Effects of Soy Isoflavone Extract Treatment on Morphology and Morphometry of the Urinary Tract of Overiectomised Female Sprague Dawley Rats

By

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A thesis submitted in partial fulfillment of the requirements of the degree of
MASTER OF VETERINARY SCIENCE IN ANATOMY

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Abstract

The effect of soy isoflavone extract on the morphology and morphometry of the urinary tract of ovariectomised Sprague Dwaley rats were evaluated using histological methods. Twenty four virgin adult Sprague Dawley rats were placed into three groups treated as follows: sham operate + distilled water, ovariectomy + soy isoflavone and ovariectomy + distilled water for 65 days. A 125µg isoflavone/g body weight/ day dosage was administered to the experimental group with ad libitum soy-free feed and water. The means for variables muscularis thickness, lamina propria thickness, muscle nuclei density, epithelium thickness and number of blood vessels were compared using ANOVA at 95% confidence interval. The sham operated group recorded significantly higher values for muscularis thickness and muscle nuclei density in all organs compared to the other two groups. Lamina propria thickness was lowest in the sham-vehicle treared group for all organs. No significant differences in the lamina propria thickness were recorded between the ovariectomy + isoflavone treated and the ovariectomy + distilled water treated groups in the urethra and bladder (p=0.74; p=0.20) respectively. Urethral blood vessel numbers were significantly higher in the ovariectomy+ soy isoflavone group compared to the control groups (p=0.03). Ovariectomy resulted muscle depletion and vacuolation copled with an increase in lamina propria thickness in all organs. Soy isoflavones slightly reversed the effects of ovariectomy on muscle and connective tissue components of the bladder, urethra and ureter. Soy isoflavones were able to weakly positively influence the morphology and morphometry of the urinary tract.

Dedication

This work is dedicated to my lovely gifts from the almighty. Ruvarashe, Rufaro and Kupakwashe, my angels, you give me a reason to survive, fight, search and strive for the best in life.

Acknowledgements

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CHAPTER 1

1.0. INTRODUCTION

Urinary incontinence is the uncontrollable loss of urine. This can occur singly as stress incontinence, urge incontinence or overflow incontinence. Cases of mixed incontinence can also occur. Stress incontinence, idiopathic or hypoestrogenic incontinence is usually experienced when pressure is exerted on the bladder during such activities as coughing, sneezing, laughing, lifting, exercising and sexual relations. The amount of leakage is usually small (drops), but can increase. Hypoestrogenic incontinent patients are now seeking safer and natural alternatives to Hormone Replacement Therapy (HRT) in view of the health risks and side effects associated with it. An increasing number of patients and clients are requesting non-hormonal treatment for menopausal symptoms. Some practitioners have advocated for natural supplements including soy isoflavones or other herbs containing phytoestrogens. Reports of successful herbal treatment in incontinence caused by weak bladder sphincter have been described (Ahmad *et al*., 2010). Male prostate cancer patients have responded positively to soy isoflavones used in conjunction with radiation therapy by showing a reduction in urinary incontinence, urgency and improved erectile function (Ahmad *et al*., 2010). Studies by some authors have suggested that isoflavones have the ability to modify by reducing the frequency and severity of menopausal symptoms such as hot flushes and osteoporosis (Adlercreutz and Mazur, 1997; Albertazzi *et al*., 1998). However, other authors reported no overall benefits of soy phytoestrogens in the urinary incontinence and bladder and urethra morphology (Messina, 1994). To date, studies on the effect of soy isoflavone consumption on menopausal symptoms have reported mixed results.

The presence of estrogen receptors in the proximal and distal urethra, vesical trigonum, pelvic floor, brain areas involved in the regulation of urination and the pubocervical fascia (Santos *et al*., 2010) also suggests the involvement of hypoestrogenism in the origin or worsening of post menopausal and post ovariectomy urinary incontinence. Further evidence by Blakeman *et al*. (2007) shows that ovarian steroids are responsible for biosynthesis of collagen and connective tissue metabolism. Estrogen can cause an increase in epithelial proliferation in the bladder and urethra thereby influencing several vesical parameters. Rud (1980) also suggested that estrogen increases urethral closure pressure and improves pressure transmission to the proximal urethra actions that promote continence This evidence further supports the involvement of hypoestrogenism in the origin of urinary incontinence as well as the role of estrogen supplementation in the relief of this condition in the rabbits(Aikawa *et al*., 2003).

Patients with hypoestrogenic urinary incontinence commonly respond to hormone replacement treatment (HRT). Daily oral and topical administration of estrogen replacement therapy (ERT) for three months resulted in a significant reduction in urinary frequency and nocturia in hysterectomized post-menopausal women (Long *et al*., 2006). The ERT increased the blood flow around the bladder neck and periurethral vessels and relieved the symptoms of the bladder overactivity and stress incontinence (Long *et al*., 2006). ERT also induced lower urinary tract proliferation of the atrophic urethral mucosa and increased the thickness of epithelium and lamina propria of both urethra and bladder (Ulmsten and Stormby, 1987) of post-menopausal incontinent women. Long term use of HRT has been credited with protecting against osteoporosis and cardiovascular disease and has been documented as the most consistently

effective therapy for vasomotor symptoms (Weiss, 1975). Despite these effects, adverse effects of HRT which include an increase in the risk of stroke, breast cancer, gall bladder disease and endometrial cancer have been reported (Rossouw *et al*., 2002; Vickers *et al*., 2007; Hilard *et al*., 1991). Estrogen replacement therapy (ERT) also provoked metaplasia, hyperplasia and increased occurrence of stratified epithelium (Suguita *et al*., 2000) in ovariectomised rats.

Urinary incontinence has been observed as a post menopausal symptom in women and a post operative complication in ovariectomised pets. Epidemiologic studies have implicated estrogen deficiency after menopause as leading to urogenital atrophy. This manifests as recurrent lower urinary tract infections, irritative symptoms of urinary urgency, frequency, and urge urinary incontinence (Elia and Bergman, 1993). Estrogen deprivation also resulted in atrophic urethritis (Elia and Bergman, 1993), and a higher frequency of pseudo-stratified and transitional epithelium in the proximal urethra and urethero-vesical junction. Females are most susceptible to a weakened bladder sphincter due to low estrogen levels along with other factors and can be affected at any age after spaying (Arnold *et al*., 1989; Thrusfield, 1985; Thrusfield *et al*., 1998).

In a study done on rabbits by Aikawa *et al*. (2003), ovariectomy resulted in a 50% decrease in circulating estrogen whereas estradiol treatment resulted in a 5-fold increase in the serum estrogen levels. Estradiol treatment resulted in significant increases in bladder capacity and bladder weight. Morphologically, ovariectomy resulted in significant urothelial apoptosis whilst estradiol treatment resulted in the appearance of large cytoplasmic vacuoles in the urothelium and a significant smooth muscle hypertrophy (Aikawa *et al*., 2003). This study investigated the

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effects of soy isoflavones extract treatment on the morphology and morphometry of the urinary tract of ovariectomised rats.

1.1. Justification

Estrogen therapy has been effective in treating hypoestrogenic urinary incontinence. However, adverse effects are associated with its use. Some patients are not responsive to it. Phytoestrogens have become the most preferred alternative due to fewer side effects compared to hormonal treatment. Although the commercial world has suggested that soy isoflavones can cure urinary incontinence, very little scientific evidence supports these claims. Some morphological and morphometric studies on the effects of phytoestrogens on incontinence say they are effective whilst some say they are not. Literature on the effects of soy isoflavones on the urinary tract is limited, more studies are required to explain the claims of epidemiological data. The current study sought to contribute to the validation of claims by the existing pool of literature which is still inconclusive. Inorder to simulate the hypoestrogenic status of post menopause and post spay, ovariectomised rats were used in this study.

1.2. Research Question

What histologically discernable anatomical changes in the urinary tract of hypoestrogenic incontinent patients can be attributed to soy isoflavone extract treatment?

1.3. Hypothesis (H$_0$)

It was postulated that treating ovariectomised female rats with soy isoflavones will not result in any histologically distinguishable changes in the morphology and morphometry of the urinary tract.

