2 and they bind the same DNA response elements (48). More recent genomic data shows that ERα and ERβ can share many commonly regulated genes in a specific cell type but also regulate unique sets of target genes depending upon the ligand used to activate the receptor and the cell or tissue type (75).

ERs are expressed in the male lower urinary tract and reproductive tissues such as the bladder (23), prostate (23), urethra, and testis (85) but the cellular distribution between ER subtypes in estrogen target organs are understudied. ERα and ERβ are expressed in the bladders and prostates of humans and rabbits (23, 132). The use of ER knockout (ERKO) mice null for either ER-α or ER-β has been helpful in elucidating the roles of these receptors in the prostate and other estrogen target organs (25). In general, the prostate develops normally in estrogen receptor-α knockout (αERKO) mice, and the prostates of adult αERKO mice are basically normal suggesting that ER-α has a minor role in normal prostatic development and growth (26, 94). In mice that lack ERα and ERβ (αβERKO), both sexes are infertile, and the males display disrupted mating behavior (24, 34, 64). More recently, it has been demonstrated that a subtle, but perhaps important prostatic phenotype exists in αERKO mice. These mice contain fewer ductal branches, and individual ducts appear to be larger in size (94). In prostate cancer and BPH, ER-α appears to be

essential, because αERKO mice do not undergo disease progression (85, 109). Estrogen receptor-β was initially cloned from the prostate and was subsequently shown to be highly expressed in adult prostatic epithelium. Subsequent studies have focused on determining the function of ER-β in the prostate. Surprisingly, prostates of ER-β knockout mice are also normal. It was reported that aged prostates from βERKO mice exhibited basal epithelial cell hyperplasia and reduced apoptosis (53). However, these observations could not be verified by other laboratories (34). Additionally, αERKO are reportedly insensitive to the activating effects of exogenous estradiol or androgen treatment (24, 91, 92). One area in which use of αERKO and βERKO mice has been informative regarding estrogen action in the prostate concerns the induction of squamous metaplasia of prostate. A well-recognized effect on the prostate of chronic exposure to estrogens is squamous metaplasia of the epithelium (111). Squamous metaplasia of the prostate is a benign condition and is the consequence of estrogen induction of proliferation and squamous differentiation in prostatic basal epithelial cells. Studies using wild-type (wt) and αERKO and βERKO mice indicate that estrogen induction of prostatic squamous metaplasia requires estrogen signaling through ER-α but not ER-β (110). For example, long-term treatment of wt and βERKO mice with diethylstilbestrol (DES) elicited pronounced prostatic squamous metaplasia, while similar treatment of αERKO mice had no such effect.

Classical estrogen signaling results from binding of an estrogen to an ER, dimerization of the ligand-bound receptors, followed by its association with target genes and recruitment of transcriptional coregulator proteins. The ensuing recruitment of many components of the transcription machinery, chromatin remodelers, and histone modifiers triggered by gene-specific binding of ERs impacts the efficiency of transcription from linked promoters (**Fig. 1-1**). In membrane-initiated classical estrogen signaling, estrogen receptors can translocate to the plasma membrane where the receptors are tethered to the cytoplasmic face of the bilayer (16, 62, 99, 108). While the exact nature of this signaling mechanism remains controversial, it has been proposed that estrogen can bind to membrane ERα outside of the nucleus and reportedly collaborate with nuclear ERα to modulate transcription (98, 101). More specifically, membrane ERα can act as a G-protein coupled receptor by inducing rapid activation of

signaling transduction pathways that can impact genomic and non-genomic functions (65, 99, 101). This rapid signaling can either activate or inhibit nuclear ER function based on the cofactors that are recruited (10). Transgenic rodent models were generated to help researchers understand the role of membrane and nuclear ERα in development. Nuclear-only ERα (NOER) mice, which have a point mutation (C451A) to prevent palmitoylation and membrane trafficking of ERα, were used to determine the importance of nuclear only ERα in development (100). NOER mice show loss of membrane ERα signaling and they develop abnormalities such as infertility, hyperplastic organs, and abnormal hormone regulation (100). Heterozygous mating of NOER male and females was found to rescue the infertility phenotype in NOER mice (100). Membrane only domain of ERα (MOER) were generated to determine the role of nuclear and cytoplasmic ERα. MOER mice express the ligand binding domain of ERα at the plasma membrane, display infertility, and adiposity (98, 101). Use of these two mouse models to probe the role of the membrane initiated signaling pathway in BPH/LUTS holds considerable potential.

Non-classical estrogen signaling occurs when ER dimers indirectly regulate transcription through interactions with other transcription factors such as AP1 and SP1, bind to their own response elements but utilize the tethered ligand-bound to impart hormone-regulated transcription upon a subset of their direct target genes ultimately utilizing mechanisms outlined above. The significance of non-classical estrogen signaling is of physiological significance given the fact that nonclassical target genes can be stimulated by ER antagonists which suggests that selective estrogen receptor modulators (SERMs) could act as agonists in this pathway (55, 96, 137). While sequences within the ER DNA binding domain (DBD) appear to be necessary there is little to no evidence of ER binding to DNA (55, 83, 125). The nonclassical ERα knock-in mouse (NERKI) mouse model is used to investigate the nonclassical ERα signaling because of a mutation in mouse ERα (E207A/G208A) which abolishes ERE binding and activation of ERE reporter genes while preserving the nonclassical estrogen signaling pathway (22, 55). NERKI females are infertile and they display distinct differences in estrogen target tissues (55, 126). This phenotype is similar to the effects of SERMs used for clinical therapy and provides evidence that these distinct differences could have arose from varied contributions of classical vs non-classical estrogen signaling in target tissues

(126). However, it is important to note that NERKI male mice displayed delayed disruptions in testis and duct morphology and they are infertile because of defects in male mating behavior (81). Estrogen-nonresponsive ERα knock-in (ENERKI) mice were also used to help differentiate between the ligand induced and ligand independent actions of ERα in an *in vivo* model (121). ENERKI mice were generated using a knock-in of the *Esr1* gene with a mutation in the ligand binding pocket of ERα to disrupt the ability of ERα to bind estradiol thus eliminating the possibly of classical estrogen signaling to occur (121, 122). ENERKI females are infertile but ENERKI male mice are sub fertile (121). In contrast to ERαKO and NERKI mice, ENERKI male mice do not display disruption of the testis or ducts (121). Overall, these rodent models highlight the significance of estrogen signaling in development and they provide a means to study the estrogen signaling pathway. Irrespective of the abnormalities of these mouse models, use of these nonclassical and classical estrogen signaling rodent models hold great potential to help determine the mechanisms associated with estrogen and environmental estrogen driven BPH/LUTS.

1.10 Non-genomic ER signaling and orphan receptors

Non-genomic estrogen signaling involves rapid (i.e., within minutes), transcription-independent cellular responses including increased levels of intracellular calcium levels, production of cAMP, epidermal growth factor receptor (EGFR)-phosphorylation, and activation of MAPK/PI3K pathways (35). Multiple studies have suggested the possibility that a specific 7-transmembrane G protein-coupled receptor GPER (aka:GPR30) is involved in one form of non-genomic estrogen signaling (40) but this is still a highly controversial topic (68). GPER is expressed in the human brain, prostate, bladder, urethra and various other tissues (17, 106). Currently, GPER function is implicated in reproductive, endocrine, urinary, nervous, immune, musculoskeletal, and cardiovascular systems (40, 105). A generally accepted mechanism of non-genomic estrogen signaling includes E_2 signaling through GPER to cause transactivation of EGFR after the protease-mediated release of tethered ligands [e.g., TGFα or heparin-bound EGF (HB-EGF)] promoting signal transduction activation of Erk -1/2 and MAPK pathways. Others have suggested GPER regulation via ER-36, a 36-KDA cell membrane associated form of ERα

(72). Finally, there is considerable evidence for a non-genomic role for intact ERs that localize to the plasma membrane (70, 139). The role of the non-genomic ER signaling in BPH/LUTS is largely unknown. Genetic ablation of the endogenous estrogen receptors using rodent models could provide a means to determine the role of non-genomic signaling in the etiology of BPH/LUTS. Several mouse models with ablation of GPR30 have been generated (54, 60, 77, 119) but these mice exhibit adiposity and age-related glucose intolerance in both male and females (118, 120).

Estrogen receptor-related receptors (ERRs) belong to the nuclear receptor subfamily III and are classified as "orphan nuclear receptors". There are three ERR isoforms, ERRα, ERRβ, and ERRγ, and they share similar homology with ERs, but they do not bind to estrogens or physiologically relevant ligands. A generally accepted mechanism derived from *in vitro* studies suggest that ERRs are constitutively transactivated in ERR target cell lines, bind to the EREs, and compete with ER, or other nuclear receptors, for the same coactivators (21). It has been proposed that ERRs can modulate or crosstalk with the classical estrogen signaling pathway (49). ERRs are expressed in estrogen sensitive tissues such as the prostate (21), bladder (67), and the brain (129). Estrogen-related receptor-α (ERRα) is the most abundant subtype of among ERRs (129) and aromatase, a key enzyme in estrogen in biosynthesis, is a direct target gene of this receptor (90). ERRα and estrogen-related-γ (ERRγ) are reportedly responsible for regulating cellular metabolism and they target a common set of gene promoters related to muscle contraction (33). *ERRα* knockout mice have smaller hearts and decreased mitochondrial levels but they display normal development under unstressed conditions (52, 67). *ERRγ* knockout mice display 100% neonatal lethality and cardiac defects after 48h (1, 136). Mice lacking cardiac *ERRγ* and ERR*α* display normal development and cardiac function while mice lacking both *ERRγ* and *ERRα* die from heart failure within the first month of life (136). ERRβ has been proposed to have an inhibitory effect on estrogen dependent cell function because it reduced the mobility of ERα in a live-cell imaging assay (129). However, ablation of the ERRβ gene is associated with fetal lethality and impaired placentation (73, 129, 130) thus future *in vivo* studies would require conditional expression of ERRβ. While there is a large amount of evidence pertaining to the role of ERRs in many estrogen driven disease processes, the

function of ERRs in estrogen sensitive tissues such as the prostate and urogenital tract is an understudied area. *In vitro* studies using four human immortalized epithelial prostatic cell lines and seven cancer cell lines suggests that ERRs and ERs control the overlapping regulatory pathways in prostate cell lines (21). More specifically, ERβ displayed similar expression patterns as ERRα and ERα exhibited similar expression patterns as that of ERRγ (21). This is particularly intriguing given the opposing roles of ERs in prostate disease, but these similarities could be a direct result of competition from DNA binding sites and cofactors. Future studies aimed towards probing the crosstalk between these co-expressed nuclear receptors which differ in their ligand dependence but somehow still share regulatory pathways are required. Furthermore, probing the role of ERRα in BPH/LUTS holds considerable potential given the fact that it reportedly regulates genes related to muscle contraction and enzyme aromatase is a direct target gene of this receptor.

1.11 ER Action in BPH-LUTS

ERα and ERβ are detected in both the prostatic stroma and epithelium of BPH and normal prostate tissues. We previously investigated the tissue-based expression of ERα and AR in human BPH (88). AR expression increased in BPH compared to normal prostate. ERα expression was also distinct in human BPH compared to normal prostate, with epithelial ERα increased and stromal ERα decreased. Moreover, cells expressing both receptors were more prevalent in human BPH, underscoring the increased hormone sensitivity of BPH. This work is important in establishing the relevance of investigating markers of interest in the human disease process prior to embarking on mechanistic studies with BPH-LUTD models. Using the male mouse treated with T+E_2, we demonstrated that ERα, but not ERβ is necessary for the development of urinary retention and bladder hypertrophy, common bladder complications of BPH (85). In addition, ERα but not ERβ, was necessary for hormonal induction of prostate growth in male mice treated with T+E_2.

Many questions remain regarding ERα action in BPH. For example, is stromal or epithelial ERα required for induction of BPH-LUTS in the mouse model? Using the conditional ERαKO mice with

selective loss of ERα in prostatic epithelium compared to selective loss of ERα in fibroblasts, it was demonstrated that epithelial, but not stromal, ERα is required for squamous metaplasia induced by diethylstilbestrol (19). Although subtle, loss of stromal ERα had less branching morphogenesis compared to their WT littermates (19). These results implicate that ERα expressed by stromal fibroblasts may play a key role in prostate development, as well as in new glandular growth associated with BPH. In addition, it is also possible ERα expressed in other prostate stromal cell types (such as myocytes or myofibroblasts) could mediate the effects of estrogens in the induction of BPH. Collectively, these data suggest that targeting ERα may be an important aspect to treating BPH.

1.12 SERMS as Therapeutics for BPH-LUTS

Estrogens have long been implicated in BPH-LUTS, but no current therapies directly target estrogen action. We recently tested the SERMs raloxifene and tamoxifen for prevention of bladder complications in male mice treated with T+E_2. While raloxifene prevented both bladder enlargement and prostate growth, and ERβ antagonist did not (85). These results support that ERα is both a key mediator and therapeutic target in BPH. There are currently five FDA-approved SERMs in clinical use, tamoxifen, raloxifene, clomiphene and toremifene. These drugs act as competitive inhibitors of estrogen binding to estrogen receptors and are relatively selective for estrogen receptor α and have mixed agonist and antagonist activity. Common side effects of SERMs are related to estrogen withdrawal such as vasomotor events (hot flashes) and these drugs carry a black box warning for increased risk of thromboembolic events, including deep venous thrombosis and pulmonary embolism. Despite these risks, because these are already FDA-approved medications, these drugs are attractive candidates for translation into ERα antagonist therapies for BPH-LUTS.

Tamoxifen was the first SERM to reach the market, and is FDA approved for adjuvant breast cancer therapy and risk reduction in pre and post-menopausal high-risk women (12, 133). It is also approved for estrogen-receptor positive breast cancer treatment in men and appears to be well tolerated in this patient population. Tamoxifen has estrogen agonist activity in endometrium and therefore carries increased risk

for endometrial hyperplasia and carcinoma, which is not the case for the second generation SERM, raloxifene. Raloxifene is approved for treatment of osteoporosis and for reduction in the risk of invasive breast cancer in postmenopausal women (133). In a small cohort of elderly men, raloxifene was well tolerated but did not affect markers of bone turnover or lipid levels (32). While we did not compare efficacy of raloxifene and tamoxifen in BPH prevention directly, we demonstrated that raloxifene decreased both bladder and prostate mass in male mice treated with $T+E_2$ (85). It is unknown whether treatment with raloxifene will reverse BPH-LUTS, once established, in animal models. It also remains unknown whether raloxifene inhibits the growth of human BPH, and this is an important area for future investigation.

Clomiphene is a SERM that is approved for ovulatory dysfunction in women. Because it acts as an antagonist at hypothalamic and pituitary ERs, clomiphene is also used off-label to stimulate gonadotrophin release in men with hypogonadotropic hypogonadism. In these men, clomiphene treatment induces increased serum T, E_2, and luteinizing hormone, improves testosterone to estradiol ratio, increases bone mineral density and improves symptoms of hypogonadism.(61, 84, 117). While clomiphene therapy achieves lower serum testosterone compared to exogenous testosterone supplementation via patches or gels, patients on both treatment strategies achieve similar levels of satisfaction (107). While it is unknown whether clomiphene therapy improves spermatogenesis in this population, exogenous testosterone should be avoided in men desiring future fertility due to suppression of the hypothalamic pituitary testis axis, consequent inhibition of spermatogenesis. A pure stereoisomer of clomiphene, enclomiphene, is currently in phase III trials for use in men (50). In a small proof of concept study, enclomiphene improved sperm counts following 3 and 6 months of treatment (57). An unanticipated beneficial effect of enclomiphene in hypogonadal men is an improvement in fasting glucose. Given the demonstrated efficacy in improving serum T in hypogonadal men, clomiphene or enclomiphene would be especially attractive as personalized therapies for men with comorbid hypogonadism and BPH-LUTS. Much of the current literature on the use of clomiphene pertains to men

of reproductive age; it remains unknown whether clomiphene or enclomiphene therapy in older men with hypogonadism and BPH-LUTS will also improve urinary function.

Toremifene is FDA-approved for use in metastatic breast cancer. It has also been evaluated for treatment of side effects of androgen deprivation therapy in men with metastatic prostate cancer (PrCa) and has been shown to decrease the incidence of pathologic fractures, improve vasomotor symptoms, prevent gynecomastia and improve lipid profiles in this population (123). More recently, Toremifene was tested for the prevention of prostate cancer in men with high-grade prostatic intraepithelial neoplasia (127). While this recent trial did not demonstrate efficacy in PrCa-free survival, few side effects were observed in this patient population, supporting the concept that SERMs, if used to treat BPH-LUTS, would be well tolerated by men.

We have previously demonstrated that raloxifene prevents bladder complications and prostate growth in male mice treated with $T+E_2$ (85). Because BPH is a disease process that likely begins in the fourth and fifth decades of life (8), potentially decades earlier than the development of LUTS, using a SERM as a preventative measure may prove challenging. Many natural compounds (so-called nutraceuticals) have estrogenic/SERM activity, and the use of herbal supplements for BPH has gained mass appeal. However, despite considerable promise, nutraceuticals have overall not been successful as BPH-LUTS therapies. A meta-analysis of saw palmetto (an extract of *Serenoa repens*) trials showed no benefit over placebo in reducing LUTS (5) or PSA (2), and β-sitoserol has also been proven to have limited benefits. Likewise, there is no demonstrated benefit of *Serenoa repens*, over placebo in improving LUTS (74). Perhaps the most promising nutritional therapy for BPH consists of less animal protein. less simple carbohydrates, and more vegetables, which would likely also be helpful in mitigating obesity and diabetes, two common comorbid conditions (41).

1.13 Synopsis of Research

The goal of this dissertation research was to examine the role of environmental estrogens in Benign Prostate Hyperplasia (BPH) and Lower Urinary Tract Symptoms (LUTS) using a novel BPH/LUTS

mouse model, state-of-the-art rodent urinary function testing, and estrogen receptor (ER) knockout mice to understand the relative contribution of ERα and ERβ to many of the lower urinary tract dysfunction (LUTD) characteristics recapitulated in this mouse model. This is based on the hypothesis that environmental estrogens signal through the estrogen receptors, which are expressed in the male lower urogenital tract, and that exposure to these compounds is a concern for aging men who are susceptible to developing BPH/LUTS. The research described in this dissertation encompasses three aims: 1) gain knowledge regarding the effects of Bisphenol-A exposure on urinary voiding behavior of male mice, 2) determine the role of Bisphenol-A analogs in LUTD, and 3) examine the contributions of ERα and ERβ in Bisphenol-A induced LUTD. The first aim describes the use of a novel LUTD mouse model to demonstrate that Bisphenol-A can induce lower urinary tract dysfunction in wildtype mice. Previous research by our group and many others demonstrate that male mice treated with Testosterone (T) in combination with 17β-estradiol (E_2) recreates many aspects of BPH/LUTS including urinary retention and bladder hypertrophy. Therefore, we used a similar approach with the environmental estrogen, Bisphenol-A. This aim also describes the use of uroflowmetry to determine the frequency and volume of urination in unrestrained mice. In addition to bladder hypertrophy, an intermediate voiding pattern was observed in T+BPA treated mice after four months of treatment. These data provide evidence that Bisphenol-A is an environmental estrogen of concern given the fact that it induces BPH/LUTS endpoints in male mice similar to the more potent endogenous estrogen E_2. It is important to note that phenotypes observed in this mouse model are highly variable and does not impact 100% of mice exposed to T+BPA or T+E_2. Thus, researchers should aim to control factors such as dominance, solubility of the compounds with respect to the length of treatment and reduce background contamination given the ubiquitous of nature of these environmental estrogens. Regardless, this mouse model serves as a tool to study the effects of exposure to environmental estrogens in the context of BPH/LUTS. The second aim was designed to evaluate the role of Bisphenol-A analogs in BPH/LUTS. Previous research by other groups indicate the estrogenic activity of several Bisphenol analogs and their increased use in many consumer products. Due to the increased use of bisphenol-S and bisphenol-F in consumer products and their reported estrogenic activity, it is important

to understand the hazards these compounds could present to aging men who are susceptible to developing BPH/LUTD. This aim also describes the use of cystometry to measure changes in urine flow rates and bladder pressure and compliance in adult male mice. Mice exposed to T+bisphenols developed enlarged bladders and prostates in addition to increased glandular growth in the prostatic urethra. Regardless, of the bisphenol used, these BPA analogs induced endpoints associated with BPH/LUTD such as bladder hypertrophy, urinary retention, and increased glandular growth in the prostatic urethra. Similar to T+E_2, T+bisphenol treatment caused voiding dysfunction manifested by detrusor instability in adult male mice. While these studies demonstrated high variability within treatment groups the negative effects induced after environmental estrogen exposure should not be ignored. Additional studies using isolated bladder strips in contractile assays could provide more information about the effects of BPA analogs on bladder contraction and the specific receptors responsible for this phenotype. The relative contribution of ERs in bisphenol-F and bisphenol-S induce LUTD should also be explored to identify a potential mechanism. Aim three was designed to determine the relative contribution of ERα and ERβ in Bisphenol-A induced LUTD as an attempt to identify a mechanism for environmental estrogen induced LUTD. Because Bisphenol-A has reported estrogenic activity we used estrogen receptor knockout mice to determine the contribution of each estrogen receptor (ERα and ERβ) in Bisphenol-A induced LUTD. Similar to E_2, ERα was identified as a key mediator of BPA induced LUTD. Given the fact nongenomic ERs are still present in mice that lack ERα or ERβ additional studies are required. These additional, *in vivo* studies include the use of mice that lack classical, nonclassical, or nongenomic estrogenic receptors (124) (**Table 1-2**) to help target the appropriate ER using a selective estrogen receptor modulator (SERMs) or another pharmacologic agent.

As demonstrated in this dissertation, environmental estrogens can induce LUTD in rodent models through an ERα dependent mechanism. The use of additional transgenic mouse models may allow for re-evaluation of therapies, such as SERMs, in the clinic.