1.4. Main objective

To assess the histomorphometric and histomorphologic changes in the urinary tract which might be attributed to soy isoflavone extract treatment in the relief of hypoestrogenic urinary incontinence.

1.5. Specific objectives

- To determine morphological and morphometric changes in the ureter, bladder and urethra of ovariectomised Sprague Dawley rats.
- To determine morphological and morphometric changes in the ureter, bladder and urethra of ovariectomised Sprague Dawley rats treated with soy isoflavone extract.

CHAPTER 2

2.0. LITERATURE REVIEW

2.1. SOY ISOFLAVONES

Soy isoflavones are phytoestrogens which are defined as structurally and/or functionally similar to mammalian estrogens (Figure 2.1). Isoflavones are abundant in legumes, especially soybeans, with trace levels in whole grain products, potatoes, fruits, vegetables, and alcoholic beverages (Boue *et al*., 2003). Isoflavones can also be found in cows' milk and meat (US Departament of Agriculture, 2008; Ward *et al*., 2010; Kuhnle *et al*., 2008). Other dietary sources of isoflavones include chick pea, alfalfa, and peanut (Dixon, 2004). There are three isoflavones in soy, namely genistein, daidzein and glycetin (Setchell *et al*., 1984). Genistein is the predominant phytoestrogen in soy which accounts for approximately two thirds of the soy isoflavone content in soy bean (Setchell *et al*., 1997) whilst daidzein and glycetin, are less abundant (Setchell *et al*., 1997; Setchell, 1998). Daidzein and glycetin are therefore relatively minor contributors to the total estrogenic effects of soy. Processing of legumes to obtain legume derived food, such as tofu, hardly alters their isoflavone content, except fermented miso in which they increase. Isoflavones exist naturally as glycosides which need to be hydrolyzed to the active form.
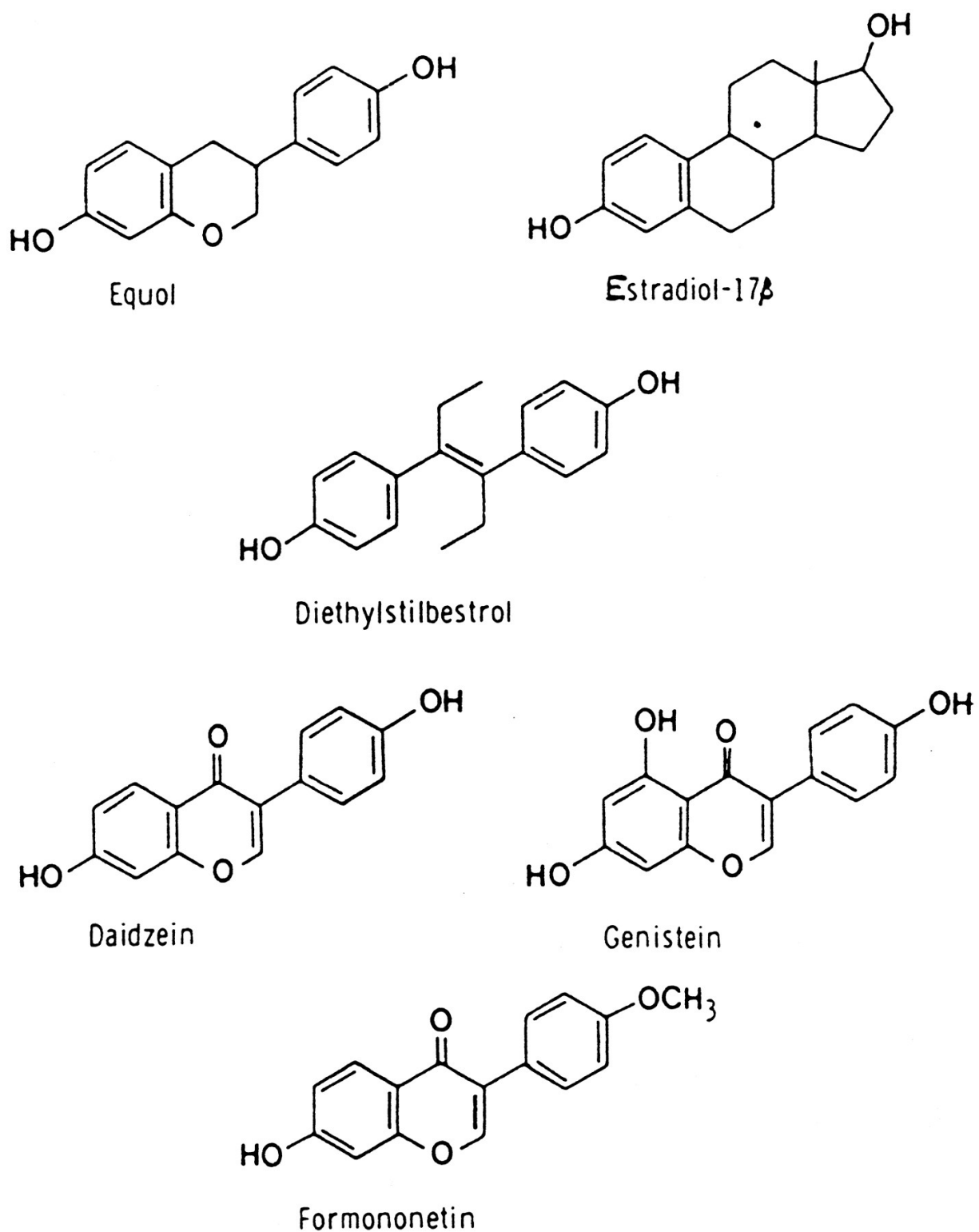
Figure 2.1. Chemical structures of Estradiol- 17β, Diethylstilbestrol, Daidzein, Genistein, Formononetin and equol. (Adapted from Setchell *et al*., 2002)

2.1.1. Soy Isoflavone metabolism and bioavailability

Soy isoflavones show complex metabolism in both animals and humans that involves mammalian and gut microbial processes. In their natural state, isoflavones occur as biologically inactive glycosides, malonates or the more water soluble acetyl conjugates (Long-ze Lin, 2000). The active forms are the aglycones which are unconjugated. Glucosides namely genistin, daidzin, and glycitin are hydrolyzed by intestinal glucosidases, to release the aglycones genistein, daidzein, and glycitein respectively (Barnes *et al*., 1994). Plasma genistein and daidzein may also be derived from precursors biochanin A and formononetin (Figure 2.2), respectively, after their breakdown by intestinal glucosidases (Setchell *et al*., 1984). Glucosidase activity also produces metabolites, such as the daidzein metabolite, equol (Setchell *et al*., 2002) (Figures 2.1 and 2.3). Equol is the end product of daidzein and is a more potent isoflavone. However, not all mammals are able to produce equol. Studies on urinary equol excretion after soy consumption indicate that only about 33% of individuals from western populations metabolize daidzein to equol (Setchell *et al*., 2002). Isoflavone concentrations rise slowly and reach maximum values of micromolar range at 7-8 hours (King and Bursill, 1998) after a single meal in humans. Genistein and daidzein have also been detected in human plasma (Adlercreutz *et al.*, 1994), urine (Adlercreutz *et al.*, 1991), and milk (Franke and Custer, 1996), as well as in saliva, breast aspirate and prostatic fluid (Finlay *et al.*, 1991). Serum dietary isoflavones are almost entirely conjugated, even in portal blood just after their absorption from the intestine. Unconjugated isoflavones are only 1–5% of the total isoflavones in the blood (Sfakianos *et al*., 1997). Unconjugated form of isoflavones is the active one which binds to estrogen receptors. Low circulating unconjugated isoflavones compete poorly with endogenous estradiol for receptor sites which is a disadvantage on the activity of isoflavones. This disadvantage is counteracted by