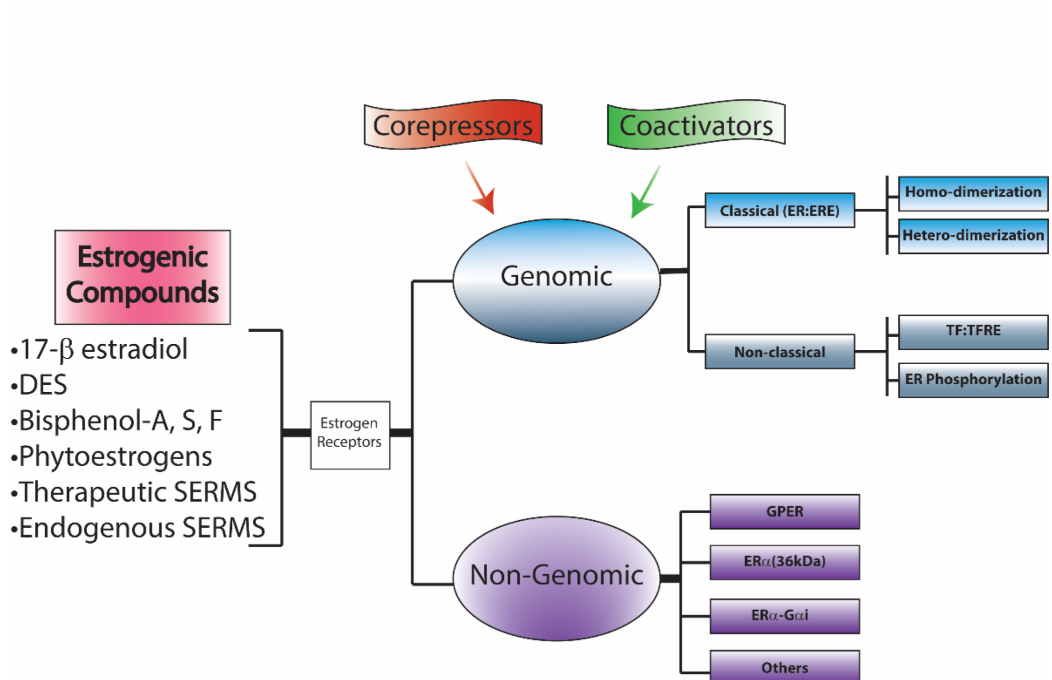
The data described in this dissertation collectively provides a foundation for further evaluation of environmental estrogen induced BPH/LUTS, as well as the use of SERMs as potential therapeutics. Two

bisphenol analogs, bisphenol-F and bisphenol-S, induced BPH/LUTD in wildtype mice and future studies to determine the contributions of ERs are warranted.

1.14 Figures and Legends

Figure 1-1 Common estrogens and their receptors.

Estrogens from a wide range of sources serve as ligands for a number of different estrogen receptors (ERs). Various estrogens can bind to ERs to facilitate genomic and non-genomic responses. Genomic ERs signaling can be enhanced or repressed through interactions with coactivators and corepressors, respectively. Classical ER signaling occurs as a result of ERs binding directly to estrogen response elements (ERE) and nonclassical signaling results from ERs binding to other transcription factors (TF). Similar to classical ER signaling, these transcription factors bind to their own transcription factor response elements (TFRE). Non-genomic estrogen actions occur as a result of interactions with G protein-coupled estrogen receptor (GPER), ERα36, ERα-Gαi (ERα interactions with Gαi binding domain), or other unknown ER pathways. Whereas genomic signaling results in transcription which may take hours to observe biological responses, non-genomic generally causes rapid ER signaling within seconds to minutes.



1.15 Tables

Table 1-1: Physiochemical properties and structure of selected bisphenols based on EPIWEB 4.1

Structure	Chemical	Acronym	CAS	MW (g/mol)	$LogK_{ow}$ [a]	$LogK_{aw}$ [b]	Water solubility mg/L (25°)	Reference
	Bisphenol-A	BPA	80-05-7	228.29	3.32[c]	-9.427	172.7	
	Bisphenol-S	BPS	80-09-1	250.27	1.65[d]	-12.957	504.9	Hansch, C et al. 1995 (46)
	Bisphenol-F	BPF	620-92-8	200.24	2.91[c]	-9.67	542.8	

Chemical structures images obtained from ChemIDPlus (https://chem.nlm.nih.gov/chemidplus/rn/57-88-5)

[a] Kow=octanol-water partition coefficient

[b] Kaw=air-water partition coefficient

[c] experimental [d] estimated

Table 1-2: Common *In vivo* models used to target estrogen signaling pathway

Name		Specification	Estrogen pathway	References
Estrogen receptor α knock-out mouse	*ERαKO*	knock-out ERα gene	classical	25,26,94
Estrogen receptor β knock-out mouse	*ERβKO*	knock-out of ERβ gene; aged mice develop basal cell hyperplasia and reduced apoptosis	classical	53
Double estrogen receptor knock-out	*DERKO or α/βERKO*	knock-out of estrogen receptors α and β; mice infertile	–	24,36,64
Nuclear-only ERα	*NOER*	ERα is still expressed but there is a point mutation that prevents palmitoylation and membrane trafficking of ERα; homozygotes are infertile $ER^{NERKI/WT}$	classical	100
Membrane only domain of ERα	*MOER*	knock-out of estrogen receptorα gene but not domain E. The ligand binding domain of ERα is still epxressed at the plasma membrane.	classical	98, 101
Nonclassical ERα knock-in mouse	*NERKI* $^{NERKI/WT}$ $^{NERKI/KO}$	mutation in ERα which abolishes ERE binding and activation of ERE reporter genes.Heterozygote of ERα gene with one dysfunctional allele and second of the wildtype or Heterozygote of ERα gene with one dysfunctional allele and second knockout; infertile	non-classical	22,55,55,125
Estrogen-nonresponsive ERα knock-in	*ENERKI*	knock-in of *Esr1* gene with a mutation in the binding pocket of Erα to block the binding of estradiol. Subfertile	non-classical	121,122
Estrogen-related receptor-α knockout	*ERRα*	knockout of ERRα gene; small hearts	orphan receptors/non-genomic	52,67
Estrogen-related-γ knockout	*ERRγ*	knockout of ERRγ gene; cardiac defects and neonatal lethality	orphan receptors/non-genomic	1,135
Estrogen-related-β knockout	*ERRβ*	knockout of ERRβ gene; fetal lethality	orphan receptors/non-genomic	73,128,129
G-protein-coupled receptor knockout	*GPR30KO*	knockout of GPR30; develop adiposity and age-related glucose intolerance	orphan receptors/non-genomic	54,60,77,118,119,120

Table adapted and expanded from Czekaj et al. 2013 (124)

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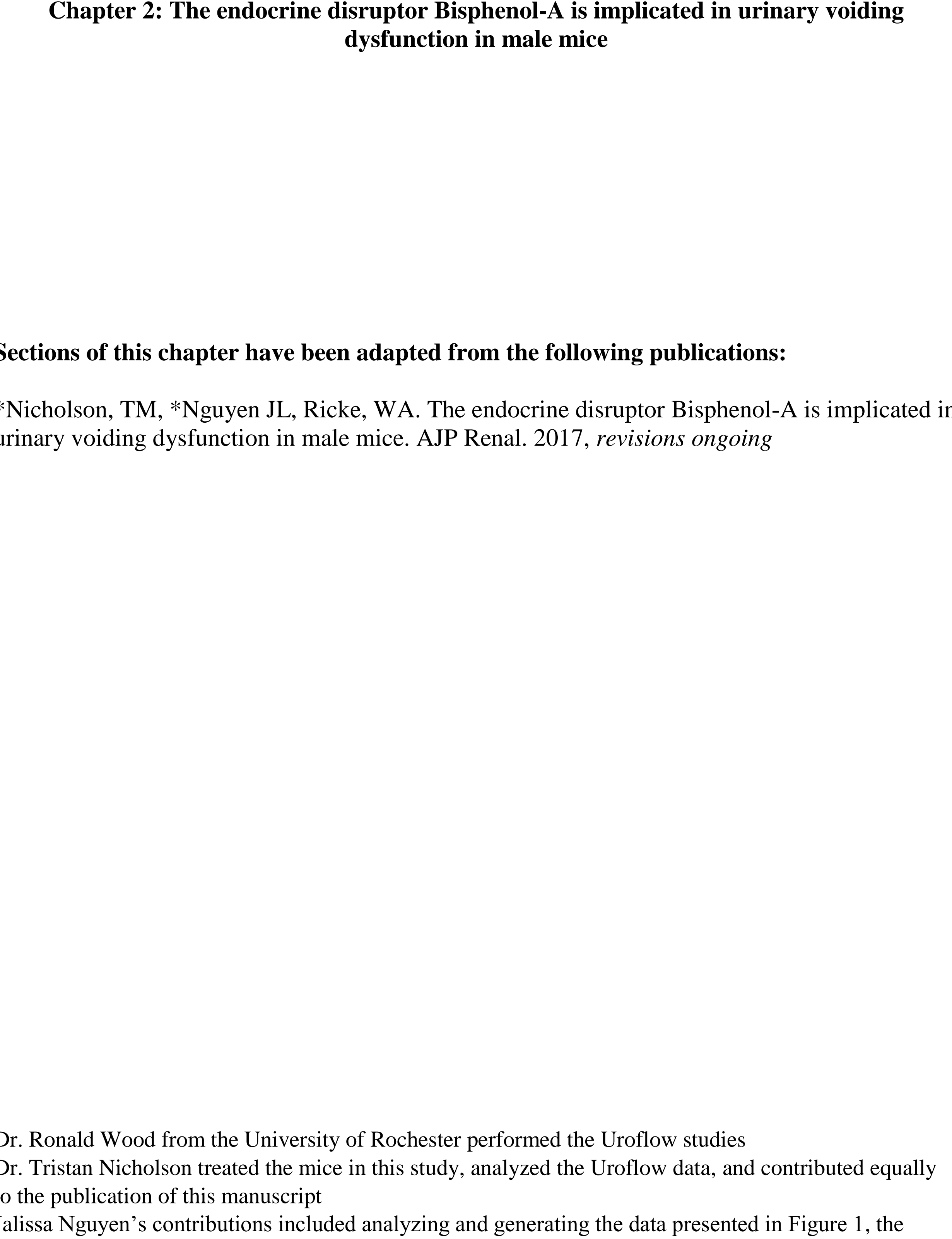
Chapter 2: The endocrine disruptor Bisphenol-A is implicated in urinary voiding dysfunction in male mice



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Dr. Ronald Wood from the University of Rochester performed the Uroflow studies
Dr. Tristan Nicholson treated the mice in this study, analyzed the Uroflow data, and contributed equally to the publication of this manuscript
Jalissa Nguyen's contributions included analyzing and generating the data presented in Figure 1, the supplementary video, and contributing equally to the publication of this manuscript.

2.1 Abstract

Estrogens, acting synergistically with androgens, are known from animal experiments to be important in lower urinary tract symptoms (LUTS) and benign prostate enlargement. Human exposure to environmental estrogens occurs throughout the lifespan, but the urologic health risks in men are largely unknown. Bisphenol-A (BPA) is an endocrine disruptor implicated in male urogenital malformations. Given the role of estrogens in male LUTS, we studied the effects of BPA administered in combination with testosterone (T) on the urinary voiding behavior of adult male mice. Adult male mice underwent subcutaneous implantation with slow release pellets of 25 mg BPA or 2.5 mg estradiol-17β (E_2), plus 25 mg T and were compared to untreated (UNT) mice that underwent sham surgery. We studied urinary voiding behavior noninvasively for one month prior to treatment and for four months after treatment. After euthanasia, we evaluated bladder volume and mass. Mice treated with T+BPA had increased bladder volume ($p < 0.05$) and mass ($p < 0.01$) compared to UNT mice. After four months of treatment with T+BPA, three of five mice developed voiding dysfunction in the form of droplet voiding or an intermediate pattern of voiding different from both UNT and T+E_2 treated mice. Treatment of male mice with BPA or estradiol induces voiding dysfunction that manifests at later time points, implicating the endocrine disruptor, BPA, as a contributor to male LUTS.

2.2 Introduction

Bisphenol-A (BPA) is a monomer in polycarbonate plastics that can leach from containers used to store food and beverages, dental implants, and epoxy resins in container linings. While toxicokinetic studies indicate that BPA is rapidly metabolized by the liver after oral exposure, human biomonitoring data support that exposure to BPA is ubiquitous and found in over 90% of individuals in the USA (1; 2). While the assumption has been that the majority of BPA exposure occurs via ingestion from food packaging (3), substantial non-food exposure routes are also common in humans, including inhalation and transdermal routes (4-6). The estrogenic activity of BPA was first reported in 1936 (7). It is often stated that BPA is a weak estrogen compared to estradiol, because the binding affinity of BPA for classical nuclear estrogen receptors is lower than that of estradiol. It is well recognized that potency is impacted by tissue-specific co-regulatory proteins and does not simply reflect affinity for nuclear receptors. Additionally, non-nuclear estrogen receptors associated with rapid-response enzyme cascades show equal potency for BPA and estradiol (8). BPA is now well accepted as an endocrine disruptor (9; 10). While precise mechanisms in addition to its estrogenic activity remain unclear, BPA has been implicated in a wide range of health problems in humans, including peripheral vascular disease, insulin resistance, and obesity (11-15). Occupational exposure of adult men in China to BPA is associated with alterations in serum hormone levels and male sexual dysfunction (16; 17). United states BPA occupational studies indicate that workers in plants that use BPA have higher serum levels of BPA than workers in China (18). In experimental animals, BPA exposure during development has been linked to male reproductive system abnormalities involving the testes, epididymis, seminal vesicles, and the prostate (19; 20). BPA exposure has also been linked to changes in gene expression within the prostate and the lower urinary tract of rodents (21).

Lower urinary tract symptoms (LUTS) affect at least 30% of men over the age of 50, and the prevalence increases with age (22). Multiple factors, including age, prostate enlargement, comorbid medical conditions, and bladder dysfunction contribute to male LUTS (23). The standard of care for the better half of the 20th century consisted of surgical intervention to manage complications due to LUTS

associated with BPH. In the 1990s noninvasive urodynamic testing, clinical history, and examinations were recommended before medical or surgical intervention (24; 25). Noninvasive testing for patients with LUTS includes uroflow, which is used to measure the flow rate during urination (26). The goal of this test is to improve clinical decisions, therefore, reducing the number of men undergoing surgery for management of complications due to LUTS. While the molecular mechanism of BPH related LUTS are unclear, the altered hormone environment in aging men has been explored (27-29). As men age, serum estrogens increase or remain constant, while testicular androgens decline (27). Although precise mechanisms remain unknown, estrogens have been implicated in benign prostatic enlargement and LUTS (30). Specifically, several studies have suggested a link between serum estradiol and LUTS (31), prostate size (32) and risk of benign prostatic hyperplasia (BPH) (32). Despite the well-characterized estrogenic activity of BPA, the possibility that adult BPA exposure could contribute to male LUTS has not been previously evaluated. Therefore, the objective of this project was to use uroflow to determine the effects of adult BPA exposure on urinary voiding behavior in male mice.

2.3 Results

Bladders

Due to unequal variances among the groups for bladder volume (Bartlett's statistic 30.44, $p < 0.0001$) and bladder mass (Bartlett's statistic 9.132, $p=0.0104$) data were transformed using reciprocals to satisfy assumptions of ANOVA. As shown in **Figure 2-1A**. There was a significant decrease in body mass in the $T+E_2$ groups relative to the UNT and T+BPA groups (overall ANOVA $*P<0.05$; UNT vs $T+E_2$. ($p<0.01$; $T+E_2$ vs T+BPA $p<0.05$). The urogenital tracts from UNT, $T+E_2$, and T+BPA treated mice for four months are shown in **Figure 2-1B**. Bladder volume was significantly altered by treatment ($p<0.01$) and was significantly larger in mice treated with $T+E_2$ ($p < 0.05$) and T+BPA ($p<0.05$) compared to UNT (body weight did not account for a significant component of the variance, $p=0.52$); in addition, when $T+E_2$ and T+BPA groups were compared there was no significant difference (**Figure 2-1C**). **Figure 2-1D**, Bladder mass significantly altered by treatment ($p<0.0001$), and body mass was not a significant covariate

(p=0.86). Specifically, bladder mass was increased relative to untreated (UNT) among mice treated with $T+E_2$ ($p<0.0001$) and T+BPA($p<0.01$). However, the average bladder mass was significantly higher among the mice treated with $T+E_2$ compared to T+BPA ($p<0.01$), indicating that BPA-induced bladder hypertrophy was less severe than the $T+E_2$ treatment.

Uroflow

To compare general patterns of urinary voiding in individual mice, we used the uroflow curves (**Figure 2-2A and 2-2B**) for all sessions in a time window of interest (**Figure 2C**) to create median uroflow curves for each mouse (**Figure 2-2D**). We compared median uroflow curves that reflect the one-month time period prior to hormone implantation, to median uroflow curves reflecting 1-4 months after treatment. The two UNT mice exhibited median uroflow curves consistent with sustained voiding pattern prior to sham surgery that was similar to median uroflow curves following surgery. One of two UNT mice (Ms 7) did display a minor droplet voiding component to the median uroflow curve, that was present both prior to and post-surgery for subcutaneous pellet implantation (**Figure 2-3A**). Among mice treated with $T+E_2$, median uroflow curves prior to hormone pellet implantation were similar to UNT mice (**Figure 2-3A&B**). In the four months following hormone implantation, five of five mice treated with $T+E_2$ displayed median uroflow curves consistent with exclusively droplet voiding pattern. Among mice treated with T+BPA, two mice (Ms 1 and Ms 5) displayed predominantly droplet voiding prior to treatment, while the remaining three mice had median uroflow curves consistent with sustained voiding similar to the UNT mice. Post-treatment, all mice treated with T+BPA displayed a mixture of median uroflow curves consistent with droplet voiding (Ms 4) or an intermediate pattern of voiding (Ms 8 and Ms 11) different from both UNT and $T+E_2$ treated mice (**Figure 2-3C**).

Urinary voiding patterns

To quantify differences in voiding patterns, we examined the frequency of sustained voids, droplet voids and the proportion of droplet voids (defined as the number of droplet voids divided by the total number of voids per session, including sustained and droplet). For each mouse on each testing day (**Figure 2-2A**), every voiding event was classified as either a sustained void or a droplet void (**Figure 2-2B**) and the

median number of each type of void was computed for the one-month time period. Analysis of the number of sustained voids showed effects for interaction ($p=0.0010$), time ($p< 0.0001$), and treatment ($p=0.0400$). As shown in **Figure 2-4A**, at all time points, UNT mice displayed sustained voids (range of median frequency 0 to 7.5). Prior to treatment, T+E_2 mice had a median frequency ranging from 1.5 – 6 sustained voids per session. In all four months following treatment with T+E_2, the median number of sustained voids decreased to zero, reaching statistical significance compared to UNT at 1 MO ($p< 0.001$) and 3 MO ($p< 0.05$) post-treatment. Among the T+BPA mice prior to treatment, there was a wide range of median sustained void counts, with one mouse (Ms 5) displaying a median number of zero sustained voids prior to treatment. Mice treated with T+BPA exhibited increased sustained voids relative to UNT mice at 1 MO post-treatment ($p< 0.05$), but other time points were not significantly different.

For the frequency of droplet voids, there were no significant effects of treatment or time ($p>0.1$). As shown in **Figure 2-4B**, a low rate of droplet voiding was observed in UNT mice at all time points, with a range of 0.5 – 3.0 median number of droplet voids per session. Mice in all groups had a similar number of droplet voids pre-treatment (T+E_2 range 1.5 – 4.0; T+BPA range 0.5 – 6.0). However, among T+E_2 mice post-treatment, there were two mice (Ms 2 and Ms 6) that exhibited an increase in the median number of droplet voids at all post-treatment time points, although the comparison of this treatment with the UNT group did not reach statistical significance. Among mice treated with T+BPA, there were two mice (Ms 4 and Ms 11) at 4 MO post-treatment that had an increased median number of droplet voids, but again, this treatment group did not differ significantly from UNT.

Because we observed differences in the voiding patterns among the groups, we determined the proportion of droplet voids in each session, and compared the median of this proportion in one-month intervals (**Figure 2-4C**). We found effects for interaction ($p=0.077$), time ($p=0.0024$), and treatment ($p=0.0228$). As shown in Figure 4C, UNT mice had consistent proportion of droplet voids over time (range 0.17 – 0.60). Prior to treatment, mice treated with T+E_2 had median proportion of droplet voids similar to UNT (range 0.31 – 0.5); however, after treatment, mice treated with T+E_2 had a significantly higher proportion of droplet voids compared to UNT (1 MO, $P < 0.01$; 3 MO, $P < 0.05$; 4 MO, $P < 0.01$). For the T+BPA

mice prior to treatment, there was a large range of median proportion of droplet voids (0.08 – 0.83). Following treatment, two of five mice treated with T+BPA (Ms 4 and Ms 5) displayed exclusively droplet voids, while two others (Ms 11 and Ms 8) displayed a high proportion of droplet voids by 4 MO (0.89 and 0.75, respectively). The remaining mouse (Ms 1) displayed a high proportion of droplet voiding prior to treatment and following treatment, decreased during the study period to display no droplet voids by the 4MO. Due to the individual variability of the voiding patterns displayed among the T+BPA mice, there were not statistically significant differences relative to UNT mice.

We also assessed median uroflow prior to hormone treatment, and at each time point following treatment. There were significant effects for time ($p=0.0252$) and treatment ($p= 0.0405$), but not for interaction ($p> 0.1$). Even though the interaction effect was not significant, it appeared as though uroflow patterns differed over time within groups. As shown in Figure 5A, median uroflow prior to treatment was similar among all groups, and the UNT mice did not change over time. However, among mice treated with T+E_2, there was a decrease in median uroflow following hormone treatment that reached statistical significance at 4 MO ($p< 0.05$). Among mice treated with T+BPA, there were no consistent differences in median uroflow compared to UNT, although four of five mice treated with T+BPA (Ms 4, Ms 5, Ms 8 and Ms 11) displayed median uroflow at 4 MO that was similar to mice treated with T+E_2. Because peak uroflow is a clinically relevant measure associated with bladder outlet obstruction in human male LUTS, we examined the changes over time in peak uroflow (**Figure 2-5B**). We found effects for interaction ($p=0.0078$) but not for time or treatment ($p> 0.1$). Peak uroflow was similar at all time points in UNT mice. Following hormone treatment, mice treated with T+E_2 exhibited decreased peak uroflow, reaching statistical significance at 4 MO post-treatment ($p< 0.05$, **Figure 2-5B**). Among mice treated with T+BPA, there were no consistent or statistically significant differences in peak uroflow compared to UNT.

2.4 Discussion

To our knowledge, we report for the first time that treatment of male mice with BPA, in an androgenic environment that models the human male hormonal milieu, induces bladder enlargement, and hypertrophy and urinary voiding dysfunction. This is consistent with bladder enlargement and hypertrophy in male mice treated with T+E_2 (38). As expected, we found droplet voiding was present in mice treated with T+E_2 in the first month following hormone treatment, while urinary voiding dysfunction manifested at later time points in mice treated with T+BPA.

After one month of hormone treatment, we observed consistent decreases in median and peak uroflow among positive control (T+E_2-treated) mice. While consistent differences in uroflow were not present in the entire group of mice treated with T+BPA, after four months of treatment, male mice treated with T+BPA showed intermediate median uroflow values that were similar to the mice treated with T+E_2, and substantially decreased relative to UNT. Decreases in median and peak uroflow that we observed in mice treated with T+E_2 are consistent with bladder outlet obstruction that is commonly observed in men with enlarged prostates. However, the late manifestations of voiding dysfunction we observed in mice treated with T+BPA are consistent with the relatively weaker estrogenic activity of BPA compared to E_2.