high circulating concentrations of the unconjugated isoflavones, 1000-fold greater than those of endogenous estradiol (Zaa and Duwe, 1997). Elimination of the majority of soy isoflavones is through the kidneys (Bingham *et al.*, 1998). Soy isoflavones can either mimic estrogen or antogonise it.
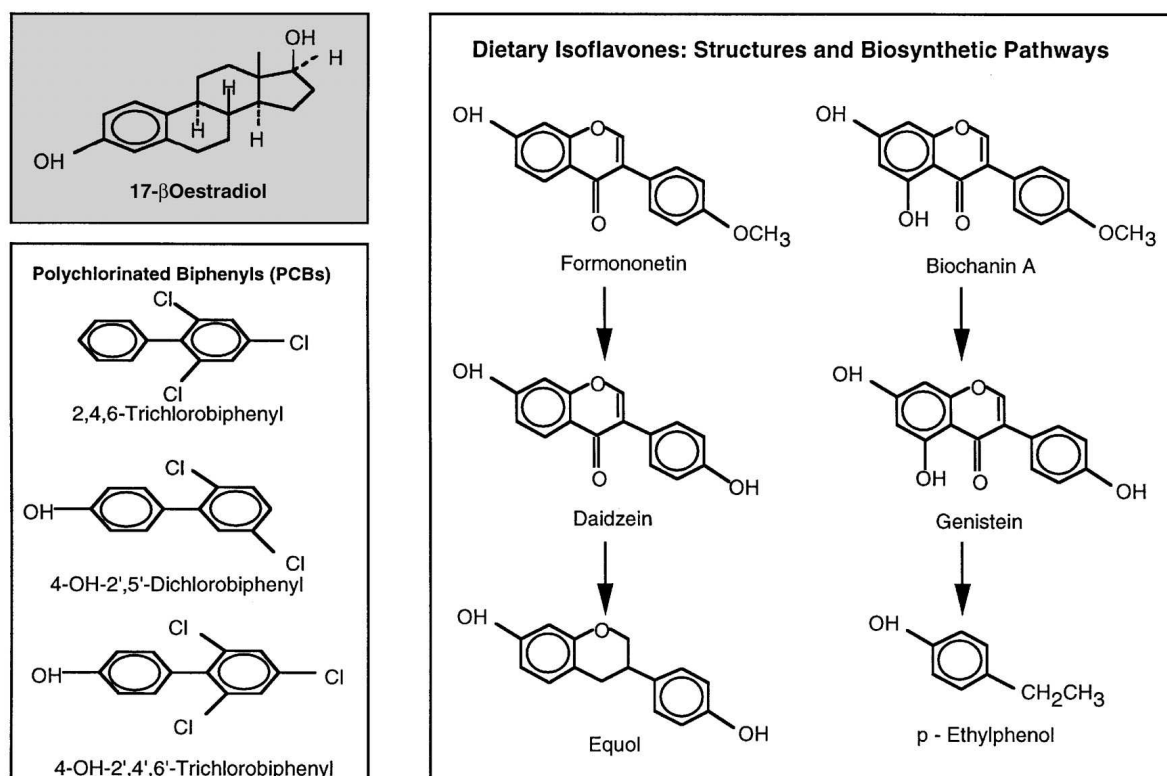
Figure 2.2 Structures of 17β-oestradiol, phytoestrogens, polychlorinated biphenyls and biosynthesis of Daidzein and Genistein from Formononetin and Biochanin A respectively. (Adapted from Reinhart *et al.*1999)
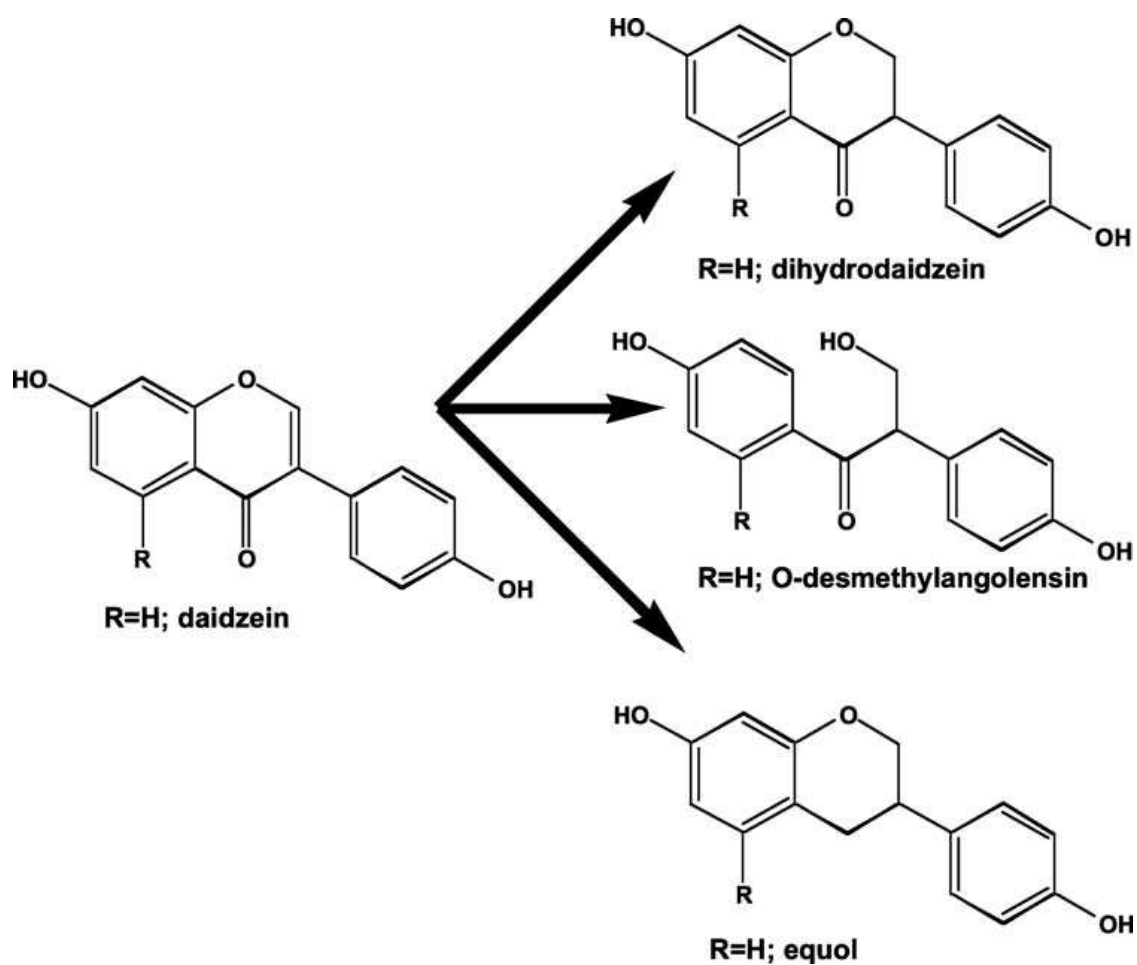
Figure 2.3 Bacterial metabolism of daidzein by intestinal microorganisms (Adapted from Setchell, 1998).

2.1.2. Soy isoflavone mechanism of action

Soy isoflavones show both estrogenic and non-estrogenic effects depending on their concentrations and the tissue being acted upon.

Estrogenic activity

Functionally, the soy isoflavones are described as "SERMS" or selective estrogen receptor modulators. These are compounds with selective estrogenic effects in that they may mimic the behavior of estrogen in some tissues (Price and Fenwick, 1985; Drane *et al*., 1980) whilst in

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others they may antagonize it or have no effect at all (Messina, 1994). They bind to estrogen receptors within cells and the estrogen-receptor complex interacts with DNA to change the expression of estrogen-responsive genes. Soy isoflavones inhibit 17-Hydroxysteroid reductase type 1, an enzyme which activates estradiol. They can also occupy the estrogen receptor sites thereby antagonising endogenous estrogen (Adlercreutz *et al*., 1994; Wang *et al*., 1994; Cassidy *et al*., 1994). Antagonism to endogenous estrogen can be a useful tool in the prevention of estrogen dependent disorders like mammary tumours (Wu *et al*., 2002; Shu *et al*., 2001). Estrogenic effects in other tissues could help maintain bone density and improve blood lipid profiles (Setchell *et al*., 2002). However, in infants, antiestrogenic effects can be deleterious (Jefferson *et al*., 2006). Some of the reported biological functions affected by soy phytoestrogens include the regulation of ovarian and oestrus cycles in female mammals,growth promotion, differentiation,as well as physiologic activities of the female genital tract, pituitary, breast, and many other organs and tissues in both sexes(Setchell, 1984). Estrogenic effects of soy isoflavones are brought about by their ability to bind to estrogen receptors.