At all time points, UNT mice exhibited, on average, less than five droplet voids per session, suggesting that some degree of droplet voiding behavior is expected in male mice. This is consistent with the known importance of scent marking behavior in male mice (40). It is known that when placed in a novel environment, dominant male mice have increased urine marks compared to subordinate animals (41). While our study did not address the dominance status of individual mice, we speculate that the droplet voiding observed in the one T+BPA mouse in the baseline period, prior to subcutaneous pellet implantation, may be explained by the dominance status and territory marking inherent in some mice. The bladder abnormalities observed in male mice treated with T+E_2 and T+BPA indicate that the droplet voiding observed in these groups following subcutaneous hormone pellet implantation is not explained by urine scent marking alone.

Given the increased variability in urinary voiding patterns among the mice treated with T+BPA, we conclude that our study was underpowered to detect differences in urinary voiding behavior compared to control mice. However, we feel that the urinary voiding dysfunction among mice treated with T+BPA observed at month four, while not consistently or statistically different, may be a clinically relevant finding in terms of the urinary voiding function of a subset of T+BPA-treated animals that appeared to be affected by BPA. Individual differences in the response to BPA could reflect differences in sensitivity that are related to stressors or other factors during development, consistent with the developmental origin of health and disease (DOHaD) hypothesis (42).

The idea that diseases present in adults may have originated very early in development is well accepted (1). Indeed, many studies suggest that humans and animals are most sensitive to endocrine disruptors, such as BPA, during development. However, our findings suggest that adult exposure to BPA, which occurs throughout the lifespan, is also of concern. Moreover, widespread, lifelong exposure of humans to BPA has only occurred in the “age of plastics” beginning in the 1970s. Therefore, men with cumulative lifetime exposure to BPA are now reaching middle age when LUTS and other prostate pathologies are prominent (43; 44). Given that male LUTS is highly prevalent and a significant burden to the healthcare system today, the implication of BPA in LUTS pathophysiology is particularly troubling. There are numerous challenges inherent in modeling human exposures to BPA in experimental animals. BPA exposure in humans occurs continuously and throughout the lifespan. In humans, the majority of BPA exposure occurs via ingestion (4), dermal (6), and inhalation (45). Ingested BPA undergoes extensive first-pass metabolism in the liver. When BPA is administered subcutaneously, it does not undergo first-pass metabolism; therefore, this route of drug administration has limitations when trying to model human exposure based only on BPA in food or beverages. In fact, BPA and a number of its replacements have been detected at very high levels in thermal receipt paper (46). The fact that there are numerous analogues of BPA that have estrogenic activity, as well as other estrogenic chemicals, must be considered in terms of cumulative exposure to environmental estrogens, given the finding that exposure to estradiol markedly impacts LUTS in male mice (47; 48).

2.5 Figures and Legends

Figure 2-1. Mice treated with T+BPA displayed enlarged bladder mass and volume.

A. Post necropsy, the body mass was determined. There was a significant decrease in body mass in the $T+E_2$ treated mice relative to the UNT ($p<0.01$) and T+BPA ($p<0.05$) treated mice. **B**. Urogenital tracts from UNT, $T+E_2$, and T+BPA treated mice for four months. **C**. At the time of necropsy, bladders were measured *in situ* with a precision caliper in three dimensions and the volume estimated as an ellipsoid. There was a significant increase in bladder volume in the $T+E_2$ ($p<0.05$) and T+BPA ($p<0.05$) groups, relative to UNT. Body weight did not account for a significant component of the variance ($p=0.52$). **D**. The bladders were carefully dissected, emptied of urine, blotted dry, and the mass determined. There was a significant increase in bladder mass in the $T+E_2$ ($p<0.0001$) and T+BPA ($p<0.01$) groups, relative to UNT. Body mass was not a significant covariate ($p=0.86$). Bl= bladder, Ur= urethra, SV= seminal vesicle

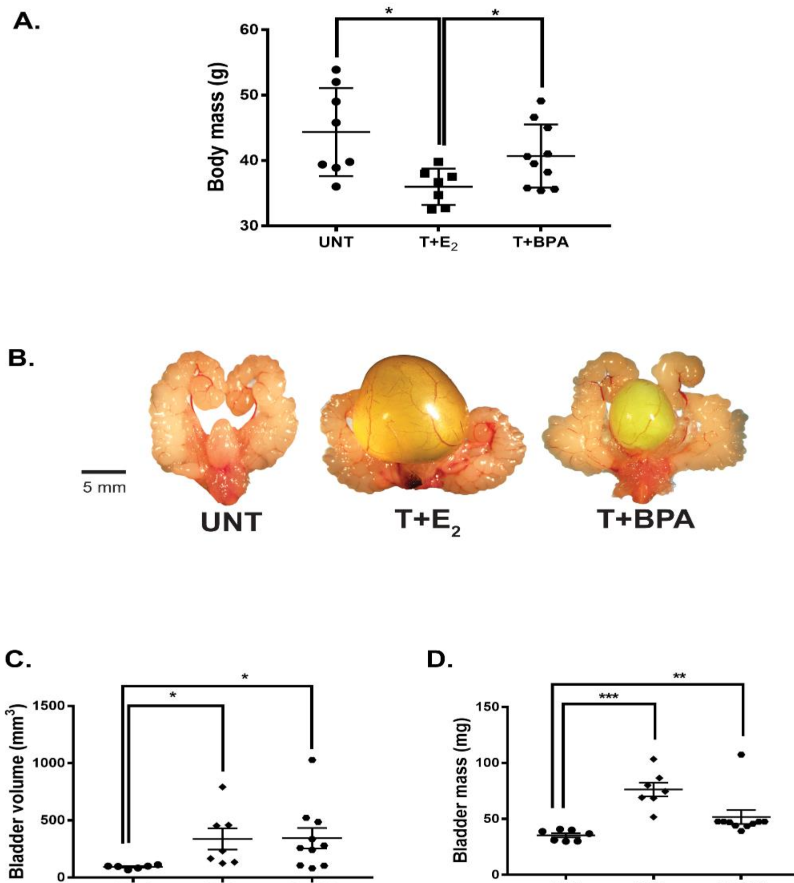


Figure 2-2 Classification of Uroflow patterns measured noninvasively.

A. All uroflow curves for one session are plotted. **B**. Voids are qualitatively classified by the shape of the uroflow curve into sustained void vs. droplet void (quantitative threshold for a droplet void as duration less than 3 sec). **C**. Uroflow curves of each void for all sessions show a mixed pattern of sustained and droplet voiding. **D**. The median uroflow curve is generated from all curves in panel C. At shorter durations, more voids contribute to the median uroflow curve.

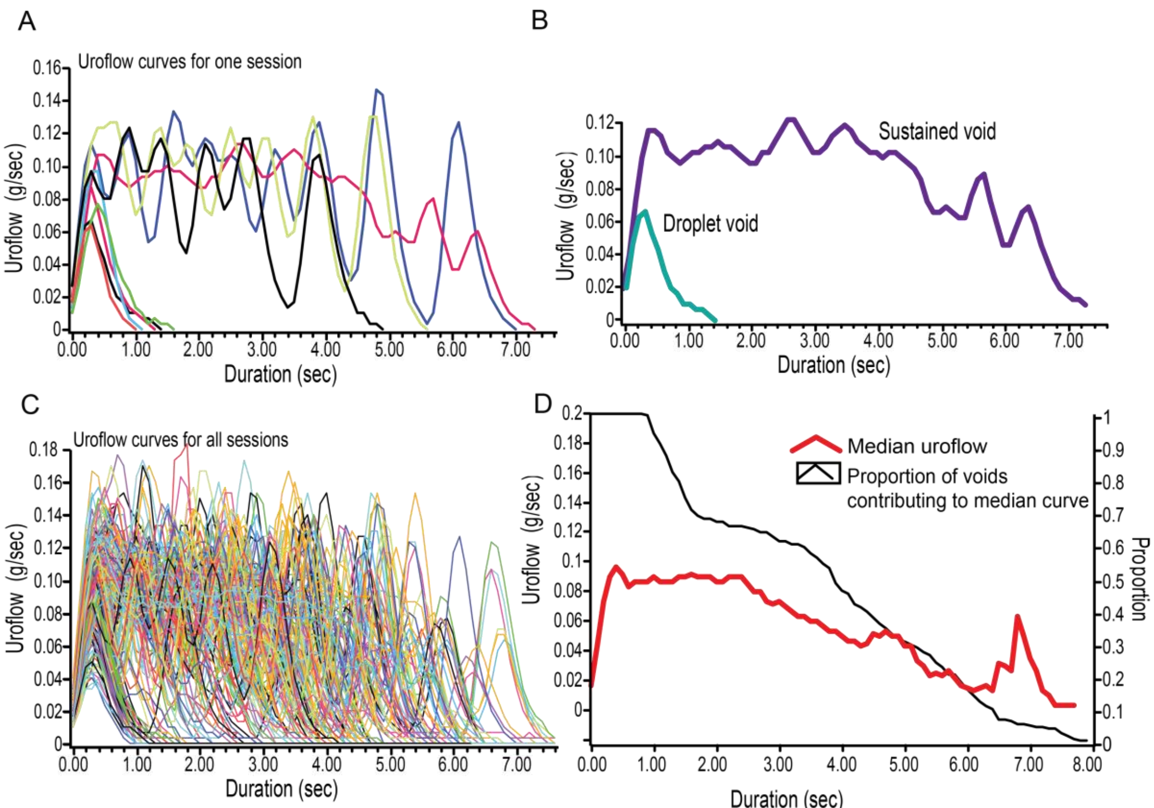


Figure 2-3 T+BPA treated mice displayed voiding dysfunction in the form of droplet or an intermediate pattern of voiding after four months.

To compare individual mice before and after hormone treatment, uroflow curves generated by each urinary void were summarized into median uroflow curves. **A**. Pre-treatment and post-treatment uroflow curves were similar in two UNT mice. **B**. While Pre-treatment uroflow curves were similar among the mice treated with T+E_2 and UNT, post-treatment, all five of the mice treated with T+E_2 displayed predominantly droplet voiding. **C**. Among mice treated with T+BPA, pre-treatment uroflow curves were similar to UNT, while uroflow curves from all T+BPA-treated mice displayed a droplet void component and void components intermediate of the UNT and T+E_2 treated mice.

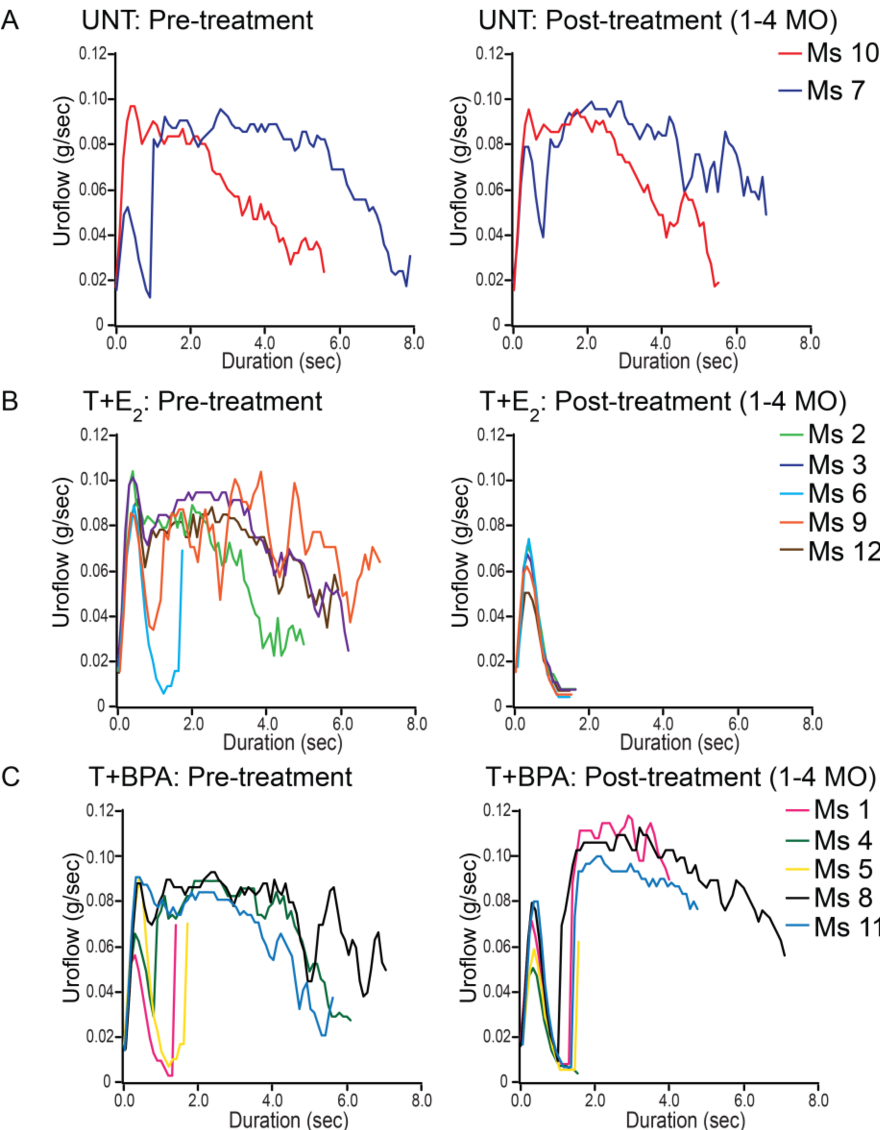
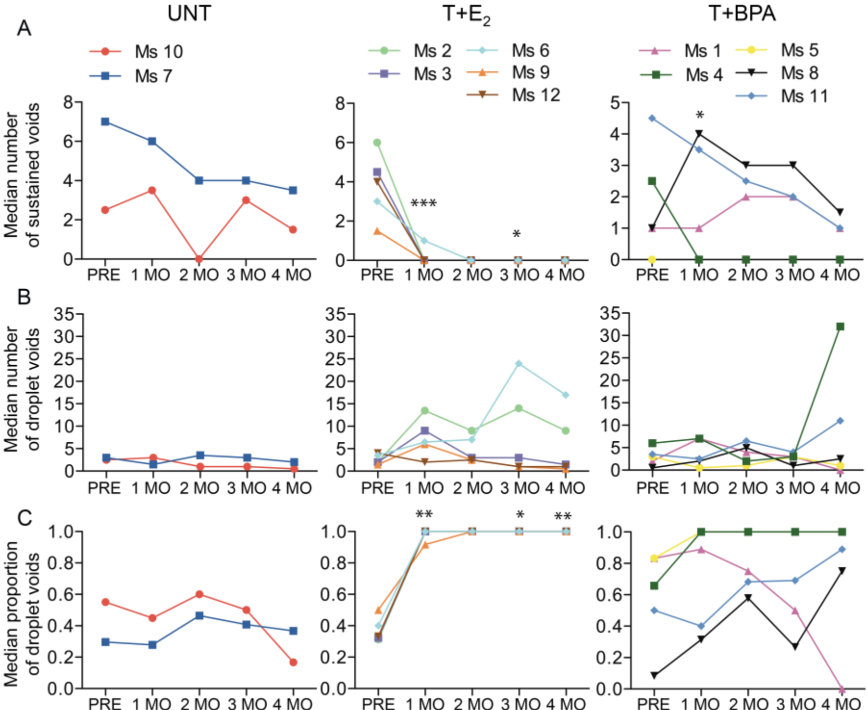

A
UNT: Pre-treatment
UNT: Post-treatment (1-4 MO)
Ms 10
Ms 7
B
T+E2: Pre-treatment
T+E2: Post-treatment (1-4 MO)
Ms 2
Ms 3
Ms 6
Ms 9
Ms 12
C
T+BPA: Pre-treatment
T+BPA: Post-treatment (1-4 MO)
Ms 1
Ms 4
Ms 5
Ms 8
Ms 11
Uroflow (g/sec)
Duration (sec)

Figure 2-4. T+BPA caused voiding dysfunction in male mice over four months of treatment.

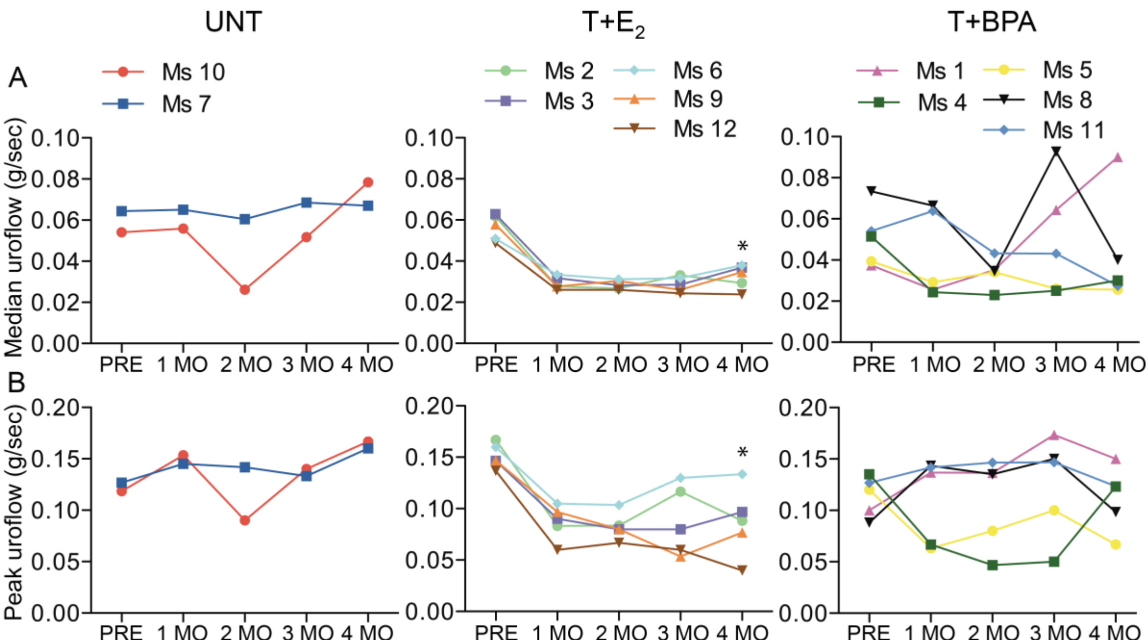
For each session, individual voids from each mouse were classified as sustained or droplet (defined as > 3 sec duration), expressed as a frequency per session, or proportion of droplet voids, and summarized by median for each month. **A**. Among UNT mice, the median number of sustained voids did not change significantly over time, while there was a marked decrease in sustained voids among the mice treated with T+E_2 following hormone treatment. Among mice treated with T+BPA, there was an increase in the number of sustained voids at 1 MO, but not at later time points interaction (P = 0.0010), time (P < 0.0001), treatment (P = 0.0398). **B**. The median number of droplet voids was consistent among all mice prior to treatment. While there were not consistent differences in the frequency of droplet voids, 2 of 5 mice treated with T+E_2 showed markedly increased frequency of droplet voiding at 3 and 4 MO, and 2 of 5 mice treated with T+BPA displayed substantially increased median droplet voids at the final month after treatment interaction (P = 0.6007), time (P = 0.8056), treatment (P = 0.5082). **C**. The median proportion of droplet voids was consistent among UNT mice over time, while it increased substantially among all mice treated with T+E_2. Among mice treated with T+BPA, four of five mice display a proportion of droplet voids greater than UNT interaction at 4 MO (P = 0.0077), time (P = 0.0024), treatment (P = 0.0228). * P < 0.05, **P < 0.01, ***P < 0.001 in post-hoc testing.



A
B
C
UNT
T+E2
T+BPA
Ms 10
Ms 7
Ms 2
Ms 3
Ms 6
Ms 9
Ms 12
Ms 1
Ms 4
Ms 5
Ms 8
Ms 11
Median number of sustained voids
Median number of droplet voids
Median proportion of droplet voids
PRE
1 MO
2 MO
3 MO
4 MO
***
*
**

Figure 2-5. T+BPA did not significantly alter median or peak uroflow in male mice.

A. Among UNT mice, there were no changes over time in median uroflow, contrasted to mice treated with T+E_2, who had a significant decrease by 4 MO. Among mice treated with T+BPA, 4 of 5 displayed decreased uroflow, although the difference was not statistically significant in post-hoc testing interaction (P = 0.2696), time (P = 0.0252), treatment (P = 0.0405). **B**. Peak uroflow (measured in the first 0-2 sec of the urinary void) did not change over time among UNT mice but decreased at 4 MO among mice treated with T+E_2. Peak uroflow among mice treated with T+BPA did not change over time interaction (P = 0.0078), time (P = 0.1703), treatment (P = 0.1757). *P < 0.05 in post-hoc testing.



2.6 Materials and Methods

Steroid implantation

C57BL/6 male mice (6-8 weeks of age) were obtained from Charles River (Wilmington, MA) and maintained under standard laboratory conditions using 1/8 performance and microisolator caging (12:12 light/dark cycle) with free access to food (laboratory autoclavable diet 5010) and reverse osmosis water from Hydropac™. All animal experiments and procedures were conducted under a protocol approved by the University of Rochester's University Committee on Animal Resources. BPA (99% pure), E_2 and T were obtained from Sigma Chemical Co. (St. Louis, MO). Mice underwent surgical implantation of compressed hormone pellets containing 25 mg T and either 25 mg BPA or 2.5 mg E_2 as described previously (33; 34). Control mice underwent the surgical procedure, but no pellet was implanted. For bladder measurements, UNT n=7, T+E_2 n=7, T+BPA n=10. Uroflow studies were done on a subset of mice (UNT n=2, T+E_2 n=5, and T+BPA n=5). At the completion of the experiment, mice were euthanized with CO_2 and the body mass was measured. Laparotomy was then performed, and bladder volume and mass were determined as previously described (35).

Uroflow

One month prior to hormone implantation, we began assessment of urinary voiding behavior (2-3 sessions per week) and continued until four months after steroid hormone implantation (36; 37). Details of our uroflow testing apparatus have been previously described (37; 38). Briefly, mice were placed in individual metabolic cages suspended over milligram-resolution balances without fecal separation screens and offered a preferred solution to drink. Perturbations in the balance were monitored ten times per second, and the character stream was received by our custom program (LabVIEW, National Instruments, Austin, TX) to generate displays of void mass, duration and uroflow, that were verified by synchronized videos of voiding events.

Statistical Analysis

For each individual mouse uroflow testing session, the median uroflow (g/sec), peak uroflow in the first two seconds of the void (g/sec) and median voided mass (g) was determined. During each uroflow testing

session, individual uroflow curves for each mouse were examined by a single observer blinded to treatment condition, counted, and classified as either a sustained void (≥3 sec duration) or droplet void (<3 sec duration). For all uroflow parameters, the median of each measure was reported for the 1-month period prior to treatment, and 1, 2, 3 and 4 months after treatment. Data analysis, using GraphPad Prism 7 (La Jolla, CA) and SAS (9.4) for comparisons of continuous variables consisted of one-way ANOVA where appropriate and repeated measures two-way ANOVA with Bonferroni *post hoc* comparisons for uroflow measures that continued over time. Data were evaluated for homoscedasticity with the Bartlett's unequal variance test and when necessary, rank transformation was performed. For body mass and bladder measurements, graphs show means ± SEM of non-transformed data, although the bladder data were subjected to analysis of covariance (ANCOVA), with body weight as the covariate. For uroflow parameters, graphs show medians for one month time period. In all analyses $p < 0.05$ was considered statistically significant.

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Chapter 3: Bisphenol-A analogues induce lower urinary tract dysfunction in adult male mice

Sections of this chapter have been adapted from the following publications:

Nguyen JL, Lamarre N, Bjorling, D Ricke, WA. Bisphenol-A analogues induce lower urinary tract dysfunction in adult male mice. *Ready to be submitted to the Journal of Endocrinology*

Dr. Neil Lamarre, Peiqing Wang, and Zunyi Wang performed Cystometry on every mouse in this study.

Tom Pier and Ella Ward from the UWCCC Experimental Pathology laboratory performed paraffin embedding and sectioning of the mouse urethras.

Dr. Roberto "Roy" Gerona performed the mass spectrometry analysis for this study.

Sally Griffith-Oh from UW-Madison's School of Pharmacy produced the scientific illustration of shown in Figure 3-2.

Yuchen "Vanesssa" Ying helped with BioVis and urethra morphometric measurements.

Jalissa Nguyen's contributions included all the *in vivo* work, all the data analysis, tissue collections, and agar embedding for this study.

3.1 Abstract

A change in the androgen/estrogen ratio has been implicated in the pathogenesis of benign prostate hyperplasia (BPH) and lower urinary tract symptoms (LUTS), but the consequences of exposure to environmental estrogens has largely been unexplored in rodent models. Bisphenol-A is an environmental estrogen implicated in lower urinary tract dysfunction, as well as adverse effects on reproduction and fetal development. Bisphenol-F (BPF) and bisphenol-S (BPS) are analogues of BPA reported to have *in vitro* and *in vivo* estrogenic and anti-estrogenic effects similar or more potent than BPA. We studied the effects of bisphenol analogues, in combination with 25 mg Testosterone (T), on urinary function of adult male mice by treating mice with subcutaneous pellets containing 25 mg BPA, BPF, or BPS. Mice, treated with environmental estrogens were compared to control mice that underwent sham surgery. Cystometry was performed before necropsy to evaluate the effects of exposure to environmental estrogens on urine storage and voiding after two months of treatment. We evaluated urethral morphometrics in addition to bladder and prostate mass. Mice treated with bisphenols displayed increased bladder ($p<0.05$ and $p<0.01$) and prostate ($p<0.0001$) mass compared to control mice. Mice exposed to T+bisphenols developed an increased number of prostatic ducts or glandular tissue in the proximal urethra ($p<0.01$) that correlated with a decrease in the size of the urethra lumen ($p<0.05$). Treatment of male mice with bisphenol analogs induced voiding dysfunction manifested by detrusor instability. Our results indicate that adult mice treated with Testosterone in combination with bisphenol analogues develop alterations in the structure of lower urinary tract as well as urinary function. These data support the concept that environmental estrogens can induce prostate hyperplasia and negatively impact urinary function.

3.2 Introduction

Benign prostate hyperplasia (BPH) is a histologically benign disease characterized by enlargement of the prostate gland. More than 50% of men over the age of 50 suffer from BPH, which is closely associated with lower urinary tract symptoms (LUTS). LUTS are characterized as obstructive symptoms associated with increased voiding frequency, irritative symptoms associated with decreased voiding volume and decreased voided volume (16, 27). Clinically significant BPH/LUTS affects 50-90% of men over the age of 50 (16). If left unmanaged, BPH/LUTS can lead to bladder outlet obstruction (BOO), which can cause urinary retention, renal impairment, and ultimately death. Several mechanisms of BPH-induced BOO have been proposed that may be influenced by various factors such as age, hormones (3, 5, 33), genetics (1), lifestyle or diet (20), and inflammation (15, 34). This urologic disease severely impacts the quality of life for aging men and represents a tremendous financial burden on the healthcare system, with direct costs estimated at $3.9 billion annually (40).

Diagnosis of BPH/LUTS includes evaluation of medical history to gauge the symptom severity using a questionnaire referred to as the International Prostate Symptom Score index (IPSS), physical examination, imaging, and laboratory tests. Urodynamic functional tests are often performed to evaluate bladder capacity, urine flow rates, post void residual measurement (PVR), and pressure-flow rates. Cystometry allows simultaneous measurement of bladder pressure and urine flow rates, bladder compliance, capacity, and sensation in humans (7).

While the molecular mechanisms associated with BPH/LUTS are largely unknown, hormones have been implicated in this disease (3, 5, 33). Considering the reported importance of hormones such as testosterone (T) and 17β-estradiol (E_2) (8, 32, 36, 45, 54) in the development of BPH/LUTS, we sought to evaluate the effects of exposure to environmental estrogens such as bisphenol A (BPA) and two of its analogues, bisphenol-F (BPF) and bisphenol-S (BPS), on lower urinary tract structure and function. BPA is monomer used in the production of polycarbonate plastics and is structural similar to the potent manmade estrogenic drug diethylstilbestrol (DES) and the potent endogenous estrogen,17β-estradiol (E_2)

(2, 52). BPA is classified as an environmental estrogen that binds to estrogen receptors and alters the expression of estrogen-regulated genes in estrogen sensitive tissues (48) such as the bladder (11), urethra and the prostate (29). The aim of this study is to evaluate the effects of exposure to bisphenols using a novel mouse model that recapitulates endpoints associated with BPH/LUTS such as increased glandular growth, bladder outlet obstruction, and voiding dysfunction (31).

3.3 Results

Serum levels of bisphenols

Analysis of BPA, BPA glucuronide, BPA sulfate, and seven other bisphenol analogues (AF, AP, B, F, P, S, Z) in mice serum was performed using LC-MS/MS. Each serum sample was injected twice, and the average was reported in ng/mL. Synthetic human serum was used as the matrix blank. The method's limit of detection (LOD) for free BPA, BPA glucuronide, and bisphenol F was 0.05 ng/mL, while the method's limit LOD for BPA sulfate was 0.0025 ng/mL, and 0.02 ng/mL for bisphenol S. Each analyte has a lower limit of quantification (LLOQ) of 0.10 ng/mL except for BPS which had a LLOQ of 0.05 ng/mL. The serum levels of T+BPF, BPF, and UNT mice serum levels were below the limits of detection while the mice treated with T+BPA, T+BPS, BPA, and BPS had detectable levels of each analyte (**Table 3-1**). The LC-MS/MS panel also included four additional bisphenol analytes, BPAP, BPAF, BPB, BPP, and BPZ, for which serum levels were below limits of detection. The method's limit of detection for the additional bisphenol analytes was 0.10 ng/mL while the LLOQ was 0.20 ng/mL.

Bladders

As shown in **Figure 3-1**, bladder mass was significantly greater in mice treated with T+E_2, T+BPA, T+BPS, and T+BPF compared to controls ($p<0.0001$), but there were no differences in bladder mass among mice treated with T+BPA, T+BPS, or T+BPF. **Table 3-2** provides additional prostate and bladder volume measurements for all treatment groups.

Histopathology

The urethra combined with the urethral prostate from each mouse was agar embedded to form an array that was then paraffin embedded. Paraffin blocks were serial sectioned at 5 µm intervals. As previously described by Nicholson et al. 2012, every 20th section was stained with hematoxylin and eosin (H&E)(31). **Figure 3-2** illustrates identification of the midpoint of the prostatic urethra, and we evaluated the histologic appearance of T+E_2, T+BPA, T+BPF, and T+BPS treated mice to that of control mice. We used an automatic contour program, BioVis 3D software (Montevideo, Uruguay), to trace the cross-sectional area of prostatic urethral lumen. The weight of the hemiprostate mass, primarily through the ventral prostate, was increased with T+E_2, T+BPA, T+BPF, and T+BPS treatment when compared to control mice (**Figure 3-3A, *****$p<0.0001$). An increased number of prostatic ducts (glandular tissue) was observed in the proximal urethra in mice treated with T+E_2, T+BPA, T+BPF, and T+BPS compared to control mice (**Figure 3-3B**; $p<0.01$). T+E_2 and T+BPA treated mice had significant narrowing of the urethral lumen compared to control mice (**Figure 3-3C,** $p<0.05$), and there was a negative correlation between the number of prostatic ducts and the size of the urethral lumen (**Figure 3-3D**; $p=0.0045$). The negative correlation between the number of prostatic ducts (glandular tissue) and the urethral lumen was determined to be independent of treatment (**Figure 3-3E;** $p=0.3169$) which can be attributed to high variability within each treatment group.

Cystometry

The patterns of cystometric tracings were different between controls and mice treated with T+E_2, T+BPA, T+BPF, and T+BPS (**Figure 3-4A-G**). Non-voiding bladder contractions were observed in E_2, T+E_2- and T+bisphenol-treated mice (**Figure 3-4C-G**). Baseline pressure (BP) was significantly increased in mice treated with E_2, T+E_2 and T+BPF (**Table 3-3**, $p<0.01$). Mice treated with T+BPF exhibited a decreased peak flow rate (**Table 3-3**, $p<0.05$). T+E_2, and E_2 treatments were associated with increased threshold pressure (TP). Voided volumes (**Table 3-3**) and inter-contractile intervals (**Table 3-3**) were increased in T+E_2 and E_2 treated mice (**Table 3-3,** $p<0.001$ and $p<0.0001$, respectively). T+BPS treated mice had

decreased TP (**Table 3-3,** $P<0.0001$) and increased compliance when compared to control and T+BPA-treated mice (**Table 3-3**, $p<0.01$).

3.4 Discussion

Humans are continuously exposed to environmental estrogens like BPA analogues, but the urologic risks associated with prolonged exposure to exogenous estrogens are largely unexplored. The findings in our rodent model are similar to the clinical observations found in men who suffer from BPH/LUTS (30) and supports the concept that estrogens may contribute to the pathogenesis of BPH/LUTS and by others (3, 8, 33). These findings are consistent with increased glandular prostatic growth, bladder outlet obstruction, and voiding dysfunction observed in T+E_2 treated mice (31).

T+BPA exposure induced bladder and prostate enlargement that were associated with non-voiding contractions. Non-voiding contractions are thought to indicate detrusor instability, and this may be further promoted by narrowing of the urethral lumen. Furthermore, we observed a significant increase in the number of prostatic ducts (glandular tissue) in the prostatic urethra of T+bisphenol treated mice compared to control mice. There was a negative trend between the number of prostatic ducts in the prostatic urethra and the size of the urethral lumen for all treatment groups. Post treatment, as the number of prostatic ducts increased, the size of the urethral lumen decreased. Because of the high variability within treatment groups we were unable to determine if the negative trend between the number of prostatic ducts and the size of the urethral lumen was treatment specific. Among the T+bisphenol-A analogue treated mice, there was greater variability per animal in prostatic duct number and urethral lumen size. More specifically, BPA analogue-induced glandular growth did not lead to decreased urethral luminal size, which suggests either that more mice should be studied or that these analogues induce BPH/LUTS in rodents through another mechanism such as fibrosis (19). To probe this further, future studies will focus on evaluating the extracellular matrix surrounding the urethra lumens of T+bisphenol treated mice (51).

We evaluated bladder function by performing anesthetized cystometry after two months of treatment. The cystometric tracings for treated mice differed significantly from the controls. Most notably, we found that

there were more non-voiding contractions in T+E_2- and T+bisphenol-treated mice compared to control mice (20). However, when we evaluated the cystometric measurements using quantitative methods, T+BPS and T+BPF induced significant changes in baseline pressure, peak urine flow, or compliance. More specifically, T+BPF induced increased baseline pressure that was associated with a decrease in peak flow rate. These results appear to indicate that T+BPF treatment caused an increase in non-voiding pressure leading to a decrease in the rate at which urine is expelled through the urethra. Taken together, our data suggests that T+BPF treatment induced bladder and prostate enlargement leads to decreased peak flow rate. Our results further indicate that T+BPS treatment induced bladder and prostate enlargement associated with increased compliance. Future studies using isolated bladder strips in contractile assays would provide further information on the effects of T+BPS on bladder contraction and the specific receptors responsible (6, 10, 20). T+E_2 treatment resulted in increased baseline and threshold pressures that were associated with increased voided volume and ICI. It appears that urinary dysfunction associated with T+E_2 treatment is primarily the result of the effects of estrogen treatment considering similar findings with E_2 only treatment (**Table 3-3**). These results of estrogen induced bladder outlet obstruction are consistent with other reports (20, 43, 46)

Very few studies have evaluated the urologic effects that develop because of exposure to environmental estrogens. Our results clearly demonstrate that common environmental estrogens can alter the function and structure of the lower urinary tract in adult male mice. Our findings suggest that exposure to BPA or BPA analogues could pose a concern for aging men relative to development of BPH/LUTS. While, results generated in rodent models are not directly translatable to humans; our data highlight the potential risks to humans as a result of exposure to environmental estrogens. BPA exposure is associated with reproductive disorders, metabolic syndrome, and prostate cancer (4, 22, 47, 53). Given these adverse findings in various scientific studies along with widespread public scrutiny of BPA, bisphenol alternatives such as BPF and BPS (26) were introduced as potential replacements in many consumer products (12, 17, 56). These bisphenol analogues offer enhanced structural benefits for various industrial applications, and they are currently being used in thermal paper (13, 18), packaging (38), and

various other products (35, 50). Humans are exposed to BPA and its analogues on a daily basis through various routes of exposure via ingestion (9, 39, 56), dermal (13, 24), and inhalation(25, 37). More specifically, the reported averages of BPF, BPS, and BPA in human urine are 0.08, 0.13, and 0.72 ng/mL respectively (55). At the time serum was collected in our experiment, the mean levels of total BPA were 3.37 ng/ml. The mean level of BPS was 0.64 ng/ml, and the level of BPF was below the limits of detection (0.05 ng/ml). These rodent serum levels are within in the range of the levels measured in adult men and women (23, 25, 49). The estrogenic activity of these analogues (21, 28, 44) should be taken into consideration given the fact that estrogen is implicated in BPH/LUTS development and progression.

3.5 Figures and Legends

Figure 3-1. T+bisphenol or T+E_2 exposure induced increased bladder mass

A. Urogenital tracts from Control, T+BPA, T+BPF, T+BPS, and T+E_2 treated mice for two months. **B**. At the time of necropsy, bladders were carefully dissected, emptied of urine, blotted dry, and the mass determined. There was a significant increase in bladder mass in the T+E_2, T+BPA, T+BPF and T+BPS groups, relative to control. Bl=bladder, Ur=urethra, SV=seminal vesicle; ***p<0.05, ***p<0.0001**

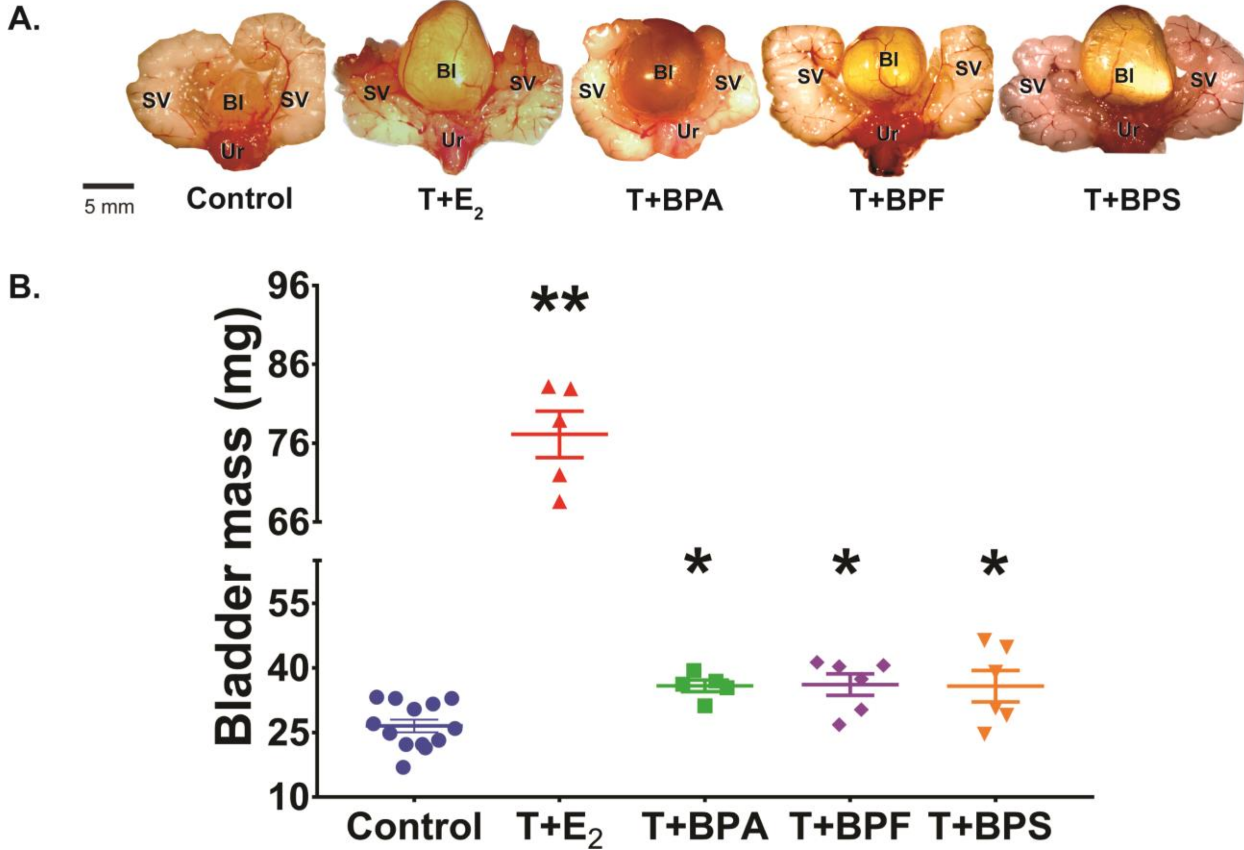


Figure 3-2. Identifying the midpoint of the prostatic urethra

The midpoint of the prostatic urethra was determined as previously described by Nicholson et al. 2012. **1.** The start site of the prostatic urethra (PRS-U) was determined when the rhabosphincter (Rb) is present <1% of the prostatic urethra. **2.** The midpoint was determined when the seminal vesicles (SV), ejaculatory ducts (ED), prostatic tissue (PT), and urethral lumen (UL) are present with a fully enclosed rhabdospincter. **3.**The endpoint was determined when the rhabdospincter is present with the membranous urethra (MBU). All histologic analysis were performed using the midpoint image for each treatment group. Prostate cancer and prostate intraepithelial neoplasia were not observed in the urethra. * Vas deferens (VD), bladder neck (BN), periurethral space (PUS), periurethral gland (PUG), anterior prostate lobe (AP), dorsolateral prostate lobe (DLP), ventral prostate lobe (VP).