2.1.3. Estrogen receptors

Estrogen receptors (ERs) are proteins which are activated by the hormone estrogen (17 β Estradiol). They occur in two classes namely ER, nuclear hormone member intracellular receptor and GPER, a G protein coupled receptor. The ER is also divided into two distinct subtypes i.e. ERα, and ERβ, with 95% homology in the DNA-binding region but only 55% homology in the ligand-binding domain (Kuiper *et al*., 1996; Tremblay *et al*., 1997; Mosselman *et al*., 1996). Some in vitro relative binding assays were conducted and they revealed that ERβ was able to bind to estrogen with the same affinity as ERα (Mosselman *et al*., 1996; Kuiper *et al*., 1998).

Two hundred and thirty chemicals were assayed for binding to the estrogen receptor (ER), 46 of these were phytoestrogens from six different chemical structure classes. Seven isoflavones bound to the ER with measurable affinity, ranging from a relative binding affinity (RBA) of 0.45 for genistein to 0.0013 for formononetin, with the RBA for estradiol being 100. Equol, a metabolite of the phytoestrogen daidzein, had an RBA for ER that was 33% that of genistein (Medlock *et al*., 1995). Binding studies using the purified receptor proteins show that the isoflavone genistein is a very active competitor for both receptor sub-types, but bind more to the beta than to the alpha subtype. A recent study using human hepatoma cells has also further asserted that daidzein binds to ERβ with a higher affinity than ERα (Medlock *et al*., 1995). Differential affinity is likely to be of functional significance, as the two receptor subtypes have different distributions across estrogen responsive tissues. Estrogen receptors are found distributed in various parts of the body.

2.1.4. Estrogen receptor distribution in body tissues

Most of the literature that is available on the distribution of estrogen receptors in tissues has been mainly referring to the classical ER α. Following the discovery of ERβ, a revisit of the distribution of ERs was done to include ERβ (Couse *et al*., 1997). Both ERs are found distributed in various tissues but with notable expression differences (Couse *et al*., 1997). ERα is found in the endometrium, ovarian stroma, hypothalamus and breast cancer cells and the efferent ducts of males (Hess, 2003). ERβ expression has been reported in the kidney, brain, bone, heart, lungs, intestinal mucosa, prostate gland and endothelial cells as shown in Table 2.1.

Table 2.1. Distribution of estrogen receptors progesterone receptors and androgen recptors in the female urogenital tract.

	Estrogen receptors	Progeste-rone receptors	Androgen receptors
Urethra	+		+
Urethral sphincter	+		
Periurethral venous plexuses	+		
Vagina	+	+	+
Trigone	+	+	+
Pelvic floor support			
▪ pubo-cervical muscle	+		
▪ levator muscle	? (+/-)		
▪ cardinal ligament	+	+	
▪ sacrouterine ligament	+ (α, β)	+	
▪ pubourethral fascia	+ (α, β)	-	
▪ perivaginal tissue	+	-	

(Adapted from Reichberger and Skorupski, 2007)

2.1.5. Estrogen receptor independent functions of soy isoflavones

Soy isoflavones and their metabolites also have biological activities which are independent from their interactions with estrogen receptors (Barnes *et al.*, 2000). By inhibiting the synthesis and activity of certain enzymes involved in estrogen metabolism, soy isoflavones may alter the biological activity of endogenous estrogens and androgens (Kao *et al.*, 1998; Whitehead *et al.*, 2002; Holzbeierlein *et al.*, 2005). Soy isoflavones can inhibit tyrosine kinases (Akiyama *et al.*, 1987) which are important enzymes in the signaling pathways that stimulate cell proliferation.

Soy isoflavones were also observed to have antioxidant properties through in vitro studies (Ruiz-Larrea *et al*., 1997).

2.2. Anatomy of the urinary tract passages

2.2.1. Gross anatomy and histology

The urinary tract passages consist of a pair of ureters, a bladder and a urethra. Estrogen effects have been observed in these structures. Their function is to collect urine and drain it out of the body.

2.2.2. Ureter

The ureter begins as an expanded part at the renal pelvis. It courses saggittaly against the abdominal roof as the tubular part. It reaches the pelvic cavity and bends to enter the male genital fold and the female broad ligament (Dyce *et al*., 2002; Getty, 1975). It attains the dorsal surface of the bladder and opens into it near the neck. It penetrates the wall of the bladder obliquely after an intramural course and together with small folds of bladder mucosa which act as flap valves; this arrangement prevents urine reflux into ureters when the bladder contracts (Dyce *et al*., 2002; Getty, 1975). The mucosa is lined by transitional epithelium (Mescher, 2010). The lamina propria is composed of dense connective tissue which becomes loose at sites in contact with the adjacent smooth muscle layer. The mucosa has prominent folds which are only absent at the renal pelvis. The folds give the lumen of the ureter a characteristic stellate shape (Mescher,

2010). The upper two thirds of the ureter have two layers of smooth muscle i.e. inner longitudinal and outer circular layers. In the lower third, there is a third muscle layer which forms an outer longitudinal layer. The outer coating (adventitia) consists of fibroelastic connective tissue, associated blood vessels and nerves (see Figure 2.4 below).
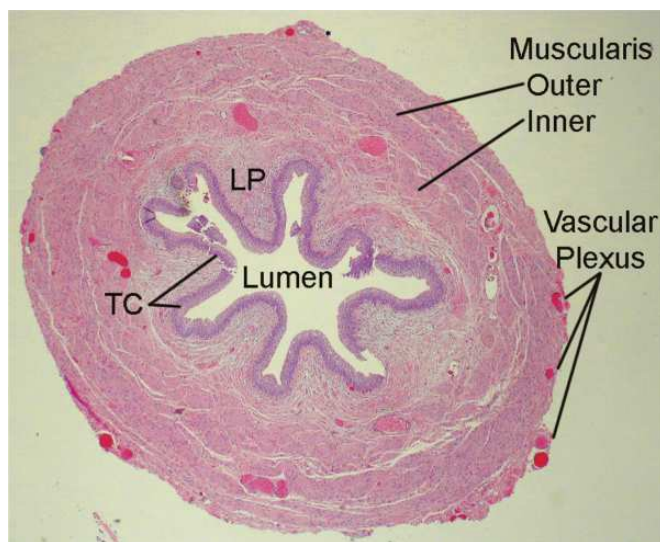
Figure 2.2 Histological structure of the ureter. (Adapted from Mescher, 2010)
LP= lamina propria, TC=transitional epithelium

2.2.3. Bladder

The bladder can be divided into two main components: the bladder body, which is located above the ureteral orifices, and the base, consisting of the trigone, urethrovesical junction, deep detrusor, and the anterior bladder wall (Figure 2.5). The bladder is a hollow smooth muscle organ lined by a mucous membrane and covered on its outer aspect partly by peritoneal serosa and partly by fascia. Its muscular wall is formed of smooth muscle cells, which comprise the

detrusor muscle (Dyce *et al*., 2002; Getty, 1975).The empty contracted organ rests on the pubis and is confined to the pelvic cavity. Distension results in a part of the bladder entering the abdomen but the neck remains in the pelvis. Bladder filling occurs continuously with no immediate increase in internal pressure (Dyce *et al*., 2002; Getty, 1975). The pressure continues to build up until it rises sharply creating the urge to void urine. The external layer of the bladder consists of connective tissue. This layer is continued three vesical folds; paired lateral folds and a medial fold. The lateral folds house the round ligaments of the bladder. The medial fold is devoid of structures in the adult but houses the urachus in the fetus. Topographically, the bladder is related to reproductive organs and their supporting tissue dorsally. Its ventral surface contacts pelvic and abdominal wall (Dyce *et al*., 2002; Getty, 1975).