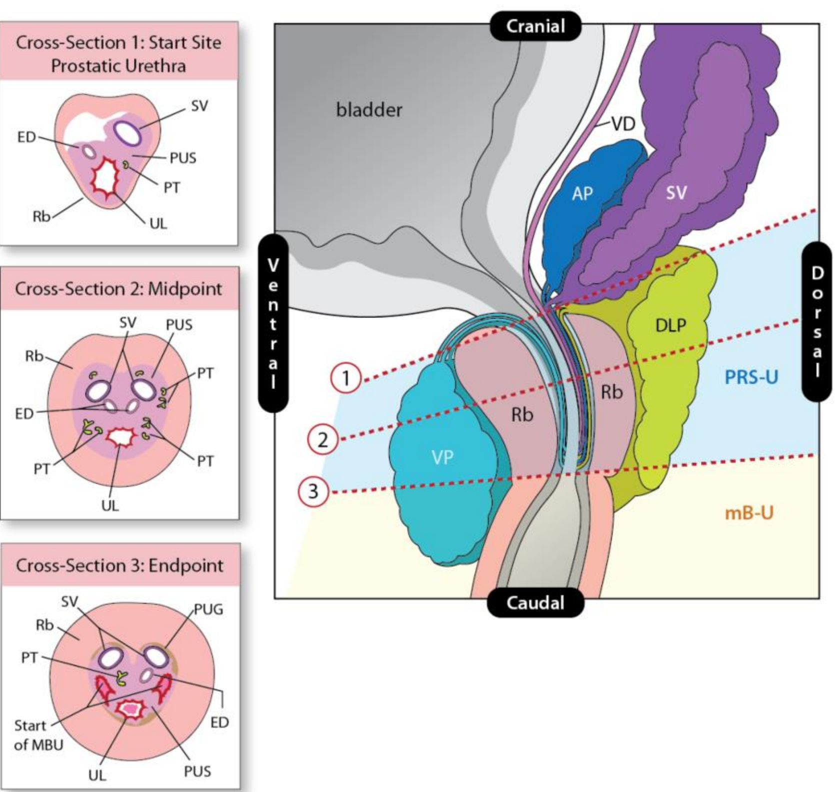


Figure 3-3. T+ bisphenol exposure caused prostate enlargement and glandular growth in the prostatic urethra

A. T+bisphenol exposure caused a significant increase in the number of prostatic ducts or glandular tissue in the proximal urethra compared to the control group (p<0.01). **B**. Mice treated with T+E_2 and T+BPA had a significantly smaller average cross-sectional area of the prostatic urethral lumen compared to the control group (p<0.05). **C**. We observed a moderate correlation in which the prostatic duct number (glandular tissue) increases as the cross-sectional area of the urethral lumen decreases between all treatment groups (p-0.005). **D**. We determined that this negative correlation between the prostatic duct number (glandular tissue) and the cross-sectional area of the urethral lumen is independent of treatment (p=0.3169). ***p<0.05, **p<0.01**

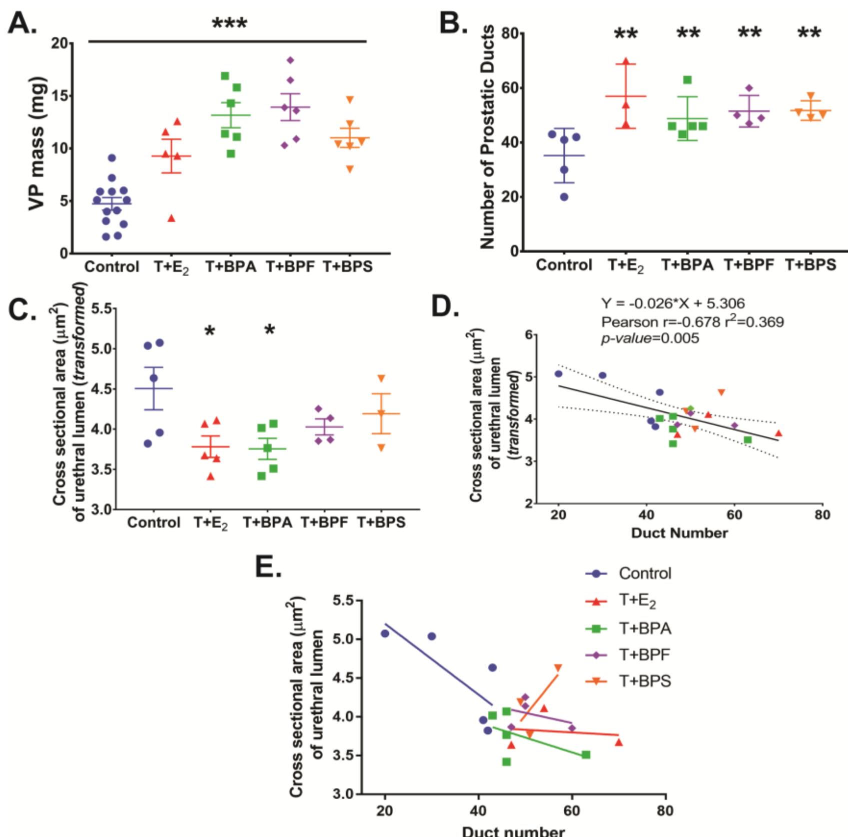
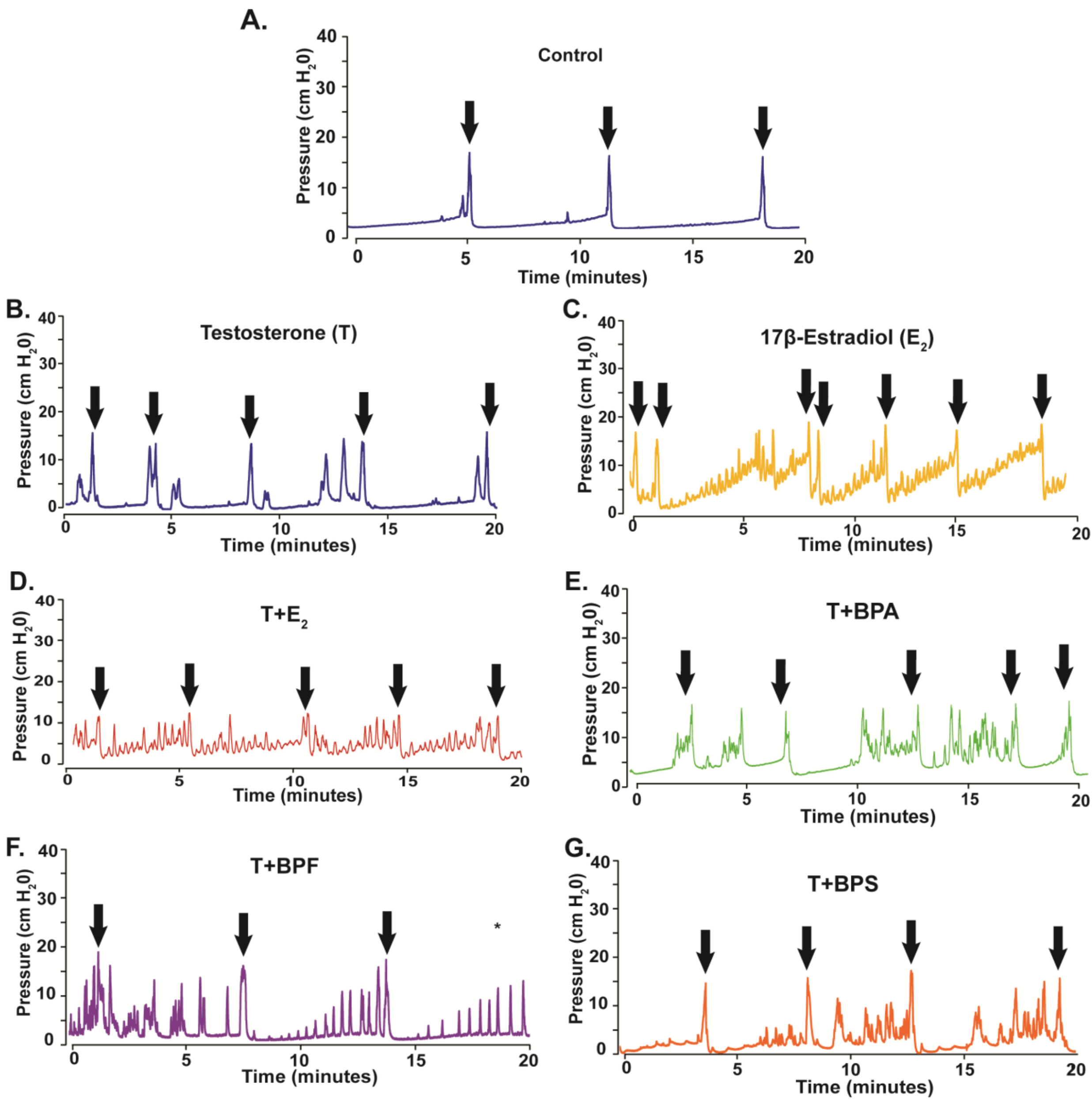


Figure 3-4. T+bisphenol exposure induces notable differences in cystometric tracings

Representative cystometric tracings for T+bisphenol treated male mice. Compared to control mice (***panel A-C***), T+E_2 (***panel D***), and T+bisphenol (***panel E-G***) treated mice displayed non-voiding contractions that were not associated with urinary voiding. The black arrows indicate a voiding event. *indicates that the mouse is leaking, or the bladder was no longer contracting.



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3.6 Tables

Table 3-1. Serum levels of Bisphenols in adult male mice

Values are reported as means in ng/mL. UNT, untreated controls. LOD, limit of detection. [a]0.05ng/mL [b]LOD=0.025 ng/mL [c]0.02ng/mL

Treatment	Free BPA[a]	BPA glucuronide[a]	BPA sulfate[b]	BPF[a]	BPS[c]
UNT (n=2)	<LOD	<LOD	<LOD	<LOD	<LOD
T+BPA (n=5)	<LOD	3.37	<LOD	<LOD	0.21
T+BPF (n=6)	<LOD	<LOD	<LOD	<LOD	0.03
T+BPS (n=4)	<LOD	<LOD	<LOD	<LOD	0.64
BPA only (n=2)	8.45	63.15	0.47	<LOD	<LOD
BPF only (n=2)	<LOD	<LOD	<LOD	<LOD	<LOD
BPS only (n=2)	<LOD	<LOD	<LOD	<LOD	0.02

Values are reported as means in ng/mL. UNT, untreated controls.
LOD, limit of detection.[a]/*LOD*=0.05 ng/mL [b]LOD=0.025 ng/mL, LOD= [c]0.02 ng/mL

Table 3-2. Effects of T+Bisphenol exposure on the lower urinary tract of adult male mice

Results are reported as mean± SEM, with the number of replicates shown in parentheses. *Significantly different from control (Untreated+Cholesterol groups combined)

***p<0.05; **p<0.01, ***p<0.0001**

Treatment	Body weight (g)	Bladder mass (mg)	Bladder Volume (mm^3)	Hemiprostate mass (mg)	Ventral prostate (mg)	Anterior Prostate mass (mg)	Dorsolateral Prostate (mg)
Control	34.3 ± 0.9 (13)	26.5 ± 1.5 (13)	38.9 ± 20.5 (13)	28.2 ± 1.9 (13)	4.7 ± 0.6 (13)	18.4 ± 1.0 (13)	5.0 ± 0.7 (13)
T+E_2	30.1 ± 1.2* (5)	77.1 ± 2.9*** (5)	305.0 ± 188* (5)	39.2 ± 4.6 (5)	9.3 ± 1.6* (5)	22.0 ± 3.8 (5)	8.0 ± 1.3** (5)
T+BPA	33.5 ± 1.2 (6)	35.8 ± 1.3* (5)	66.1 ± 44.2* (6)	42.9 ± 2.1*** (6)	13.2 ± 1.2***(6)	22.8 ± 1.1 (6)	6.9 ± 0.7 (6)
T+BPF	36.3 ± 1.2 (6)	36.1 ± 2.5* (6)	17.2 ± 4.2 (6)	44.0 ± 3.1*** (6)	13.9 ± 1.3*** (6)	22.6 ± 2.5 (6)	7.5 ± 1.2** (6)
T+BPS	34.9 ± 1.5 (6)	35.8 ± 3.7* (6)	52.7 ± 22.1* (6)	40.6 ± 3.7** (6)	11.0 ± 0.9***(6)	21.8 ± 3.4 (6)	7.8 ± 1.0** (6)
T	38.3 ± 0.8* (6)	34.7 ± 2.1 (6)	53.1 ± 41.8 (6)	39.7 ± 4.8* (6)	9.5 ± 1.6** (6)	24.9 ± 2.9 (6)	5.4 ± 0.7 (6)
E_2	29.6 ± 1.0** (5)	59.8 ± 4.3*** (5)	61.8 ± 33.8* (5)	5.7 ± 1.9*** (5)	0.8 ± 0.3*** (5)	4.0 ± 1.5***(5)	0.9 ± 0.4** (5)
BPA	34.7 ± 1.0 (7)	27.4 ± 1.4 (7)	41.9 ± 32.9 (7)	26.7 ± 3.5 (7)	5.4 ± 0.9 (7)	16.7 ± 3.4 (7)	4.6 ± 0.7 (7)
BPF	33.0 ± 1.3 (7)	24.2 ± 2.6 (7)	10.8 ± 4.6 (7)	21.8 ± 1.8 (7)	3.9 ± 0.7 (7)	14.0 ± 0.7 (7)	3.9 ± 0.8 (7)
BPS	30.8 ± 1.7 (7)	27.0 ± 2.2 (7)	60.5 ± 23.1 (7)	23.4 ± 2.4 (7)	4.5 ± 0.4 (7)	14.7 ± 2.0 (7)	4.2 ± 0.3 (7)

Results are reported as mean ± SEM, with the number of replicates shown in parentheses.
*Significantly different from control (UNT+CHO) ***p<0.05**; ****p<0.01**; *****p<0.0001**

Table 3-3. Effects of T+bisphenol exposure on rodent cystometric parameters

Results are reported as mean±SEM, with the number of replicates shown in parentheses.

*Significantly different from control (Untreated+Cholesterol groups combined)

***p<0.05; **p<0.01, ***p<0.0001**

Treatment	Baseline pressure (cmH$_2$0)	Threshold pressure (cmH$_2$0)	Normalized Threshold pressure (cmH$_2$0)	Maximum voiding pressure (cmH$_2$0)	Amplitude (cmH$_2$0)	Peak flow rate (µl/sec)	Voided volume (mg)	Compliance (µl/cmH$_2$0)	Inter-contractile interval (minutes)
Control	4.8 ± 0.4 (21)	9.3 ± 0.7 (22)	4.7 ± 0.4 (22)	32.1 ± 1.3 (22)	28.0 ± 1.7 (16)	32.9 ± 3.0 (14)	64.4 ± 6.8 (14)	13.4 ± 1.4 (21)	2.7 ± 0.2 (22)
T+E_2	**9.0 ± 0.7** (4)**	**20.0 ± 5.6*** (3)**	**10.0 ± 5.2** (3)**	34.5 ± 1.7 (4)	26.0 ± 1.5 (4)	29.2 ± 3.3 (3)	**142 ± 24.0* (3)**	5.5 ± 0.9 (2)	**7.1 ± 2.9** (2)**
T+BPA	4.4 ± 0.8 (7)	8.0 ± 0.4 (7)	3.6 ± 0.7 (7)	29.4 ± 2.1 (7)	25.0 ± 2.3 (7)	27.6 ± 5.7 (5)	81.3 ± 18.0 (6)	13.6 ± 2.3 (6)	2.8 ± 0.4 (7)
T+BPF	**8.0 ± 1.2** (6)**	10.6 ± 1.9 (6)	3.0 ± 1.2 (6)	36.9 ± 4.6 (6)	26.0 ± 2.0 (6)	**15.8 ± 3.5** (4)**	75.0 ± 20.0 (4)	15.6 ± 1.6 (4)	3.3 ± 0.8 (5)
T+BPS	3.6 ± 0.9 (6)	**5.7 ± 0.8* (6)**	**2.1 ± 0.7*** (6)**	27.0 ± 1.8 (6)	22.2 ± 2.2 (6)	20.2 ± 5.7 (4)	80.5 ± 10.7 (4)	**38.0 ± 14*** (5)**	3.3 ± 0.3 (6)
T	4.4 ± 1.0 (7)	9.1 ± 1.3 (7)	4.7 ± 0.4 (7)	29.1 ± 3.0 (7)	25.0 ± 2.5 (7)	31.3 ± 4.8 (6)	77.3 ± 11.0 (5)	13.2 ± 2.3 (7)	3.3 ± 0.7 (7)
E_2	**9.8 ± 1.7*** (8)**	**21 ± 1.8*** (7)**	**12.7 ± 1.1*** (7)**	35.7 ± 2.3 (8)	25.9 ± 3.0 (8)	25.0 ± 9.0 (7)	**204 ± 60.1***(6)**	9.4 ± 2.1 (7)	**6.5 ± 1.1*** (7)**

Results are reported as mean ± SEM, with the number of replicates shown in parentheses.
* Significantly different from control (UNT+CHO) ***p<0.05; **p<0.01; ***p<0.0001**

3.7 Materials and Methods

Pellet implantation

C57BL/6 male mice (6-8 weeks of age) were obtained from Charles River (Wilmington, MA) and reared under standard laboratory conditions (12:12 light/dark cycle) with free access to food and water. All animal experiments and procedures were conducted under a protocol approved by the University of Wisconsin's Animal Care and Use Committee. Bisphenol A (BPA), bisphenol S (BPS), 17β-estradiol (E_2) and testosterone (T) were obtained from Sigma Chemical Co. (St. Louis, MO), and bisphenol F (BPF) was purchased from MP Biomedicals, LLC (Solon, OH). Positive controls consisted of mice that underwent surgical implantation of compressed hormone pellets containing 25 mg T and 2.5mg E_2 as described previously (31). Experimental mice received 25mg T and 25mg BPA, 25 mg T and 25 mg BPS, or 25mg T and 25 mg BPF. Each 25 mg bisphenol pellet included 22.5 mg of cholesterol (CHO) which served as a binding agent to control for solubility issues. Negative controls consisted of mice who underwent the surgical procedure, but in which no pellet was implanted or mice that received a single 22.5 mg CHO pellet. Additional controls included mice treated with 25 mg T only, 25 mg E_2 only, 25 mg BPA only, 25 mg BPF only, and 25 mg BPS only. Mice were housed 4-5 per cage, separated by treatment group. For bladder and prostate mass measurements, UNT n =6; T+E_2 n=6; T+BPA n=6; T+BPF, n=6; and T+BPS, n=6. Histopathology of the prostatic urethra was performed on a subset of mice (UNT n=5; T+BPA, n=5; T+BPF, n= 5; and T+BPS, n=3). Another group of mice underwent anesthetized cystometry testing (UNT n = 21; T+E_2 n = 4; T+BPA n =8; T+BPF, n=6; and T+BPS, n=6). Serum BPA, BPA metabolites, and BPS and BPF measurements were also made in a subset of mice (n = 2-5 per group).

Serum measurements of BPA analogues

Mice were euthanized with CO_2, and cardiac puncture was performed to collect blood. Serum was collected by allowing blood to clot followed by centrifugation at 12,000 rpm for 15-20 minutes at 4°C. Serum was collected (200-300 µl/mouse), transferred to a new Eppendorf tube, and stored at -80°C until serum measurements were performed. Serum bisphenol analogues were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described by Gerona et al (14).

Direct simultaneous analysis of BPA, BPA glucuronide and BPA sulfate, and seven other bisphenol analogues (AF, AP, B, F, P, S, Z) were measured by LC-MS/MS using an Agilent LC 1260 (Agilent Technologies, Sta. Cruz, CA)- AB Sciex 5500 (AB Sciex, Foster City, CA). Each analyte was ionized using electrospray ionization in the negative mode and was monitored by multiple reaction monitoring using two transitions for each analyte and BPA-d16 as internal standard. The limit of detection (LOD) for BPA and BPA glucuronide is 0.05ng/mL while the LOD for BPA sulfate is 0.025 ng/mL. The LOD for all bisphenol analogues is 0.1 ng/mL except for BPS which is 0.02 ng/mL. Quantitation of each analyte was done by isotope dilution method using a 10-point calibration curve. Each analyte has a limit of quantitation (LOQ) of 0.1ng/mL except BPS (0.05 ng/mL) and BPAF, BPAP. BPB, BPP, and BPZ (0.2 ng/mL).

Serum was prepared for LC-MS/MS analysis by solid phase extraction using Waters Oasis HLB cartridge (1mL). Each SPE cartridge was washed with 5 column volumes of methanol to get rid of its reported BPA contamination. The cartridges were then activated with water (1mL) before 250 µL of cord serum was loaded. The column was washed with 5% methanol (1 mL) before each analyte was eluted by methanol (1mL). The methanol eluates were evaporated under a stream of nitrogen gas after which they were reconstituted in 10% methanol for column injection (250 µL).

A 25 µL aliquot of the extract was used for each of the replicate injections of the sample.

Chromatographic separation of the analytes were achieved by gradient elution using water with 0.05% ammonium acetate (pH=7.8) as mobile phase A and methanol with 0.05% ammonium acetate (pH=7.8) as mobile phase B. The elution gradient employed was- 0-1 min= 5%B; 5 min= 98%B; 5-7 min= 98%B; and 7-8.5 min= 5% B. AB Sciex Analyst and MultiQuant were used for data analysis.

Bladder measurements and prostate lobe collections

Bladder volume and weights of the bladder and prostate were determined after collection of blood as previously described (31). To eliminate or reduce bias, the mice treatments were masked from the researchers during necropsy. A caliper was used to measure bladder volume by taking transverse plane measurements and then the ellipsoid equation was used to calculate bladder volume. Bladders were

excised, blotted, and wet bladder weight was measured with an analytical balance. During necropsy, the prostate lobes were dissected, and the weights were recorded for the left half of the prostate. The left anterior prostate lobe (AP), the left ventral prostate lobe (VP), and the left dorsolateral (DLP) prostate mass were recorded for each mouse. Additionally, the hemiprostate mass (anterior prostate, ventral prostate, and dorsal lateral prostate) was calculated by adding the masses for each lobe of the prostate together.

Cystometry

Cystometry was performed to assess bladder function as detailed on University of Wisconsin's O'Brien Center for Urologic Research Rodent Urinary Function Testing Core (RUFT) website (www.urology.wisc.edu/researchU54-george-m-obrien-center-for-benign-urology-research/; accessed 1/12/2018). As previously described by Bjorling et al., mice were anesthetized with urethane (1.43 g/kg, subcutaneous injection), an incision was made into the caudal abdomen, a purse string suture was placed in the bladder dome, and a PE 50 cannula was inserted into the bladder via an incision made in the center of the purse string suture (7). The suture was tightened and tied to secure the cannula. The cannula was connected to a physiological pressure transducer (Memscap AS, Norway) and infusion pump (Harvard Apparatus, Holliston, MA). Room temperature saline was infused (1.5 mL/hour) for 60-90 minutes to elicit repetitive micturition cycles. Intravesical pressure was recorded continuously using a PowerLab data collection system (ADInstruments, Colorado Springs, CO) connected to a PC computer. At least 6 reproducible micturition cycles were recorded and analyzed to determine baseline pressure, intercontractile interval (ICI; time between micturition events), threshold pressure (intravesical pressure at which voiding began), maximal voiding pressure (peak intravesical pressure during voiding), void volume, void duration, and bladder compliance. Bladder compliance was determined by calculating the ratio of the change in volume over the first 10% of bladder filling divided by the change in pressure during this time as previously described (41, 42). Urine flow was calculated by dividing void volume by void duration.

Statistical analysis

For all measurements data analysis for comparisons among control and treated mice was performed using one-way ANOVA (GraphPad Prism, LaJolla, CA) or Student's t-test when appropriate. Data were evaluated for homogeneity of variance using Bartlett's unequal variance test, and when necessary, logarithmic and/or rank transformation was performed. For all measurements, the graphs show means ± SEM, unless otherwise noted. In all analyses $p < 0.05$ was considered statistically significant. We observed no significant difference between cholesterol (CHO)-treated and untreated mice, therefore these groups were combined into one negative control group as indicated on all graphs, unless otherwise noted. ***p<0.05**, ****p<0.01, ***p<0.0001**

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Chapter 4: Bisphenol-A elicits mouse urinary dysfunction through an *Esr1* dependent mechanism

Sections of this chapter have been adapted from the following publications:

Nguyen JL, Lamarre N, Bjorling D, Ricke, WA. Bisphenol-A induces mouse lower urinary tract dysfunction through *Esr1* dependent mechanism. *Preparing for submission to* PNAS.

Dr. Neil Lamarre, Peiqing Wang, and Zunyi Wang performed Uroflow measurements on every mouse in this study.

Tom Pier from the UWCCC Experimental Pathology laboratory performed paraffin embedding and sectioning of the mouse urethras.

Dr. Clara Jeong and Samuel Thomas performed the mass spectrometry analysis for this study.

Yuchen "Vanesssa" Ying helped with BioVis and urethra morphometric measurements.

Jalissa Nguyen's contributions included all the *in vivo* work, all the data analysis, tissue collections, and agar embedding for this study.

4.1 Abstract

While steroid hormones such as Testosterone (T) and 17β-estradiol (E_2) are essential in prostate growth and development, they have also been implicated in benign prostate hyperplasia (BPH), the molecular mechanisms are poorly understood. Estrogens act via estrogen receptor 1 (*Esr1 or ERα*) and estrogen receptor 2 (*Esr2 or ERβ*) and they reportedly play a role in BPH. The environmental estrogen, bisphenol-A (BPA), has weak estrogenic activity and we have previously reported its ability to induce lower urinary tract dysfunction (LUTD) in a rodent model. In this study, we evaluated the relative contributions of ERα and ERβ in BPA induced LUTD using a rodent model that recapitulates prostate and bladder complications that may be associated with BPH. We also evaluated changes in rodent urinary voiding behavior using noninvasive uroflowmetry (uroflow) and measured changes in steroid hormone levels in rodent serum via LC-MS/MS after two months of treatment. Adult male C57bl/6 mice received implants of slow release pellets that contained 25mg testosterone (T) and 2.5mg 17β-estradiol (E_2) or 25mg T and 25mg BPA, and untreated (UNT) controls underwent sham surgery. To determine the relative contributions of estrogen receptor (ER) subtypes in BPA induced lower urinary tract dysfunction, we used mice null for Estrogen receptor 1 ($Esr1^{-/-}$) or Estrogen receptor 2 ($Esr2^{-/-}$) compared to their respective untreated (UNT) littermates. While *Esr1* and *Esr2* wildtype littermates treated with T+E_2 and T+BPA developed enlarged bladders ($p<0.0001$ and $p<0.05$ respectively). $Esr1^{-/-}$ mice treated with T+E_2 or T+BPA did not. $Esr2^{-/-}$ mice treated with T+E_2 or T+BPA also developed bladder enlargement ($p<0.0001$ and $p<0.05$ respectively). In *Esr1* and *Esr2* wildtype littermates treated with T+BPA we observed an increased in the number of prostatic ducts or glandular tissue ($p<0.01$) while $Esr1^{-/-}$ mice treated with T+BPA showed no significant change when compared to untreated littermates. Additionally, $Esr2^{-/-}$ mice treated with T+E_2 or T+BPA displayed an increased in the number of prostatic ducts or glandular tissue in the prostatic urethra ($p<0.01$ *and* $p<0.0001$ respectively), but only mice treated with T+BPA developed a decrease in the cross-sectional area of the urethral lumen ($p<0.01$). In wildtype mice, T+E_2 or T+BPA treatment caused increased urinary retention which correlated with a decrease in the amount of urine voided (p=0.0338). Additionally, wildtype and $Esr2^{-/-}$ mice treated with T+E_2 or T+BPA

appear to display an inverse relationship between the number of voids and voided volumes. Essentially, wildtype and *Esr2*$^{-/-}$ mice treated with T+E_2 or T+BPA appear to have increased voiding frequency and decreased in voided volumes (*p=0.0279* and *p=0.0408* respectively), while *Esr1*$^{-/-}$ mice treated with T+E_2 and T+BPA appear to have a direct relationship between the number of voids and voided volumes like untreated littermates (*p=0.0001*). In this study, *Esr1* wildtype littermates treated with T+BPA had a significant increase in serum levels of T and 3α-Androstanediol (3α-diol)/3β-Androstanediol (3β-diol) while *Esr1*$^{-/-}$ treated with T+BPA did not. *Esr2* wildtype littermates treated with T+BPA displayed increased serum levels T, Dihydrotestosterone (DHT), and 3α/3β-diol while *Esr2*$^{-/-}$ mice displayed increased 3α/3β-diol serum levels. With T+E_2 treatment, *Esr1*$^{-/-}$ mice treated with T+E_2 displayed an increase in serum levels T, DHT, and 3α-diol/3β-diol. Taken together, our results indicate that *Esr1*, but not *Esr2*, is a key mediator of BPA induced bladder complications and urinary dysfunction associated with BPH/LUTS. Additionally, we propose a model where BPA, an environmental estrogen, alters testosterone metabolism through a possible estrogen receptor β (ERβ/*Esr2*) and inactive androgen driven mechanism.

4.2 Introduction

Benign prostate hyperplasia (BPH) impacts more than 50% of men over the age of 50 and its closely associated with lower urinary tract symptoms (LUTS) which impacts 50-90% of men over the age of 50 (11). Clinically significant BPH/LUTS can manifest as a constellation of symptoms such as enlargement of the prostate, hyperplasia of glandular and stromal tissues surrounding the prostatic urethra, or lower urinary tract dysfunction (LUTD) which is associated with increased urinary frequency and decrease voided volumes (11, 22). If left unmanaged, BPH/LUTS can cause obstructive symptoms related to weak urine flow, urine retention, and ultimately death. The molecular mechanism of BPH is poorly understood and limited therapies exist for patients who suffer from symptomatic BPH. Current treatment for BPH and lower urinary tract symptoms (LUTS) include adrenergic receptor antagonistic blockers (alpha-blockers) to relax smooth muscle and increase urine flow and 5α-reductase inhibitors to decrease intraprostatic dihydrotestosterone (DHT) production and reduce prostate size (6, 10, 28). Unfortunately, α-blockers and 5α-reductase inhibitors only treat the symptoms of BPH and are not curative. Furthermore, there are various adverse side effects including low blood pressure, low treatment efficacy, and decreased libido.

While it is widely accepted that steroid hormones such as testosterone (T) and dihydrotestosterone (DHT) play a role in BPH, the actions of estrogens such as 17β-estradiol (E_2) have been implicated in BPH/LUTS. More specifically, as men age serum androgens decline as the prevalence of BPH-LUTS increases, while serum estrogens such 17β-estradiol (E_2) increase or remain constant. Thus, the aging male hormone environment consists of an increased Testosterone (T) to E_2 ratio which may contribute to the age associated increase in prostate size observed in men. Estrogens in men are important steroid hormones that help regulate reproductive organs and other processes by binding to estrogen receptors (ERs) to facilitate genomic and non-genomic responses. ERs are expressed in the male lower urinary tract and reproductive tissues such as the bladder, prostate, urethra, and testis (8, 18). Estrogen receptor α (ERα) is expressed in the developing prostate but it is not needed for normal development (1). However, ERα is required for the adverse effects of exogenous E_2 on prostate development and squamous

metaplasia. ERα has been shown to have a proliferative effect on the stroma and has been detected in prostate stromal and epithelial cells (30). Estrogen receptor β (ERβ) has been demonstrated to have an anti-proliferative effect on the prostate epithelium (20). ERβ has also been identified as a beneficial pro-apoptotic ER in BPH and prostate cancer (21). The differential roles of estrogen receptors (ERα and ERβ) in various cell types and their tissue specificity highlight the need to investigate their role in BPH (13). Our lab has previously evaluated the contribution of E_2 and ERα/ERβ to bladder complications associated with BPH. We have shown that ERα is the key mediator of urinary dysfunction characterized by enlarged bladders, urinary retention, increased bladder mass, and decreased peak uroflow (i.e. development of BPH/LUTD) (24, 25).

In addition to estrogens, ERs have been shown to possess affinity for environmental contaminants such as bisphenol-A (BPA), phthalates, and polycyclic aromatic hydrocarbons (5). These environmental contaminants are also known as environmental estrogens because they can mimic and/or interfere with ER-signaling in estrogen responsive tissues (3, 16, 23, 32). More specifically, BPA is a known environmental estrogen and endocrine disruptor because it mimics and or interferes with estrogen receptor signaling (ER) in estrogen responsive tissues such as the prostate, bladder, and urethra (8, 18). BPA is one of the highest volume chemicals produced worldwide with over 6 billion pounds produced each year and is estimated to be found in the urine of more than 90% of people in the United States at levels greater than 100ng/mL (relative to E2=pg/mL found in men) in the urine (34). BPA exposure has been implicated in adverse effects on human reproduction, prostate cancer, and disruption of prostate development (35, 36). Little is known about the molecular and biological action of BPA in animals and humans (40). With the large incidence of BPH (nearly ubiquitous) observed in aging men and the exposure of BPA to humankind (also ubiquitous), the objective of this study was to elucidate the role of BPA in ER hormone action and urinary function. More specifically, we evaluated the relative contributions of ERα and ERβ in BPA induced LUTD using a rodent model that recapitulates prostate and bladder complications that may be associated with BPH.

4.3 Results

ERα is necessary for bladder complications in male mice treated with T+BPA and T+E_2

After two months of treatment, bladder enlargement was observed in *Esr1* WT littermates treated with T+E_2 or T+BPA (**Figure 4-1A and 4-1B,** $p<0.0001$ and $p<0.05$ respectively). There was no change in bladder mass in *Esr1*$^{-/-}$ mice treated with T+E_2 or T+BPA (**Figure 4-1A and 4-1C**). *Esr2* WT littermates treated with T+E_2 or T+BPA had increased bladder mass when compared to *Esr2* WT UNT littermates (**Figure 4-2A and 4-2B,** $p<0.0001$ and $p<0.05$ respectively). Additionally, bladder mass was significantly increased in *Esr2*$^{-/-}$ mice treated with T+E_2 or T+BPA when compared to *Esr2*$^{-/-}$ UNT mice (**Figure 4-2A and 4-2C,** $p<0.0001$ and $p<0.05$ respectively). **Table 4-1** provides additional prostate and bladder volume measurements for all treatment groups and genotypes.

T+BPA induced glandular growth seems to be mediated by Esr1

In chapter 3, we demonstrated that there is negative trend between the number of prostatic ducts and the cross-sectional area of the urethral lumen. Essentially, as the number of prostatic ducts or glandular growth increases the size of the cross-sectional area of the urethral lumen decreased. In this study, we evaluated the number of prostatic ducts and the size of the cross-sectional area of the urethral lumen in *Esr1* or *Esr2* mice treated with T+BPA or T+E_2 compared to their respective UNT littermates after two months of treatment (**Figure 4-3)**. *Esr1* wildtype littermates treated with T+BPA displayed an increased number of prostatic ducts or glandular tissue (**Figure 4-3A**, $p=0.0157$) while *Esr1*$^{-/-}$ mice treated with T+BPA did not (**Figure 4-3B**). T+BPA treatment caused no change in the cross-sectional area of the prostatic urethral lumen of *Esr1* wildtype nor *Esr1*$^{-/-}$ mice when compared to their respective UNT littermates (**Figure 4-3C and Figure 4-3D**). T+BPA treatment caused an increase in the number of prostatic ducts or glandular tissue in the proximal urethra of *Esr2* WT mice compared to UNT WT littermates (**Figure 4-3E**, $p=0.0011$). Additionally, *Esr2*$^{-/-}$ treated with T+E_2 and T+BPA displayed an increase in prostatic ducts (glandular tissue) after two months of treatment (**Figure 4-3F**, *$p=0.0010$* and *$p=0.0001$* respectively). As shown in **Figure 4-3G,** there was no change in the size of the cross-sectional area of the urethral lumen after treatment with T+E_2 or T+BPA in *Esr1* mice. T+BPA treatment caused a

decrease in the cross-sectional area of the urethral lumen in *Esr2*$^{-/-}$ mice when compared to UNT *Esr2*$^{-/-}$ littermates (**Figure 4-3H**, *p=0.0017*).

T+BPA treatment induced urinary retention, increased frequency, and decreased voided volumes through an Esr1 dependent mechanism

We evaluated changes in urinary retention, urinary frequency, and voided volumes after two months of treatment with T+BPA or T+E_2 using noninvasive uroflowmetry (uroflow). We observed urinary retention (**Figure 4-4**) and increased bladder mass (**Table 4-1)** in wildtype mice and *Esr2*$^{-/-}$ mice treated with T+E_2 or T+BPA. We assessed uroflow using a metabolic cage system as previously described (24). As shown in **Figure 4-4A**, in wildtype mice there appears to be an inverse relationship between bladder volume and amount of urine voided (p=0.0338). In **Figure 4-4B**, wildtype mice treated with T+E_2 or T+BPA seem to have an increased number of voids (frequency) and decreased voided volumes whereas the UNT mice seem to have a consistent number of voids that may be directly related to the average void amount (p=0.0279). As shown in **Figure 4-4C**, irrespective of treatment *Esr1*$^{-/-}$ mice have a linear relationship between the number of voids and the average void amount (p=0.0001). As shown in **Figure 4-4D**, like wildtype mice treated with T+E_2 or T+BPA, *Esr2*$^{-/-}$ mice treated with T+E_2 or T+BPA seem to have a decrease in the number of voids along with decreased voided volumes (p=0.0408).

Bisphenol-A seems to alter testosterone metabolism through an estrogen receptor β (ERβ) and inactive androgen driven mechanism

Analysis of multi steroids in the serum of male mice was performed using liquid chromatography mass spectrometry (LC-MS/MS). Each sample was injected once, and reported in ng/ml. The analytes measured in the serum of male mice via liquid chromatography mass spectrometry (LC-MS/MS) include Testosterone (T), Dihydrotestosterone (DHT), Androstenedione (Androsten.), 3α-Androstanediol (3α-diol)/3β-Androstanediol (3β-diol), Estrone (E_1), 17β-estradiol (E_2), 11-Deoxycortisol (11-DOC), and Progesterone (Prog.). The method's limit of detection (LOD) is reported in ng/ml and varies for each steroid hormone as follows: T =0.01, DHT =0.16, Androsten. =0.01, 3α-/3β-diol=0.65, E_1= 0.002, E_2=0.002, 11-DOC=0.02, Prog.=0.01. **Table 4-2** provides additional analytical parameters for each

analyte and internal standard. In this study, rodent serum steroid hormone levels were altered in *Es1* and *Esr2* littermates after two months of treatment with T+E_2 or T+BPA (**Figure 4-5 and Table 4-3**). As shown in **Figure 4-5A and 4-5B**, there were significant effects for interaction (p=0.0883), treatment (p<0.0001) and genotype (p=0.0230) for serum T levels in *Esr1* and *Esr2* wildtype mice. More specifically *Esr1* and *Esr2* wildtype mice treated with T+BPA (p=0.0236 and p=0.0012 respectively) displayed increased serum T levels when compared their respective UNT control while *Esr1*$^{-/-}$ and *Esr2*$^{-/-}$ mice treated with T+BPA displayed no change in serum T levels. *Esr1*$^{-/-}$ mice treated with T+E_2 displayed an increase in serum T levels (p=0.0022). As shown in **Figure 4-5C and 4-5D**, there were significant effects for interaction (p=0.0391), treatment (p=0.0003), genotype (p=0.0033), and interaction for serum DHT levels in *Esr1*$^{-/-}$ and *Esr2* wildtype mice. More specifically, in *Esr1*$^{-/-}$ treated with T+E_2 (p=0.0049) and *Esr2* wildtype mice treated with T+BPA (p=0.0098) displayed increased serum DHT levels when compared to their respective untreated littermates. In **Figure 4-5E and 4-5F**, there significant effects for interaction (p=0.0166), treatment (p<0.0001), genotype (p=0.0187) for serum 3α-/3β-diol levels in *Esr1* and *Esr2* wildtype mice. More specifically, *Esr1* and *Esr2* wildtype mice treated with T+BPA (p=0.0418 and p=0.0005 respectively) had increased serum 3α-/3β-diol levels when compared to their respective littermates. Additionally, *Esr1*$^{-/-}$ mice treated with T+E_2 also displayed altered 3α-/3β-diol serum levels (p=0.0044) when compared to their respective UNT littermates (**Figure 4E**). **Table 4-3** provides additional steroid hormone levels measurements for all treatment groups with respect to the genotype for each mouse.

4.4 Discussion

The ubiquitous nature of BPA and various routes for human exposure places emphasis on the importance of this study. More specifically, the urologic risks associated with prolonged repeat exposure to exogenous estrogens like BPA are largely unknown. The data presented from our rodent model are similar to the clinical observations found in patients who suffer from BPH/LUTS (25) and supports other studies that suggest that estrogens may contribute to the pathogenesis of BPH/LUTS (4, 5, 27). These

findings are consistent with increased glandular prostatic growth, bladder outlet obstruction, and voiding dysfunction observed in T+E_2 treated mice (26).

Esr1 and *Esr2* wildtype littermates treated with T+E_2 and T+BPA developed enlarged bladders while *Esr1*$^{-/-}$ mice treated with T+E_2 or T+BPA did not. Additionally, *Esr2*$^{-/-}$ mice treated with T+E_2 or T+BPA also developed bladder enlargement. These findings suggest that, T+BPA induced bladder and prostate enlargement, which might contribute to increased glandular growth and ultimately a decrease in the size of the urethral lumen, occurs through an *Esr1* dependent mechanism. These findings are particularly interesting given the fact that increased glandular growth reportedly contributes to BPH/LUTS by causing urethral obstruction (31). Because of the high variability and reduced sample size due to processing, we were unable to determine which ER subtype is responsible for T+E_2 and T+BPA induced increased glandular growth. Future studies are required to refine the selection of the representative midpoint image and to account for the extreme variability and sample loss that occurs during sample processing. While we can't statistically prove that T+E_2 treatment leads to an increase in the number of prostatic ducts in *Esr1* and *Esr2* wildtype mice, the overall trend in the data shows that the T+E_2 treated mice have higher means than their respective untreated littermates. The data presented in our previous study (chapter 3) and by others from our group (26), showed that wildtype mice treated with T+E_2 or T+BPA exhibit a decrease in the size of cross-sectional area of the urethral lumen. Thus, in this study we tried to determine which ER subtype is driving this T+E_2 / T+BPA induced phenotype, but high variability and sample processing issues prevented this.

We observed urinary retention, increased frequency, and decreased voided volumes with T+E_2 and T+BPA treatment in wildtype and *Esr2*$^{-/-}$ mice, but not *Esr1*$^{-/-}$ mice. These data suggest that T+E_2/T+BPA induced urinary dysfunction occurs through an *Esr1* dependent mechanism. Future studies that incorporate the use of repeated measures as a function of time and larger sample sizes are required to strengthen our uroflow findings.

In this study, *Esr1* wildtype littermates treated with T+BPA displayed a significant increase in serum levels of Testosterone (T) and 3α-Androstanediol (3α-diol)/3β-Androstanediol (3β-diol) while *Esr1*$^{-/-}$

treated with T+BPA did not. *Esr2* wildtype littermates treated with T+BPA displayed increased serum levels of T, Dihydrotestosterone (DHT), and 3α/3β-diol while *Esr2*$^{-/-}$ mice displayed increased 3α/3β-diol serum levels. With T+E_2 treatment, *Esr1*$^{-/-}$ mice treated with T+E_2 displayed an increase in serum levels of T, DHT, and 3α-diol/3β-diol. Taken together, we propose a model where BPA, an environmental estrogen, alters testosterone metabolism through a possible estrogen receptor β (ERβ/*Esr2*) and inactive androgen driven mechanism (**Figure 4-6**). These findings are particularly interesting because Testosterone can be converted by the enzyme 5α reductase to the more potent and biologically active metabolite known as DHT. It has been hypothesized that DHT can be converted to 5α-androstan-3β, 17β-diol (3β-diol), an androgen metabolite with estrogen-like effects, through enzymes like 17β hydroxysteroid dehydrogenase (17β-HSD) or the combined enzymatic actions of 3α hydroxysteroid dehydrogenase (3α-HSD) and 3β hydroxysteroid dehydrogenase (3β-HSD)(12, 37). This is especially intriguing since, Testosterone is also metabolized by the enzyme aromatase (CYP19A1) to estradiol. The steroid hormone, estradiol, can transcriptionally activate estrogen receptors, such as estrogen receptor α (ERα) and estrogen receptor β (ERβ), and 3β-Diol can reportedly bind to ERα but it has a higher affinity for ERβ (17). This inactive androgen metabolite pathway is particularly intriguing since BPA can reportedly bind to ERs, because of the structural similarity it shares with estradiol (3, 16), and cause activation of inactive androgen metabolites like 3α/3β-Diol. We propose that BPA induces these effects through *Esr2* because we did not observe an increase in androgen metabolites or testosterone in *Esr1*$^{-/-}$ mice treated with T+BPA.

Post T+BPA treatment, *Esr1* mice displayed very low E_1 and E_2 serum levels (**Table 4-3**). Future studies that involve measuring the level of aromatase, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels, in our model, are required to probe this further. A possible mechanism for this increase in serum levels of Testosterone and androgen metabolites after T+BPA treatment is that BPA is somehow antagonizing estrogen action in our model but not enough to induce chemical castration. An increasing number of studies suggest that circulating levels of estradiol mask the effects of a local source of estradiol that is synthesized in the hippocampus through hippocampal synaptogenesis (14, 19).

Additionally, gonadotropin-releasing hormone (GnRH) reportedly regulates estradiol synthesis, from hippocampal synaptogenesis, through an aromatase-mediated mechanism (9). Antagonizing estrogen action in the hippocampus can lead to reduced levels of estrogen and some grave consequences that could impact the brain and reproduction (41). BPA can reportedly inhibit the effects of estrogen from hippocampal synaptogenesis (19) but the mechanism is largely unknown, especially given the fact that others have reported that BPA can increase aromatase/estrogen expression (2, 7, 15). BPA can bind to multiple hormone receptors including androgen (38, 39) and estrogen receptors (3). BPA has also been identified as a G protein-coupled estrogen receptor 1 (GPER) agonist which suggests that BPA can signal through a non-genomic estrogen signaling pathway (29), a signaling pathway that was unopposed in our study. Future studies will include the use of GPER knockout mice to investigate the role of this ER in BPA induced lower urinary tract dysfunction (LUTD).

4.5 Figures and Legends

Figure 4-1. Bladder enlargement in male mice treated with T+BPA is mediated by ERα.

A. Urogenital tracts from UNT, $T+E_2$, and T+BPA treated ERαWT and *Esr1*$^{-/-}$ mice after two months of treatment. (B) Bladder masses for UNT, $T+E_2$ and T+BPA treated ERαWT and *Esr1*$^{-/-}$ mice.