The mucosa is lined by transitional epithelium. The lamina propria is fibroelastic, composed of loose dense connective tissue and elastic fibres. The muscular layer is a dense woven sheath of smooth muscle (Park, 2007; Mescher, 2010). There are three layers of smooth muscle which have been described (Anderson and Arner, 2004). Cells of the outer and inner layers are arranged longitudinally, whilst those of the middle layer are circularly oriented. The orientation and interaction between the smooth muscle cells in the bladder are important, since this will determine how the bladder wall behaves and what effect activity in the bundles cells will have on its shape and intraluminal pressure. In smaller animals, e.g., rabbit, the muscles are less complex and the patterns of arrangement simpler than in the human detrusor (Anderson and Arner,2004). The individual smooth muscle cells in the detrusor are typical smooth muscle cells,long, spindle-shaped cells with a central nucleus. Their cytoplasm contains numerous packed normal myofilaments, and the membranes contain regularly spaced dense bands, with membrane vesicles (caveoli) between them. There are also scattered dense bodies in the cytoplasm and

sparse mitochondria as well as fairly sparse nucleocentric elements of sarcoplasmic reticulum. The adventitia is fibroelastic (Anderson and Arner, 2004; Park, 2007).
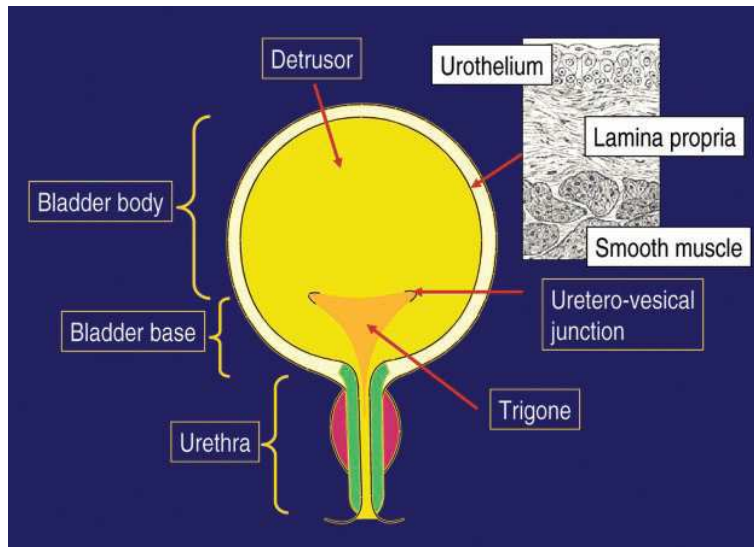
Figure 2.5 Schematic diagram of the urinary bladder. (Adapted fom Andersson and Arner, 2004).

2.2.4. Urethra

Female urethra

The urethra of the females runs caudally on the pelvic floor below the reproductive organs. It passes through the vaginal wall obliquely to open ventrally at the junction of the vagina and vestibule. In some species (sow and cow), it opens together with suburethral diverticulum. In the bitch, it opens on a hammock (Dyce *et al*., 2002; Getty, 1975). When the urethral diverticulum is present, it is found ecnclosed in the urethralis muscle. The urethralis surrounds the urethra in its entire course. Its cranial fassicles encircle the urethra. The caudal fassicle support the urethra in U shaped loops which originate and terminate on the vaginal wall (Dyce *et al*., 2002; Getty, 1975). Contraction of these loops closes the urethra by pressing it to the vagina thereby narrowing the vagina. The lumen of the urethra on cross section is crescentic in shape. The mucosa in the proximal part is lined by transitional epithelium, the middle portion by stratified

(pseudostratified) columnar whilst distally towards the external urethral orifice, it becomes stratified squamous non- keratinized epithelium (Cormack, 1987; Chapman *et al*., 1973; Park, 2007).   A thick and fibroelastic lamina propria lies beneath the epithelium. It contains a plexus of thin walled veins (Cormack, 1987; Mescher, 2010; Chapman *et al*., 1973). The muscularis comprises the inner longitudinal and outer circular layers. A urethral sphincter made up of voluntary skeletal muscle encircles the external urethral orifice (Cormack, 1987; Mescher, 2010; Chapman *et al*., 1973).

Male urethra

The male urethra stretches from the internal urethral orifice at the bladder neck to the external urethral orifice at the free end of the penis. It is divided into an internal pelvic part and an external spongy part. The pelvic part is joined by the deferent and vesicular ducts or they combine with the jaculatory duct close to its origin from the bladder. The initial part also bears a dorsal crest from the urethral orifice to a thickening (colliculus seminalis). The colliculus seminalis bears orifices for the deferent duct and prostatic ducts. Its dorsal surface is related to the rectum and various accessory sex glands (Dyce *et al*., 2002; Brooks, 2007). The end of penile urethra has a lining of stratified squamous epithelium; farther up in the pelvic urethra, nearer the bladder, the lining is still transitional epithelium. The lamina propria is also fibroelastic but sometimes contains smooth muscle fibres. Lamina propria of prostatic urethra is highly vascularized (Cormack, 1987; Anderson *et al*., 2007).

2.2.5. Developmental anatomy

Urinary Bladder and Urethra

An urorectal septum divides the cloaca into dorsally, a rectum, anal canal and anal membrane, and ventrally, a urogenital sinus and urogenital membrane. Membranes subsequently degenerate, resulting in an anus and a urogenital orifice, respectively. Cranially, the urogenital sinus connects with the urachus, the intra-embryonic stalk of the allantois (Park, 2007).

Urinary bladder develops from the cranial end of the urogenital sinus and the adjacent region of urachus. Growth expansion results in separate openings of the mesonephric duct and ureter into the dorsal wall of the urogenital sinus. Differential growth of the dorsal wall results in mesonephric duct and ureter openings being switched cranio-caudally, creating a trigone region that anchors ureters to the bladder and urethra (Park, 2007).

Urethra develops from the urogenital sinus caudal to the urinary bladder. Urethra development is gender specific. In females, the mid region of the urogenital sinus becomes the urethra. The caudal region of the urogenital sinus becomes the vestibule and the vagina growths out of the vestibule wall. In males: the pelvic urethra develops from the mid region of the urogenital sinus and the penile urethra develops from elongation of the caudal end of the urogenital sinus (Park, 2007).

Abnormal development of the ureter, bladder and urethra can occur. Developmental anomalies can result in dysfunction of the affected structures. Patent urachus (urachal fistula) results from a failure of the allantoic stalk to close at birth and vesicourachal diverticulum, urachus persists as a bladder pouch, predisposing to chronic cystitis (Schlussel and Retik, 2007). Ectopic ureter can result in a ureter that opens into the urethra or vagina instead of the bladder (Schlussel and Retik, 2007). Ectopic ureteral orifices tend to be in the urethra, vagina or perineum predisposing the urinary tract to urinary incontinence (Schlussel and Retik, 2007).

2.3. Effects of oestrogen on morphology and morphometry of the urinary tract

Effects of estradiol replacement on the bladder collagen and elastic fibers of ovariectomised rats were investigated by Dambros *et al*. (2003). No changes in the distribution of the connective tissue components within the bladder wall were observed. Estrogen administration did not alter the quantitative behavior of the main connective tissue components in ovariectomised rats. Contrary to this evidence is the result obtained by Aikawa *et al*. (2003) in which bladder function and structure were significantly affected by modulating the circulating estrogen levels. Pharmacological levels of estrogen caused hypertrophic effects on bladder smooth muscle, resulting in increased contractile function. Sartori *et al*. (2001) revealed that estrogen replacement in ovariectomised rats results in a reduction of collagen fibres in the detrusor muscle and the urethra. Collagen fibres were reduced in number and thin in the periurethral tissue of estrogen treated post-menopausal patients (Falconer *et al*., 1998).

2.4. Effects of soy isoflavones on the urinary tract morphology and morphometry

Bladder and urethra anatomical integrity has been under scrutiny in order to find an anatomical explanation of post ovariectomy and menopausal hypoestrogen linked incontinence. In these studies, the effect of isoflavone treatment on the integrity of the urinary tract was anatomically assesed. A study done by Santos *et al.* (2010) examimed the morphological structure of rat bladder post ovariectomy with and without isoflavone extract intervention. Treatment times were also varied into early and late treatment post ovariectomy. Animals treated early with isoflavone showed reversal of ovariectomy effects whilst late treatment could not reverse the effects. Transgenic and organic soy effects on the bladder of ovarictomised Wistar rats were assessed (da Silva Faria *et al.*, 2009). Both soy based diets showed an increase in collagen fibres and a decrease in smooth muscle compared to the control. Higher collagen to muscle ratios was obtained. Collagen fibres in the control group were of a different type from those of the soy diet groups. New collagen fibres were synthesised in the experimental groups suggesting possible detrusor remodeling in response to treatment.