SV=seminal vesicles, Bl=bladder, Ur=urethra. ***p<0.05; ***p<0.0001.**

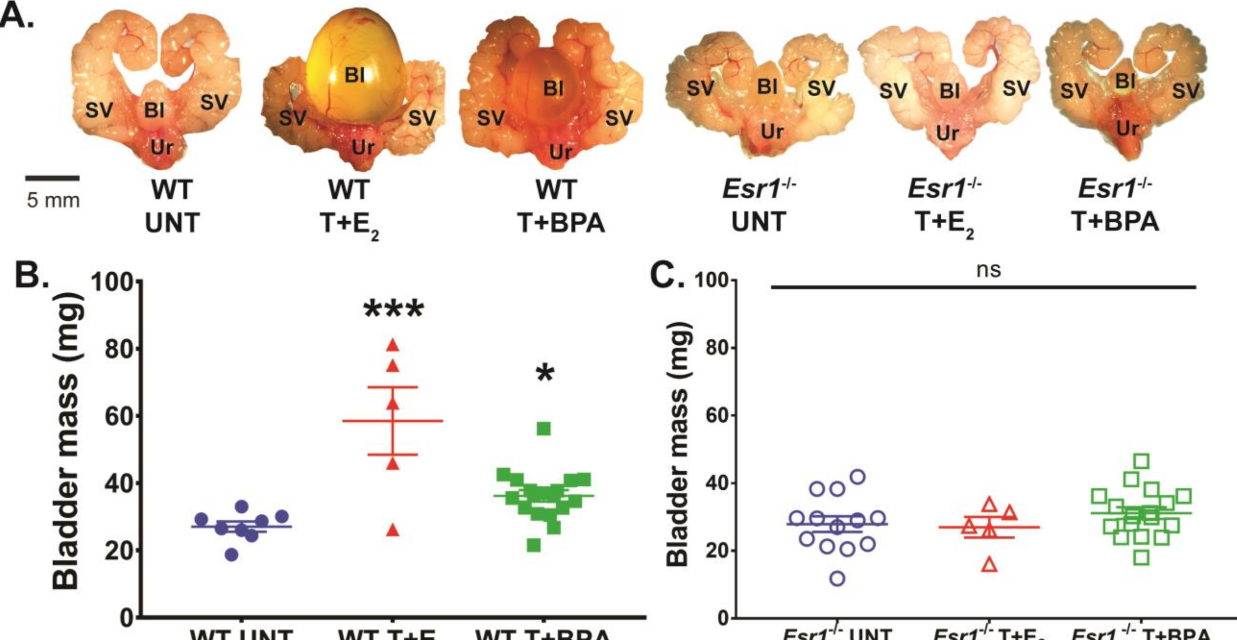


Figure 4-2. Bladder enlargement in male mice treated with T+BPA is not mediated by ERβ

A. Urogenital tracts from UNT, T+E_2 and T+BPA treated ERβWT and *Esr2*$^{-/-}$ mice after two months of treatment. **B**. Bladder masses for UNT, T+E_2, and T+BPA treated ERβWT and *Esr2*$^{-/-}$ mice. SV=seminal vesicles, Bl=bladder, Ur=urethra. ***p<0.05; ***p<0.0001.**

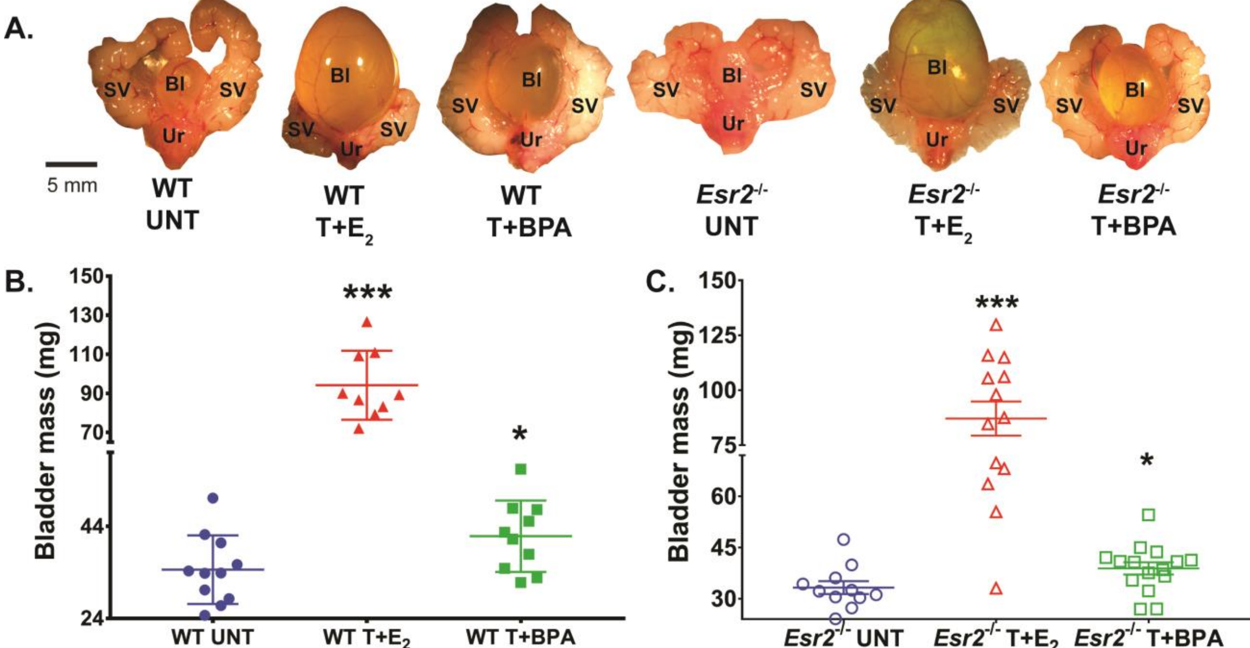
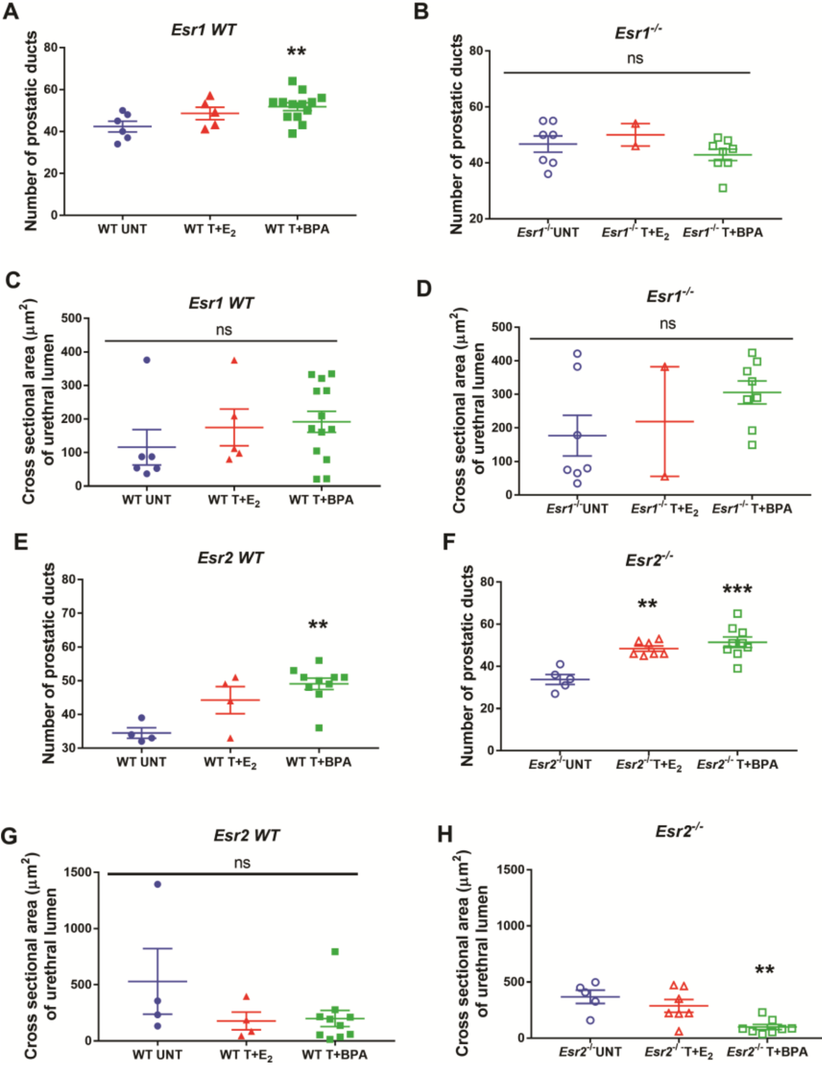


Figure 4-3. T+BPA induced glandular growth seems to be mediated by *Esr1*

A-B. T+BPA treatment caused a significant increase in the number of prostatic ducts or glandular tissue in the proximal urethra of *Esr1* wildtype mice when compared to UNT WT littermates (*p=0.0157*), but not *Esr1*$^{-/-}$ mice. **C-D.** T+BPA treatment caused no change in the cross-sectional area of the prostatic urethral lumen of *Esr1* wildtype and *Esr1*$^{-/-}$ mice when compared to their respective UNT littermates **E.** T+BPA treatment caused an increase in the number of prostatic ducts or glandular tissue in the proximal urethra of *Esr2* WT mice compared to UNT WT littermates (p=0.0011). **F**. T+E_2 or T+BPA treatment caused an increase in the number of prostatic ducts in *Esr2*$^{-/-}$ mice when compared to UNT *Esr2*$^{-/-}$ littermates (*p=0.0010* and *p=0.0001* respectively). **G**. There was no change in the size of the cross-sectional area of the urethral lumen after treatment with T+E_2 or T+BPA in *Esr1* mice. **H**. T+BPA treatment caused a decrease in the cross-sectional area of the urethral lumen in *Esr2*$^{-/-}$ mice when compared to UNT *Esr2*$^{-/-}$ littermates (*p=0.0017*).

***p<0.05; **p<0.01; ***p<0.0001**


A
Esr1 WT
Number of prostatic ducts
**
WT UNT
WT T+E2
WT T+BPA
B
Esr1-/-
ns
Number of prostatic ducts
Esr1-/- UNT
Esr1-/- T+E2
Esr1-/- T+BPA
C
Esr1 WT
ns
Cross sectional area (μm2) of urethral lumen
WT UNT
WT T+E2
WT T+BPA
D
Esr1-/-
ns
Cross sectional area (μm2) of urethral lumen
Esr1-/- UNT
Esr1-/- T+E2
Esr1-/- T+BPA
E
Esr2 WT
Number of prostatic ducts
**
WT UNT
WT T+E2
WT T+BPA
F
Esr2-/-
Number of prostatic ducts
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Esr2-/- UNT
Esr2-/- T+E2
Esr2-/- T+BPA
G
Esr2 WT
ns
Cross sectional area (μm2) of urethral lumen
WT UNT
WT T+E2
WT T+BPA
H
Esr2-/-
Cross sectional area (μm2) of urethral lumen
**
Esr2-/- UNT
Esr2-/- T+E2
Esr2-/- T+BPA

Figure 4-4. T+BPA treatment induced urinary retention, increased frequency, and decreased voided volumes through an Esr1 dependent mechanism

A. Graph shows correlation between bladder volume and the average void amount in wildtype untreated (UNT), T+E_2, and T+BPA treated mice (p=0.0338). **B**. Graph shows a correlation between the number of voids and average void amount in wildtype UNT, T+E_2, or T+BPA treated mice (p=0.0279). **C**. Graphs shows a correlation between the number of voids and average void amount in *Esr1*$^{-/-}$ untreated, T+E_2, and T+BPA treated mice (p=0.0001). **D**. Graph shows a correlation between the number of voids and average void amount in *Esr2*$^{-/-}$ that underwent sham, T+E_2, or T+BPA treatment (p=0.0408).

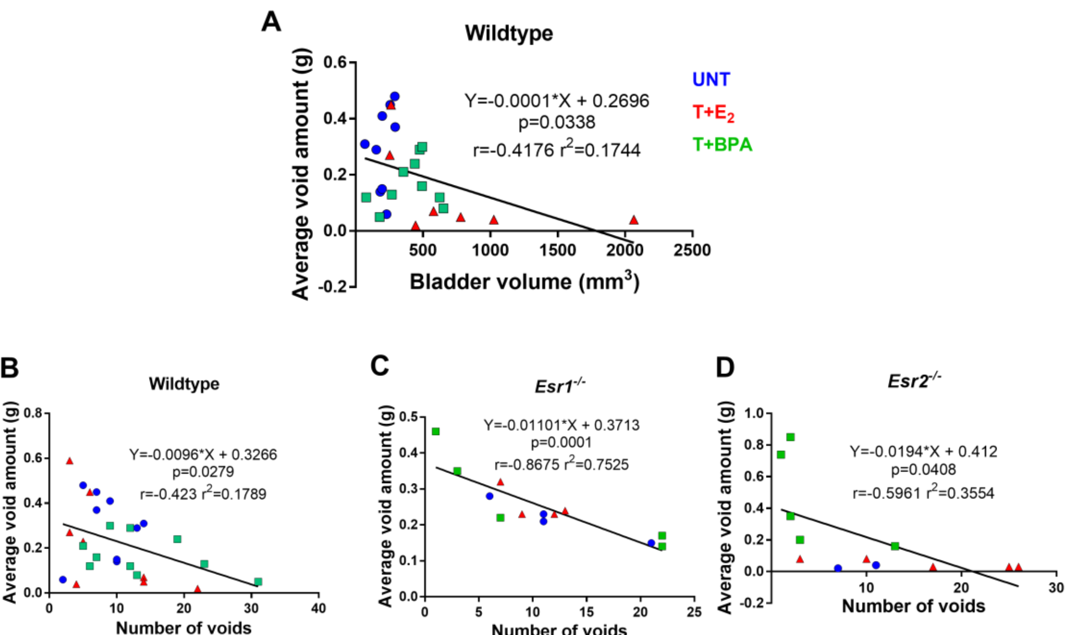


Figure 4-5. Hormone levels in mice treated T+E_2 and T+BPA compared to UNT littermates

A-B. Serum Testosterone (T) levels in *Esr1* and *Esr2* wildtype mice treated with T+BPA (p=0.0236 and p=0.0012 respectively) were increased while *Esr1*$^{-/-}$ and *Esr2*$^{-/-}$ mice treated with T+BPA displayed no change in serum T levels. *Esr1*$^{-/-}$ mice treated with T+E_2 displayed an increase in serum T levels (p=0.0022). **C-D.** Serum Dihydrotestosterone (DHT) levels were increased in *Esr1*$^{-/-}$ mice treated with T+E_2 (p=0.0049) and *Esr2* wildtype mice treated with T+BPA (p=0.0098) when compared to their respective untreated littermates. **E-F**. Serum 3α-Androstanediol (3α-diol)/3β-Androstanediol (3β-diol) (3α-/3β-diol) levels were increased in *Esr1* and *Esr2* wildtype mice treated with T+BPA (p=0.0418 and p=0.0005 respectively) when compared to their respective littermates. *Esr1*$^{-/-}$ mice treated with T+E_2 also displayed increased 3α-/3β-diol serum levels (p=0.0044) when compared to their respective UNT littermates. Graphs show means ± SEM. Compared with UNT littermates.

p<0.05*; **p<0.01*; ***p<0.0001

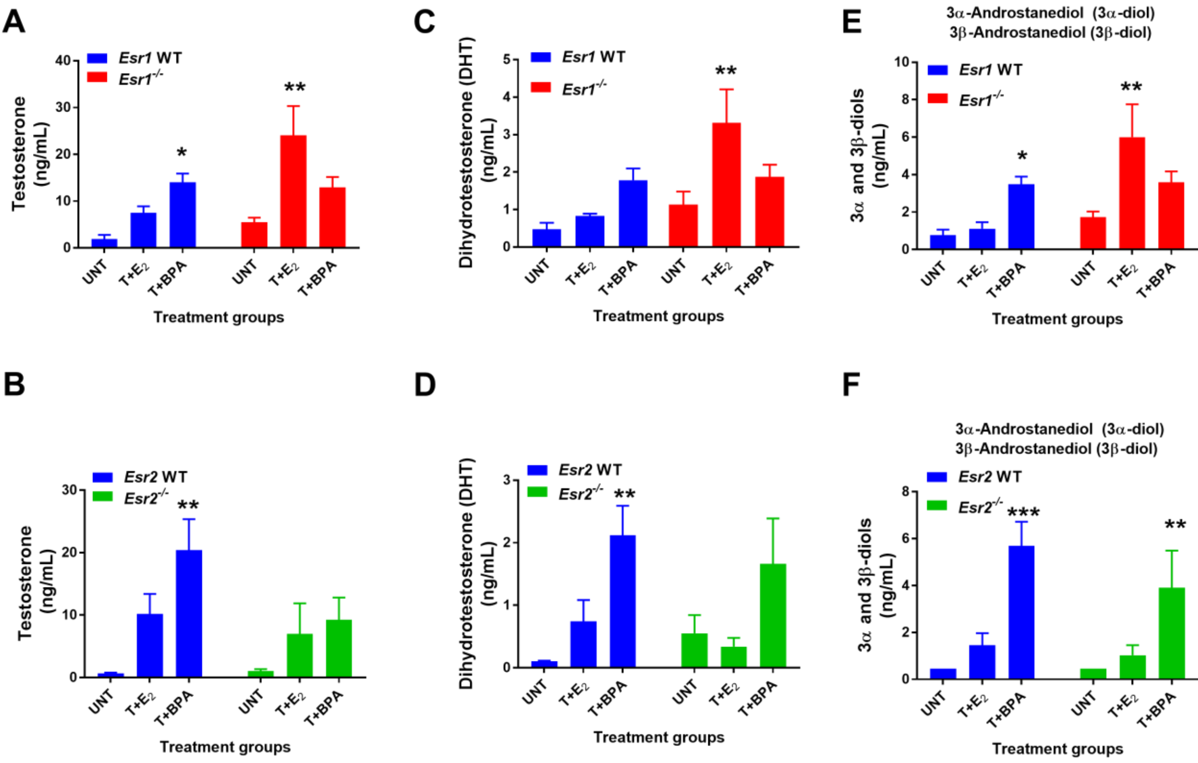
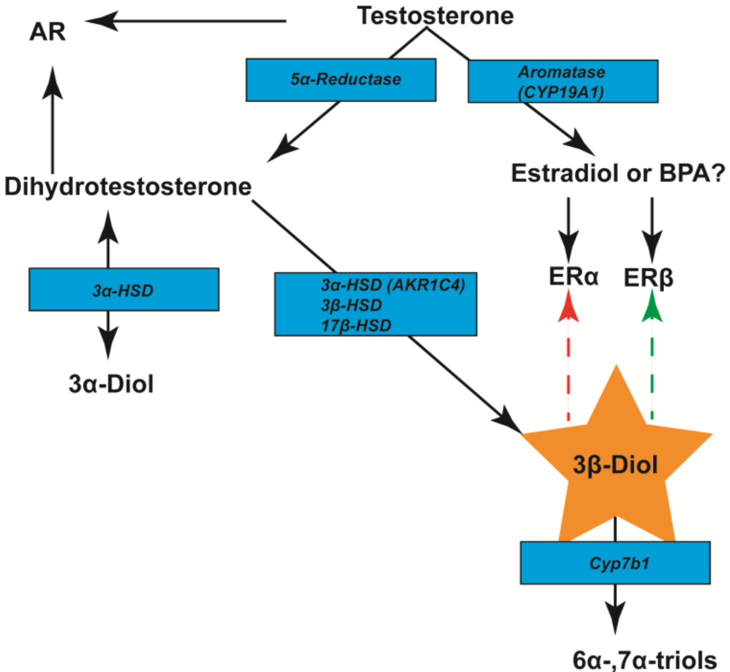


Figure 4-6. Proposed signaling mechanism in BPA induced LUTD

Diagram adapted from Handa et al. 2008 (12) depicts the metabolism actions of the natural steroid hormone Testosterone. Testosterone can bind directly to the androgen receptor (AR) and regulate the expression of AR target genes in androgen sensitive tissues. Another mechanism of AR activation includes, the conversion of testosterone by the enzyme 5α reductase to the more potent and biologically active metabolite known as dihydrotestosterone (DHT). It has been hypothesized that DHT is can be converted to 5α-androstan-3β, 17β-diol (3β-diol), an androgen metabolite with estrogen-like effects, through enzymes like 17β hydroxysteroid dehydrogenase (17β-HSD) or the combined enzymatic actions of 3α hydroxysteroid dehydrogenase (3α-HSD) and 3β hydroxysteroid dehydrogenase (3β-HSD). This is especially intriguing since, Testosterone is also metabolized by the enzyme aromatase (CYP19A1) to estradiol. The steroid hormone, estradiol, can transcriptionally activate estrogen receptors, such as estrogen receptor α (ERα) and estrogen receptor β (ERβ), and 3β-Diol can reportedly bind to ERα but it has a higher affinity for ERβ. 3β-diol regulation is caused by the ability of the enzyme Cyp7b1 to reduce 3β-diol to inactive metabolites known as 6α,-α7-triols (33, 37). The conversion of DHT to 3α-Diol is reversible mainly due to the enzyme 3α-HSD which allows for bidirectional conversion of 3α-Diol from DHT and ultimately additional production of DHT. **Taken together, we propose a model where BPA, an environmental estrogen, alters testosterone metabolism through an estrogen receptor β (ERβ) and inactive androgen driven mechanism.**



Abbreviations:
Androgen receptor (AR); 3-oxo-5α-steroid 4 dehydrogenase (5α-reductase); Aromatase (CYP19A1); bisphenol-A (BPA); 3α-hydroxysteroid dehydrogenase (3α-HSD); 3β-hydroxysteroid dehydrogenase (3β-HSD); 17β hydroxysteroid dehydrogenase (17β-HSD); estrogen receptor α (ERα); estrogen receptor β (ERβ) ;3α-androstanediol (3α-diol); 5α-androstane-3α,17β diol (3β-diol); 25-hydroxycholesterol 7-α-hydroxylase

4.6 Tables

Table 4-1. Bladder and prostate measurements for T+BPA and T+E_2 treated mice.

Results are reported as mean± SEM, with the number of replicates shown in parentheses. * Significantly different from UNT littermates**. *p<0.05*; ***p<0.01*; ****p<0.0001***

Genotype	Treatment	Body weight (g)	Bladder mass (mg)	Bladder Volume (mm^3)	Hemiprostate mass (mg)	Ventral prostate (mg)	Anterior Prostate mass (mg)	Dorsolateral Prostate (mg)
Esr1 WT	**UNT**	33 ± 1.4 (8)	27 ± 1.5* (8)	20 ± 4.4 (8)	26 ± 3.1 (8)	5 ± 0.8 (8)	16 ± 2.0 (8)	5.1 ± 0.7 (8)
	T+E_2	30 ± 1.5 (5)	**59 ± 10*** (5)**	**647 ± 279 (4)*** **	35 ± 2.6 (5)	**10 ± 0.8*** (5)**	17 ± 2.2 (5)	7.6 ± 0.9 (5)
	T+BPA	34 ± 0.6 (16)	**36 ± 1.7* (18)**	**113 ± 26** (17)**	**45 ± 1.6*** (18)**	**13 ± 1.0*** (18)**	**24 ± 1.2** (18)**	**8.4 ± 0.6** (18)**
Esr1 $^{-/-}$	**UNT**	36 ± 1.6 (12)	28 ± 2.3 (13)	27 ± 7.0 (13)	33 ± 2.6 (13)	7.5 ± 0.7 (13)	18 ± 1.8 (13)	7.9 ± 1.0 (13)
	T+E_2	**29 ± 1.1** (5)**	27 ± 3.0 (5)	17 ± 5.7 (5)	43 ± 3.3 (5)	**12 ± 1.0** (5)**	22 ± 2.3 (5)	8.8 ± 0.8 (5)
	T+BPA	34 ± 1.0 (18)	31 ± 1.7 (17)	69 ± 22 (18)	39 ± 2.0 (18)	9.7 ± 0.7 (18)	22 ± 1.3 (18)	7.9 ± 0.4 (18)
Esr2 WT	**UNT**	34 ± 1.2 (11)	35 ± 2.2 (11)	77 ± 21 (11)	28 ± 1.6 (11)	**5.9 ± 0.5** (11)**	16 ± 0.9 (11)	5.5 ± 0.6 (11)
	T+E_2	32 ± 0.7 (9)	**94 ± 6.0*** (9)**	**709 ± 192*** (9)**	**40 ± 4.8** (9)**	**12 ± 2.0** (9)**	19 ± 2.4 (9)	**8.7 ± 1.0* (9)**
	T+BPA	35 ± 1.0 (12)	**42 ± 2.4* (10)**	75 ± 17 (12)	**41 ± 2.7** (12)**	11 ± 1.2 (12)	**22 ± 1.6* (12)**	7.9 ± 0.9 (12)
Esr2 $^{-/-}$	**UNT**	40 ± 2.0 (11)	33 ± 2.0 (11)	250 ± 80 (11)	30 ± 2.3 (11)	6.5 ± 0.8 (11)	16 ± 1.0 (11)	7.7 ± 0.9 (11)
	T+E_2	**33 ± 0.8** (13)**	**87 ± 8.0***(13)**	673 ± 164 (13)	32 ± 3.0 (15)	7.3 ± 1.3 (13)	17 ± 1.4 (13)	6.8 ± 0.8 (13)
	T+BPA	37 ± 1.2 (15)	**39 ± 2.0* (15)**	130 ± 30 (15)	39 ± 3.0 (15)	7.7 ± 0.7 (15)	**24 ± 3.0* (15)**	7.8 ± 0.6 (15)

Abbreviations: *Estrogen receptor 1* (*Esr1*), *Estrogen receptor 2* (*Esr2*), wildtype (WT), untreated (UNT), Testosterone (T), 17β-estradiol (E_2), bisphenol-A (BPA)
Results are reported as mean ± SEM, with the number of replicates shown in parentheses.
*Significantly different from UNT ***p<0.05; **p<0.01; ***p<0.0001**

Table 4-2. Analytical parameters for 11 analytes in addition to analyte MRM and retention time

Mice serum samples (300 µl) and standards were transferred into a 5 ml glass extraction tube. Each sample were diluted in 500 µl of ultra-purified bottled water (Fisher Scientific) and internal standards were added. Internal standards consisted of 200 pg of deuterated 9 (d9)-Progesterone, 50 pg of d5-E_2, and 200 pg of d5-Testosterone. Q1-3 ionized fragments used quantify the compound of interest.