2.5. Functions of the urinary excretory passages

The ureter conveys urine by peristaltic contractions of its wall to the bladder. Urinary bladder stores urine without leakage for long periods of time and then ensures its rapid expulsion during micturition. These functions involve a very complex interaction between the structural/anatomic parts of the urinary tract and the nervous control systems. Both filling and emptying of the urinary bladder provide a challenge to the muscle components in the walls of the lower urinary tract. During filling of the urinary bladder, the smooth muscle cells have to relax, and to elongate and rearrange in the wall over a very large length interval (Andersson and Arner, 2004). During

micturition, force generation and shortening must be initiated comparatively fast, synchronized, and occur over a large length range. These activities require both regulation of contraction and relaxation (Andersson and Arner, 2004). In early work by Bozler (1941), two classes, "single-unit" and "multiunit," of smooth muscle were defined on the basis of contractile behaviour. Bozler's class of single-unit smooth muscles is arranged in sheets or bundles, and the cell membranes have several connection points and gap junctions. The gap junctions constitute low-resistance pathways, formed by connexin subunits, through which ions can flow from one cell to the other, and thereby an electrical signal can be spread rapidly throughout the tissue (Brink, 1998). Action potentials can thus be conducted from one area to another by direct electrical conduction. Multiunit smooth muscles are thought to be composed of discrete muscle fibers or bundles of fibers that operate independently of each other. They are richly innervated by the autonomic nervous system and are controlled mainly by nerve signals. They rarely show spontaneous contractions. Although the detrusor muscle exhibits several of the characteristics ascribed to a single-unit smooth muscle, it also shows several features ascribed to multiunit smooth muscles, being densely innervated, and functionally requiring nervous coordination to achieve voiding. An alternative functional division of the urinary bladder smooth muscle is a comparatively fast smooth muscle with characteristics of a "phasic" smooth muscle form, e.g., trigonal and urethral smooth muscle (Andersson and Arner, 2004).

The urethral sphincters control the release of urine from the body. The internal sphincter muscle of urethra is a continuation of the detrusor smooth muscle and is therefore under involuntary or autonomic control (Anderson and Arner, 2004). This is the primary muscle for prohibiting the release of urine. The external sphincter muscle of urethra is a

secondary sphincter to control the flow of urine through the urethra. It is skeletal muscle therefore it is under voluntary control of the somatic nervous system (Tanagho, 2008).

2.6. Pathophysiological adaptations of bladder and urethra

Urinary outlet obstruction can result in hypertrophic and hyperplastic changes of the smooth muscle in the urinary bladder e.g., in benign prostatic hypertrophy, or after decentralization, e.g., in spinal cord injuries. Several human studies on hypertrophic growth of detrusor muscle in response to urinary outlet obstruction have been conducted and are well documented (Gilpin *et al*., 1985). Animal models have been extensively studied in cases of hypertrophy of partial urethral obstruction (Brent and Stephens, 1975; Levin *et al*., 1984; Mattiasson and Uvelius, 1982; Mostwin *et al*., 1991; Sibley, 1985; Steers and De Groat, 1988; Williams et *al*., 1993). Gabella and Uvelius (1990) extensively characterised the fine structure of normal and hypertrophic rat urinary bladder. They showed an increase in size, and length and transverse area, suggesting hypertrophy of the cells in the absence of mitoses, although double nuclei were present in some of them. There were very few or no Gap junctions in both control and hypertrophied tissues. The bladder hypertrophy can also be associated with alterations in extracellular materials. Extracellular material and collagen content alterations were observed by Gosling and Dixon (1980) in the trabeculated bladder from patients with prostatic enlargement. In the hypertrophic rat urinary bladder, total collagen increases whilst the concentration of collagen appears to decrease (Uvelius and Mattiasson, 1984.) Several adaptive changes in the detrusor muscle have been observed which suggested that bladder wall has a

regenerative capacity after injury. Hypertrophy of the bladder wall involves thickening of epithelium, muscle layer, and serosa with an increase in tissue vascularization (Gabella and Uvelius, 1990), suggesting formation of new blood vessels in the vascular wall. Microarterial vessel size is also increased (Boels *et al*., 1996). Blood flow has been reported to increase to the rabbit bladder initially during hypertrophy (Lieb *et al*., 2000). However, continued obstruction of more than two weeks resulted in impaired microcirculation (Tong-Long *et al*., 1998). In chronic decompensative hypertrophy, a general decrease in blood supply occured (Schroder *et al*., 2001). Urinary bladder function is altered in several other (patho) physiological conditions e.g pregnancy.

2.7. Urinary incontinence

Urinary incontinence is the uncontrollable loss of urine. This can occur singly as stress incontinence, urge incontinence or overflow incontinence. Cases of mixed incontinence can also occur. Stress incontinence is usually experienced when pressure is exerted on the bladder during such activities as coughing, sneezing, laughing, lifting, exercising and sexual relations (Rogers, 2008). The amount of leakage is usually small (drops), but can increase (Nazir *et al*., 1996). Urge incontinence or overactive bladder is usually associated with increase in the frequency of urination, a need to urinate frequently at night, and an intense urge to urinate with very little warning (Nazir *et al*., 1996). This is not a bladder problem but rather a problem of abnormal signals to the bladder from the nerves and muscles. Overflow incontinence occurs when the bladder empties insufficiently. This may be caused by a blockage or narrowing of the urethra

such as an enlarged prostate in males, scar tissue, or a prolapsed bladder. It can also result from a bladder contraction problem due to medications, nerve injury, or chronic overstretching of the bladder muscle (McFall *et al*., 1997). Symptoms include dribbling urine throughout the day, a weak urinary stream, or the feeling of a need to urinate, but sometimes cannot do so. Mixed incontinence is a common combination of any of the above types (Nazir *et al*., 1996).

2.7.1. Risk Factors for urinary incontinence

Risks for urinary in continence include age, race, obesity, menopause, hypoestrogenism, child birth and pregnancy, smoking and chronic lung disease and hysterectomy.

Age

Human studies have shown a gradual increase of incontinence with age with broadpeak at middle age. A steady rise is then maintained after 65 years (Chutka *et al*., 1996). The type of incontinence may show variation with age, some studies asset that stress urinary incontince is most prevalent in women below 60 years whilst urge incontinence in older females (Gibbs *et al*., 2007).

Menopause

Menopause has been associated with an increase in the urinary dysfunction. It is however difficult to separate changes due to age with those due to menopause since the events may coincide. However, estrogen deficiency has been implicated in the genesis of postmenopausal urinary incontinence. High affinity estrogen receptors were identified in the urethra, bladder,

pubococcygial muscle (Santos *et al*., 2010). The lowering of estrogens reduces the number of periurethral vessels and urethral a-adrenergic receptors and causes atrophy of urethral and bladder supporting tissues leading to impaired function (Rud, 1980). The use of estrogen therapy has been controversial in the treatment of urinary incontinence, with some studies finding no changes in quality of life or incontinence episodes (Jackson *et al*., 1996; Fantl *et al*., 1994). However, estrogen supplementation has been found to subjectively improve symptoms of urgency and frequency. In menopausal women, oestrogen therapy improved the urinary urgency, frequency, nocturia and dysuria but did not improve incontinence (Salmon *et al*., 1941; Musiani, 1972; Schleyer-Saunders, 1976).

Hypoestrogenism

Oestrogen administration increases the contraction intensity of the periurethral muscles in female rats and rabbits in response to the adrenergic stimulus (Raz *et al*., 1972).