No.	Analyte	Abbreviation	Limit of detection (LOD) in ng/ml	Internal Standard used	Q1 (m/z)	Q3 (m/z)	Q3 (m/z)	Retention time (min)
1	Testosterone	T	0.01	d5-Testosterone	289.0	108.7	97.1	7.0
2	Dihydrotestosterone	DHT	0.16	d5-Testosterone	291.1	255.0	91.2	8.4
3	Progesterone	Prog	0.01	d9-Progesterone	314.9	109.1	97.0	13.0
4	Estrone (dansylated)	E_1	0.002	d5-Estradiol	504.1	170.8	156.1	15.5
5	Estradiol (dansylated)	E_2	0.002	d5-Estradiol	506.0	171.2	128.0	15.7
6	Androstenedione	Androsten	0.01	d5-Testosterone	287.1	109.1	97.2	8.2
7	11-Deoxycortisol	11-DOC	0.02	d5-Testosterone	347.0	109.0	96.8	7.0
8	3α and 3β-Androstanediol	3-diols	0.65	d5-Testosterone	275.3	257.3	-	11.4
9	d5 Testosterone	d5-T	-	N/A	294.1	100.2	-	9.4
10	d9 Progesterone	d9-Prog	-	N/A	324.1	113.2	100.1	12.9
11	d5 Estradiol (dansylated)	d5-E_2	-	N/A	510.9	170.8	-	15.7

Table 4-3. Effects of T+BPA and T+E_2 treatment on serum levels of steroid hormones in adult male mice after two months of treatment

Individual samples include one 300µl serum sample and sets of pooled samples indicate the number times that two or more serum tubes combined to achieve a total volume of 300µl.

Results are reported as mean ± SEM. *Significantly different from UNT littermates.

p<0.05*; ****p<0.01*; ******p<0.0001

Genotype	Treatment	Individual samples	Sets of pooled samples	Analytes ng/ml							
				T	DHT	Androsten.	3-diols	E_1	E_2	11-DOC	Prog.
Esr1 WT	UNT	2	4	1.881 ± 0.892	0.477 ± 0.170	1.057 ± 0.664	0.760 ±0.300	0.007 ± 0.003	0.002 ± 0.001	2.332 ± 0.889	2.594 ± 0.818
	T+E_2	1	3	7.512 ± 1.358	0.828 ± 0.063	1.147 ± 0.216	1.113 ± 0.348	0.010 ± 0.005	**0.298 ± 0.168***	**0.776 ± 0.391***	4.253 ± 1.794
	T+BPA	2	4	**14.001 ± 1.878***	1.784 ± 0.315	0.310 ± 0.034	**3.479 ± 0.417***	0.003 ± 0.000	0.001 ± 0.000	**0.439 ± 0.107****	1.091 ± 0.308
Esr1$^{-/-}$	UNT	4	2	5.471 ± 0.993	1.135 ± 0.346	0.825 ± 0.363	1.738 ± 0.294	0.003 ± 0.000	0.002 ± 0.001	0.882 ± 0.276	3.166 ± 0.296
	T+E_2	2	2	**24.080 ± 2.239****	**3.317 ± 0.893****	0.808 ± 0.111	**6.005 ± 1.749****	0.009 ± 0.003	0.103 ± 0.027	0.964 ± 0.328	2.609 ± 0.180
	T+BPA	2	4	13.904 ± 2.239	1.876 ± 0.323	0.424 ± 0.079	3.593 ± 0.578	0.003 ± 0.000	0.001 ± 0.000	0.861 ± 0.226	2.156 ± 0.435
Esr2 WT	UNT	3	3	0.694 ± 0.103	0.104 ± 0.009	0.245 ± 0.107	0.460 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	1.329 ± 0.556	2.536 ± 1.039
	T+E_2	2	3	10.160 ± 3.223	0.741 ± 0.344	0.362 ± 0.101	1.450 ± 0.517	0.006 ± 0.002	0.110 ± 0.018	0.769 ± 0.270	**10.047 ± 4.606****
	T+BPA	3	3	**20.384 ± 4.979****	**2.119 ± 0.473****	0.486 ± 0.111	**5.688 ± 1.037*****	0.001 ± 0.000	0.001 ± 0.000	0.295 ± 0.084	3.901 ± 1.115
Esr2$^{-/-}$	UNT	2	4	1.017 ± 0.328	0.552 ± 0.290	0.197 ± 0.083	0.460 ± 0.000	0.001 ± 0.000	0.003 ± 0.001	0.270 ± 0.094	2.445 ± 0.973
	T+E_2	3	3	6.991 ± 4.890	0.338 ± 0.142	0.426 ± 0.280	1.030 ± 0.425	**0.014 ± 0.006****	**0.235 ± 0.085*****	0.891 ± 0.172	2.381 ± 0.849
	T+BPA	4	2	9.227 ± 3.574	1.661 ± 0.728	0.223 ± 0.087	**3.911 ± 1.575****	0.001 ± 0.000	0.001 ± 0.000	0.845 ± 0.230	1.634 ± 0.523

Abbreviations: *Estrogen receptor 1* (*Esr1*); *Estrogen receptor 2* (*Esr2*); wildtype (WT); untreated (UNT); Testosterone (T); 17β-estradiol (E_2); bisphenol-A (BPA); Testosterone (T); Dihydrotestosterone (DHT); Androstenedione (Androsten.); 3α-Androstanediol (3α-diol) and 3β-Androstanediol (3β-diol); Estrone (E_1) 17β-estradiol (E_2); 11-Deoxycortisol (11-DOC); Progesterone (Prog.)

Individual samples:one 300ul serum sample; Sets of pooled samples: number of times 2 or more serum tubes were combined to achieve a total volume of 300µl

Results are reported as mean±SEM

*Significantly different from UNT ***p<0.05; **p<0.01; ***p<0.0001**

4.7 Materials and Methods

Genotyping and pellet implantation

All animal experiments and procedures were conducted under a protocol approved by the University of Wisconsin's Animal Care and Use Committee. Mice heterozygous for ERα (*Esr1*) and ERβ (*Esr2*) on a C57bl/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred to generate knockout mice (*Esr1*$^{-/-}$ and *Esr2*$^{-/-}$) for comparison with their respective wildtype littermates (*Esr1* WT and *Esr2* WT). Genotyping was performed by Transnetyx using real-time PCR (Cordova, TN 38016). Mice were reared under standard laboratory conditions (12:12 light/dark cycle) and provided food and water *ad libitum*. Bisphenol-A (BPA), 17β-Estradiol (E_2), Testosterone (T), and Cholesterol (CHO) were purchased from Sigma Chemical Co. (St. Louis, MO). Adult male mice (6-8 weeks old) underwent surgical implantation of compressed pellets containing 25 mg T and 2.5 mg E_2 mixed with 22.5mg CHO or 25mg T and 25mg BPA mixed with 22.5mg of cholesterol. Untreated (UNT) littermates (*Esr1* WT, *Esr2* WT, *Esr1*$^{-/-}$, *and Esr2* $^{-/-)}$ received sham surgery. At time of necropsy, the mice were euthanized using carbon dioxide and serum was collected by cardiac puncture. The urogenital tracts were dissected and photographed.

Bladder measurements and prostate lobe collections

As previously described, bladder mass and volume were determined after serum collections (24, 26). To reduce bias, the genotype and mouse treatments were masked from the researchers at the time of necropsy. Bladders were excised, blotted, and wet bladder weight was measured using an analytical balance. A caliper was used to take transverse plane measurements and the ellipsoid equation was used to calculate bladder volume. During necropsy, the prostate lobes were dissected, and the weights were recorded for the left half of the prostate. The left anterior prostate lobe (AP), the left ventral prostate lobe (VP), and the left dorsolateral (DLP) prostate mass were recorded for each mouse. Additionally, the hemiprostate mass, which is the summation of the left anterior prostate, left ventral prostate, and left dorsal lateral prostate lobes, was calculated by adding the masses for each lobe of the prostate together.

Histopathology

As previously described, the urethra from each mouse was agar embedded to form an array that was then paraffin embedded. As previously described, paraffin blocks were serial sectioned at 5μm intervals and every 20th section was stained with hematoxylin and eosin (H&E). We used an automatic contour program, BioVis 3D software (Montevideo, Uruguary), to trace the cross-sectional area of the prostatic urethral lumen and count the number of prostatic ducts (glandular growth) in the prostatic urethra.

Uroflow

Uroflowmetry was used to assess spontaneous urination patterns in unrestrained mice, post hormone or BPA treatment, as detailed on the University of Wisconsin's O'Brien Center for Urologic Research Rodent Urinary Functional Testing Core (RUFT) website (www.urology.wisc.edu/researchU54-george-m-obrien-center-for-benign-urology-research/:accessed1/19/2018). An automated and noninvasive technique was applied to evaluate voluntary voiding behavior in conscious mice (40). In brief, mice were offered a preferred sweet solution containing 3% glucose and 0.125% saccharin to increase urine output two days prior to and during the testing period. Mice were placed individually in metabolic cages (Model 650-0322, Nalge, Rochester, New York) with floor grids optimized to minimize urine retention for 2 hours. A waste plate was placed on an analytical balance (Mettler Toledo New Classic MF, model MS 303S) directly under the floor grid. Changes in weight sensed by balances were recorded and synchronized video movies corresponding to weight changes on balances were generated. Video recordings were subsequently reviewed to verify voiding events. Voiding frequency and void amount were then determined. Mice were returned to standard housing after testing was completed.

Steroid Hormones Liquid-liquid Extraction

Mice serum samples (300 μl) and standards were transferred into a 5 ml glass extraction tube. Each sample was diluted in 500 μl of ultra-purified bottled water (Fisher Scientific) and the internal standards were added. Internal standards consisted of 200 pg of deuterated 9 (d9)-progesterone, 50 pg of d5-E2, and 200 pg of d5-testosterone. Next, 2 ml of methyl ter- butyl ether (Fisher Scientific) was added, vortexed vigorously for 5 min, and centrifuged at 1,500 rpm for 3 min at 4 °C. The top organic phase which

contained the steroid hormones was transferred into a new glass test tube using a glass pipette, followed by evaporation to dryness using an air stream and heated water bath (60°C). Dried samples were re-suspended in 200 µl of ethanol and diluted with 500 µl of water. A second liquid–liquid extraction was performed using 1 ml of dichloromethane (Fisher Scientific). The bottom organic phase containing steroid hormones was transferred into a clean glass test tube and evaporated to dryness using an air stream and heated water bath (60°C). The Dried samples were re-suspended in 25 µl of 0.1 M $NaHCO_3$ buffer and estrogens (E_1 and E_2) were derivatized using 25 µl of dansyl chloride (200 mg/ml in acetonitrile; Fisher Scientific), heated at 40°C for 4 min. The Final samples were transferred into minivials (TruView LCMS Certified Clear Glass 12 x 32mm Screw Neck Total Recovery Vial, Waters) and stored in 4 °C until LC-MS/MS analysis were performed.

LC-MS/MS analysis

The LC-MS/MS analysis of the extracted mice serum samples was performed using Waters Acquity binary pump UPLC system (Milford, MA) interfaced to an AB Sciex QTRAP 5500 (Framingham, MA) mass spectrometer with a Turbo V ™ source. Waters Acquity UPLC Console 1.50 software was used to control the UPLC and Analyst 1.6 software (AB Sciex) was used to control the mass spectrometer. 10 µl of sample was injected onto a Phenomenex Kinetex 2.6u C18 100A, 100 × 2.1 mm column (Phenomenex) for separation using a mobile phase: water with 0.1% formic acid (Solution A) and methanol with 0.1% formic acid (Solution B), with a flow rate of 250 µl/min. The column temperature was 35 °C, and the samples were kept at 4 °C throughout the experiment. 45% Solution B was held for 1 min followed by an increase to 67.5% Solution B for the next 11.5 min. Solution B was increased to 85% over 0.5 min, followed by an increase to 87% over 1 min. Solution B was then increased up to 92% for 0.5 min, followed by an increase to 96% over the next 2.5 min. Finally, solution B was increased to 98% over 0.5 min and held for 0.5 min before the system was returned to the initial conditions of 45% solution B over 0.5min. Mass spectrometer results were generated in positive-ion mode using the following settings: curtain gas, 30 psi; corona discharge current, 3 V; collisionally activated dissociation gas, medium; nebulizing gas, 25 psi, and entrance potential, 10 V. The source temperature was 500°C. Quantitative

results were recorded as multiple reaction monitoring (MRM) area counts after determination for the response factor for each compound and the internal standards. D9-progesterone was used as an internal for progesterone, d5-E_2 was used for E_1 and E_2, and d5-testosterone was used for all the other analytes. The data were acquired and processed using Analyst software, Ver 1.5.1 (AB-Sciex Concord, ON) and MultiQaunt software (AB-Sciex Concord, ON).

Statistical analysis

Statistical testing was performed using Graphpad Prism (LaJolla, CA). Continuous variables were compared using one-way ANOVA with Bonferroni post hoc or Fischer's LSD comparisons and a Student's t-test when appropriate. Data were evaluated for homogeneity of variance using Bartlett's unequal variance test, and when necessary, logarithmic and/or rank transformation was performed. For steroid hormone measurements, Two-way ANOVA was used with Tukey's multiple comparison test. The graphs show means ± SEM. As indicated in the figures and figure legends,

***p<0.05**, ****p<0.01**; *****p<0.0001** was considered statistically significant in all analyses.

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Chapter 5: Conclusions and Future Directions

5.1 Conclusions

BPH impacts the lives of 50% of men over the age of 50 with direct costs estimated of $3.9 billion annually (10). BPH is reportedly associated with enlargement of the prostate, hyperplasia, inflammation, fibrosis, and urinary dysfunction. More specifically, BPH occurrence increases with age and is marked by prostate enlargement and urethral narrowing. BPH is often associated with urinary dysfunction, which includes increased frequency, urinary retention, bladder wall thickening, and could lead to acute urinary retention if left untreated. As men age, the prostate enlarges, the urethra is compressed, and the bladder is unable to empty completely. The molecular mechanisms associated with BPH is poorly understood and limited therapies exist for patients.

It is widely accepted that steroid hormones such as testosterone (T) and dihydrotestosterone (DHT) play a role in the progression of prostate diseases such as BPH. Additionally the actions of estrogens such as 17β-estradiol (E_2) are gradually being recognized as important male sex steroids in BPH due to the increase in E_2 compared to testosterone ratio in the serum of aging males (2). ERs are expressed in the male lower urinary tract and reproductive tissues such as the bladder (4), prostate (4), urethra, and testis (8). In fact, as men age, serum E_2 has been shown to be increased or unchanged, which contributes to the increased E_2 to T ratio observed in patients with BPH (2). Our lab has previously evaluated the contribution of ERα and ERβ to bladder complications of benign prostatic hyperplasia. We have shown that ERα is the key mediator of urinary dysfunction characterized by enlarged bladders with urinary retention, increased bladder mass, and decreased peak uroflow (8). These findings are important because increased ERα expression has been reported in bladder tissues of men with bladder outlet obstruction (7),(8). Environmental estrogens, also known as endocrine disruptors, can mimic and/or interfere with estrogen signaling in ER responsive tissues and organs. ER (estrogen receptors α/β) have been shown to possess affinity for environmental contaminants such as bisphenol a (BPA), phthalates, pesticides, and polycyclic aromatic hydrocarbons (5). BPA is one of the highest volume chemicals produced worldwide with over 6 billion pounds produced each year and is estimated to be found in the urine of 90% of people in the United States (14). BPA exposure has been implicated in adverse effects on human reproduction,

early onset of prostate cancer (13), and disruption of prostate development (16). However, the impacts of exposure to environmental estrogens such BPA, bisphenol f (BPF), and bisphenol s (BPS) in urinary dysfunction are poorly understood. Recent studies have stressed the importance of further analysis of these BPA alternatives as well as their metabolites based on the estrogenic potency associated with exposure to these compounds.

In chapter 2, to evaluate the role of adult BPA exposure in male LUTS, we treated male mice with BPA and supplemented with T to simulate the hormonal milieu of older men. We determined that treatment of male mice with T+BPA induces bladder enlargement, hypertrophy and in some mice, urinary voiding dysfunction, implicating this endocrine disruptor as a contributor to male LUTS. In chapter 3, we demonstrate that exposure to bisphenol analogues can lead to lower urinary tract dysfunction. This dysfunction is characterized by bladder and prostate enlargement, increased glandular growth in the proximal urethra, and increased non-voiding contractions that may indicate detrusor instability. The findings from this mouse model emphasizes the importance of hormones in the development of lower urinary tract dysfunction and highlights the applicability of this model to the study of molecular mechanisms associated with BPH/LUTS (8). In chapter 4, we found that BPA requires ERα to induce urinary dysfunction and prostate hyperplasia in male mice. More research will be required to explore which aspect of the estrogen signaling pathway BPA requires, the work described here contributes to our understanding of BPA's mechanism of action in lower urinary tract dysfunction (LUTD) through an *in vivo* model.

5.2 Future directions

The work presented in this dissertation was designed to evaluate elucidate the role of an environmental estrogen, BPA, on estrogen receptor (ER) signaling and urinary function. The present findings implicate BPA exposure in adulthood with male lower urinary tract dysfunction. These findings are consistent with our prior research showing that exposure of adult male mice to E_2 and T is associated with LUTS (9). Thus, BPA, which is a known estrogenic endocrine disrupting chemical and was

considered for use as an estrogenic drug in the 1930s, also has the capacity to cause LUTS in male mice. An important issue to consider is whether the dose of BPA and method of administration that we used results in serum BPA levels that are relevant to human levels. In chapter 3, the serum levels of BPA were ~8.45 ng/ml which is very close to the maximum levels of BPA found in adult men and women (~7.2 ng/ml) after transdermal exposure due to holding a cashiers receipt that is coated with free BPA (6). With regard to the route of drug administration, there is evidence that there are routes of exposure (inhalation, transdermal) that do not result in rapid first-pass metabolism of BPA, thus leading to much higher levels of bioactive (unconjugated) BPA in blood relative to levels found after intragastric gavage administration (17). Mean human blood levels of BPA have been reported to range from 0.3-4 ng/mL (15), but it is well recognized that there are substantial individual differences in the metabolism of estrogenic drugs and chemicals, and thus some individuals have blood levels of bioactive BPA well within the range we found in this study (6). In addition, our use of subcutaneous pellets to administer BPA would lead to continuous exposure rather than widely spaced exposure. Importantly, there is evidence from studies with human populations, including from the National Health and Nutrition Examination Survey (NHANES) conducted by the Centers for Disease Control and Prevention (CDC), that exposure to BPA in the US population is continuous (12). Additional research is necessary to determine the differences in endogenous steroid hormones that may result from BPA administration, since BPA reportedly decreases serum testosterone in men and rodents, and reduces 5α-reductase, but increases aromatase activity in the mouse and rat prostate (1, 3, 11). In chapter 4, we determined that serum levels of steroid hormones were different from the reports of others. More specifically, serum levels Testosterone and androgen metabolites increased after T+BPA treatment whiles estrogen serum levels were found to be unremarkable when compared to control (Table 4-3). Future studies that involve measuring the levels of aromatase, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), in our model, are required to probe this further. A possible mechanism for this increase in serum levels of Testosterone and androgen metabolites after T+BPA treatment is that BPA is somehow antagonizing estrogen action in our model, but not enough to induce chemical castration. Additionally, more experiments are required to

probe the relationship between estrogen receptors and inactive androgens in T+BPA induced lower urinary tract dysfunction (LUTD). These additional experiments include determining the protein expression and enzyme levels in the mouse prostates, measuring the urinary metabolites of inactive androgens, and developing an LC-MS/MS panel to differentiate between 3α and 3β-diols in mouse serum. It will also be important to investigate whether BPA causes these effects via classical or non-classical estrogen receptor pathways, and determine which ERs, as well as other receptors, mediate the action of BPA in the male lower urinary tract. Table 1-2 provides additional models that could be employed to determine if BPA treatment impacts estrogen action via non-classical or non-genomic estrogen signaling.

Our findings highlight the utility of this model to study many of the clinical features of BPH-LUTS. This model could be useful for genetic approaches to elucidate the benefit of pharmacologic intervention with selective estrogen receptor modulators (SERMS), the urologic risk other environmental estrogens might pose, and a means to probe the molecular mechanisms associated with environmental estrogen induced BPH/LUTS. The data presented in this dissertation demonstrates that ERs could serve as potential targets in future therapies for bladder complications that develop because of environmental estrogen induced BPH/LUTS.

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Appendix 1

Supplemental Figure A1-1

Video legend for Chapter 2

The uroflow video shows C57BL/6 male mice in metabolic cages undergoing noninvasive uroflow testing. Untreated male mice displayed sustained voids while the T+E_2 treated mice displayed droplet voiding. Mice treated with T+BPA treated mice displayed droplet voiding or an intermediate pattern of voiding after four months of treatment.