Since the lower urinary tract and the vagina share common embryological origin, it has been postulated that hypoestrogenism affects the sensory threshold of the urinary tract in post-menopausal patients, leading to decreased functional bladder capacities. Neutering effectively results in hypoestrogenism. Urinary incontinence reports were documented a long time ago in neutered bitches (Joshua, 1965). Other subsequent reports have been further asserting the predisposition of neutered animal to acquired urinary incontinence. A 6% prevalence of acquired incontinence was reported in 109 dogs with post- neutering complications (Okkens, 1997). It can be argued that neutering acquired urinary incontinence can occur due to mechanical damage to

tissue and nerves. However, Janssens and Janssens (1991) reported acquired urinary incontinence as a long-term complication in 12 of 72 bitches (17 %) following ovariectomy in which the cervix was left intact. This evidence supports that some cases of acquired urinary incontinence in neutered animals are probably due to a reduction in circulating oestrogen levels, caused by removal of the ovaries (Nickel, 1998), rather than an anatomical cause. Menopausal urinary symptoms influenced by hypoestrogenism include improper perception of bladder fullness, dysuria, polyuria, nocturia. Furthermore, an overactive bladder syndrome and stress or mixed urinary incontinence at least in part may be linked to the atrophy of trigone, loss of pelvic floor striated muscle tone, improper collagen metabolism leading to the urethral stiffness and decrease of the activity of -adrenergic system (Hextall and Cardozo 2001; Dessole et al., 2004). Chronic hypoestrogenism may further result in thinning of urethral mucosa and a decrease of urethral blood flow also occur leading to stress incontinence (Hextall and Cardozo, 2001).Some parameters of hypoestrogenic incontinence have been successfully alleviated using estrogen replacement therapy (see Tables 2.2 a and b).

Table 2.2 a &b. The influence of estrogens on the function of urinary bladder and urethra.

Hypoestrogenism	Estrogen replacement therapy
■ elevated volume of postvoid residual urine ■ decreased cystometric capacity of the bladder ■ decrease of maximal detrussor pressure during micturition ■ decrease of urine flow during micturition ■ urethral syndrome ■ dysuric symptoms ■ urinary incontinence ■ urinary tract infections	■ Modification of the density of muscarinic receptors in detrusor muscle ■ decreased influx of calcium ions to bladder muscle cells ■ decrease of amplitude and frequency of spontaneous bladder contractions ■ increase of the threshold of micturition reflex ■ urethral smear – increase of kariopyknotic and maturation indices ■ increase of urethral closure pressure by 33% ■ sensitization of α-adrenergic receptors in smooth musce cells of external urethral sphincter ■ increase of blood flow in periurethral plexuses ■ stimulation of collagen biosynthesis
Hypoestrogenism	Estrogen replacement therapy
■ decrease of collagen content in urogenital diaphragm and periurethral connective tissue ■ close correlation between collagen content and urethral closure pressure ■ hydroxyproline depletion by 40% in women with stress urinary incontinence	■ activation of the expression of collagen genes ■ stimulation of the fibrillar collagens synthesis in pelvic floor fascias ■ increase of periurethral tissue collagen biosynthesis

Adapted from (Reichberger and Skorupski, 2007

2.8 Methods of analyzing morphologic and morphometric changes on the urinary tract

The functional and morphological modifications of the urinary bladder in aging female rats were studied by Lluel *et al*., 1999. Morphometric measurement was based on computerized image analysis using a Nachet NS15, 000 processor (Nachet, Evry, France) driven by a microcomputer following a program written in the INSERM Unit (Veniant *et al*., 1993).

Santos *et al*. (2010) studied morphology of the urethra using a light microscope with a 10x ocular and a 40x objective lens to analyse the tissue sections. Morphometric studies were caried out by counting the numbers of nuclei, collagen fibres and blood vessels using the "test point-counting volumetric technique described by Weibel *et al*. (1966). This method made use of a 25-point integration ocular lens associated with a light microscope.

CHAPTER 3

3.0. MATERIALS AND METHODS

3.1. Study design

This was a randomised controlled experiment (RCT).

3.2. Study setting

This was a laboratory based study at the Faculty of Veterinary Science at the University of

Zimbabwe main campus in the Preclinical and Clinical Veterinary studies departments.

3.3. Animals

Experimental animals were purchased from the animal house facility, a department of the

Faculty of Veterinary Science at the University of Zimbabwe main campus. Twenty four virgin

adult female Sprague Dawley rats, approximately 90 days old, weighing 200 g on average, were

used in this study.

3.4. Housing

The animals were transferred to the preclinical veterinary studies department in plastic cages

with gridded metal covers. They were then transferred into similar cages but in groups of four

animals in each cage. Wood shavings were used as bedding and placed in plastic trays just below

the cages. They were kept in an animal room at room temperature (24-26°C), under artificial

lighting with 40 Watt daylight model Philips fluorescent bulbs. Light and dark photoperiods of

12 hours each were administered.

3.5. Feed

All the rats were fed with standard soy- free rat chow *ad libitum* for 10 days as they acclimatised before the start of the experiment. The soy- free chow contained white maize grain, maize germ meal, wheat feed, cotton meal, carcass meal and fish meal. It was specifically made for these particular experimental animals by a nutritionist of the National Foods Private Limited Company, Harare, Zimbabwe. Soy protein was substituted with fish meal and carcass meal protein. The relative volumes per 100g of nutrients in the feed are shown in table 3.1 below.

Table 3.1 Relative volumes of nutrients in the soy isoflavone free feed

VOLUME	100
DRY MATTER	89.15
OILEE	5.85
OIL AH	6.18
CPROTEIN	22.10
FIBRE	8.17
ASH	8.21
CALCIUM	2.09
PHOS	1.49
AVPHOS	1.13
SALT	0.38
SODIUM	0.19
NDF	25.68
STARCH	21.51
ME_R	11.81
SUGAR	4.44
DE PIG	11.99
MEP:M+C	9.37
CA:P	1.40
%LYSDIG	0.77
%METDIG	0.23
FISH	2.48
LYSINE/DE	0.09
TDN	74.00
MET/DE	0.04
ARG	1.96
MEP	9.98
RAPE	0.00
WHEAT	32.72
TLYSINE	1.08

3.6. Sample size and groups

A total of 24 animals were randomly placed into 3 groups of 8 animals each as follows;

- Group 1 animals were sham operation and distilled water treatment.

- Group 2 was that of bilateral ovariectomy and isoflavone extract treatment.

- Group 3 animals were bilateral overiectomy and distilled water treatment.

3.7. Ovariectomy Procedure

Bilateral ovariectomy was performed on two groups of 8 rats each as outlined by Saadat *et al*. (2008). The rats were placed in an induction chamber with halothane and oxygen only. The dorsal thoracolumbar area was prepared for an aseptic procedure. A one centimetre incision was done on the skin just caudal to the last rib close to the midline. The incision was extended into the muscle and the abdomen. The ovaries were identified and removed for the Groups 2 and 3 only. The muscle was left open whilst the skin was sutured with PDS size 3/0. The animals were allowed to recover for 10 days before the start of the treatment.

3.7.2. Sham operation

The anaesthetic and surgical preparation protocol was the same as for ovariectomy operation. A one centimetre incision was done on the skin just caudal to the last rib close to the midline. The incision was extended into the muscle and the abdomen. The ovaries were identified but were not

removed. The muscle was left open whilst the skin was sutured with PDS size 3/0. The animals were allowed to recover for 10 days before the start of the treatment.

3.8. Treatment administration

Treatment was administered by oral gavage over 65 days. During this period, the soy free meal and water were provided ad libitum. A soy isoflavone extract (Soy Balance, Nature's Resources Products, and Mission Hills, CA 91346- 9606) was used. It was administered an effective dose of 125 µg/g body weight per day after dilution in distilled water. This dose had been established in pilot study conducted by Santos *et al*. (2010). This dose was contained in 3ml of solution which was administered per rat. The treatment protocol was as shown in Table 3.2.

Table 3.2 Animal groups and the different treatments administered.

Group	1	2	3
Treatment	Sham operation and 3ml distilled water treatment per day.	Bilateral ovariectomy and isoflavone extract treatment (125µg/g body weight) in 3ml solution per day.	Bilateral overiectomy and 3ml distilled water treatment per day.

3.9. Sacrifice and tissue collection

At the end of the experimental trial, the animals were sacrificed in a suffocation chamber with ether drenched cotton wool. A ventral median incision was made on the skin, linea alba and peritoneum to access the abdominal cavity. The kidneys, ureters and the urinary bladder were

exposed, the urinary tract was then dissected out and the different parts were fixed in 10% formaldehyde solution for 24 hours.

3.10. Tissue processing

The fixed tissue were processed in an automatic processor overnight and then embedded in paraffin wax to allow for cross-sectional cuts, perpendicular to the largest axis of the structures to be analyzed.

3.11. Staining methods

Staining of the tissue sections was done using Haematoxylin and Eosin while Van Gieson's stain was used for collagen fibres.

3.12. Microscopy

A light microscope with a 10× ocular and a 40× objective was used to analyze the tissue sections. Morphological studies on the sections of the organs were done using a Leitz vario othomat 02 automatic microscopic camera (Ernst Leitzwetzlar GMBH, West Germany). Images were uploaded onto a computer and analysed.

For morphometric analysis, the numbers of epithelial lining cells and blood vessels were recorded. The thickness of lamina propia and musularis layers were measured using an integrated ocular lens/graticle (Graticles LTD, Tonbridge, Kent, England), associated with light microscopy, with a 40× lens. The intergrated ocular lens had one linear vertical and several horizontal divisions parallel to each other but perpendicular to the vertical line. These were used

to measure the thickness of the muscularis layer and the lamina propria in the ureter, urethra and bladder using magnification rates in Table 3.3.

Table 3.3 Total magnification for light microscopy morphological and morphomtrical studies of the ureter, bladder and urethra.

Tissue	Total magnification
Ureter	X400
Bladder	X100
Urethra	X200

Three fields were randomly selected and two slides were selected per section making a total of 6 random fields selected per sample per tissue. All the visible blood vessels at the tabulated magnifications in the different organs were counted using a counter.

3.13. Analysis of Results

A variance analysis was performed for the variables: number of layers in the epithelial lining, muscularis nuclei density, lamina propria thickness, number of blood vessels and muscularis thickness, recorded in the urethra, bladder and ureters of the groups studied. Descriptive statistics were used to calculate the means. The significance level chosen for rejecting the null hypothesis was 0.05 (5%). All statistical analyses were carried out using SPSS statistical package (version 8, Chicago, USA).

3.14. Ethical considerations

A license to capture and experiment on rodents was obtained from the Veterinary Research Council Board. Anaesthesia was used when conducting any surgical procedures on the rats as well as when the rats were being sacrificed.

CHAPTER 4

4.0. RESULTS

4.1. Mohphometric Study

4.1.1 .Muscularis thickness

In the ureter, the ovariectomised vehicle treated animals recorded a significantly lower muscularis thickness compared to the other two groups. However, there was no significant difference between the ovariectomised isoflavone treated rats and sham- vehicle treated rats. In the bladder, the ovariectomised vehicle treated group recorded a significantly lower muscularis thickness compared to the other two groups. No significant difference was observed between sham operated vehicle treated and ovariectomised isoflavone treated animals. In the urethra, the sham-vehicle treated rats recorded a significantly higher muscularis thickness compared to the other two groups (see Table 4.1).

4.1.2. Lamina Propria thickness

In the ureter, the lamina propria thickness was significantly different across all three groups. The ovariectomised vehicle treated group recorded a significantly higher lamina propria thickness, followed by the ovariectomised isoflavone treated rats. The sham-vehicle treated animals recorded a significantly lower lamina propria compared to the other two groups. In the bladder, the sham-vehicle treated animals recorded a significantly lower lamina propria thickness compared to the other two goups. However, no significant difference was observed between the ovariectomised isoflavone treated and the ovariectomised vehicle treated rats (see table 4.1).

4.1.3. Muscle nuclei density

In the ureter, a significantly higher muscle nuclei density was recorded for the sham-vehicle treated rats. There was however no significant difference in the muscle nuclei density between the ovariectomised isoflavone treated and the ovariectomised vehicle treated animals. In the bladder, ovariectomised vehicle treated animals recorded a significantly lower muscle nuclei density compared to the other two groups. No significant difference was recorded between the ovariectomised isoflavone treated and the sham-vehcle treated animals.In the urethra, ovariectomised vehicle treated group also recorded a significantly lower muscle nuclei density than the other two groups. There was no significant difference recorded between the ovariectomised isoflavone treated and the sham-vehicle treated rats (see Table 4.1).

4.1.4. Epithelium

No significant difference in the epithelial layer thickness was observed across all groups in the ureter. In the bladder, epithelium lining was significantly thicker in the sham vehicle treated rats compared to the other two groups. No significant difference in epithelium thickness was recorded between the ovariectomised isoflavone treated and ovariectomised vehicle treated rats. Urethral epithelium lining was significantly thicker in the sham-vehicle treated animals compared to the other two groups. There was no significant difference in epithelium lining thickness between the ovariectomised isoflavone treated and ovariectomised vehicle treated groups (see Table 4.1).

4.1.5. Blood vessels

In the ureter, there was no significant difference recorded in the blood vessel numbers of all three groups. There was also no significant difference recorded in the bladder blood vessels across all groups. In contrast, in the urethra, the ovariectomised vehicle treated rats recorded a significantly lower number of blood vessels compared to the other two groups. No significant difference was noted between the ovariectomised isoflavone treated and the sham-vehicle treated rats (see Table 4.1).

Table 4.1 Mean (95% confidence interval) muscularis thickness, lamina propia thickness, muscle nuclei density, epithelium thickness and blood vessel number in the bladder, urethra and ureter of ovariectomised and sham operated rats.

Organ	*Group Means (95% confidence interval)		
	sham+vehicle (n = 8)	ovx+soy isoflavone (n =7)	ovx+vehicle (n = 6)
Ureter			
Muscularis	381.8[a] (312.1;451.5)	327.2[a](281.8;375.7)	263.6[b](212.1;315.1)
Lamina propria	130.3[a](103.0;154.5)	190.9[b](160.6;218.2)	230.3[c](203.0;257.6)
Muscle nuclei density	3.2[a](3.0;3.4)	2.86[b](2.6;3.2)	2.83[b](2.5;3.2)
Epithelium	4.1[a](3.5;4.7)	4.0[a](3.4;4.6)	3.9[a](3.1;4.8)
Blood vessels	14.9[a](12.1;17.6)	19.0[a](16.1;21.9)	18.5[a](14.0;23.0)
Bladder			
Muscularis	7862.5[b] (7475;8250)	7587.5[b] (6950;8200)	5612.5[a](4437.5;6687.5)
Lamina propria	937.5[a](787.5;1087.5)	1537.5[b](1387.5;1725)	1337.5[b](975;1687.5)
Muscle nuclei density	2.8[b](2.4;3.0)	2.61[b](2.4;2.7)	2.06[a] (1.6;2.5)
Epithelium	3.4[a](3.1;3.7)	2.7[b](2.1;3.3)	2.3[b](1.6;2.9)
Blood vessels	166.4[a](147.7;185.1)	182.2[a](126.4;238.0)	146.6[a](121.0;172.1)
Urethra			
Muscularis	4080.7[a](3463.3;4692.2)	2934.1[b](2422.6;3345.7)	2740.1[b](3422.2;1675.8)
Lamina propria	335.2[a](264.6;394)	582.1[b](499.8;664.4)	723.2[b](505.7;940.8)
Muscle nuclei density	3.22[a](3.0;3.5)	2.71[b](2.5;2.9)	2.54[b](2.2;2.9)
Epithelium	3.6[a](3.1;4.0)	3.1[b](2.8;4.4)	3.0[b](2.5;3.5)
Blood vessels	55.3[a](41.4;69.1)	64.6[a](49.8;79.4)	37.7[b](30.9;44.5)

*Figures in the same row with a different superscript are significantly different at P < 0.05. All thickness measurements are given in micrometers.

4.2 Morphology study

4.2.1 Bladder

 Figures 4.2.1 and 4.2.2 show the morphology of the bladder for the sham-vehicle treated group(A), ovariectomised vehicle treated group(B) and the ovariectomised isoflavone treated group(C).
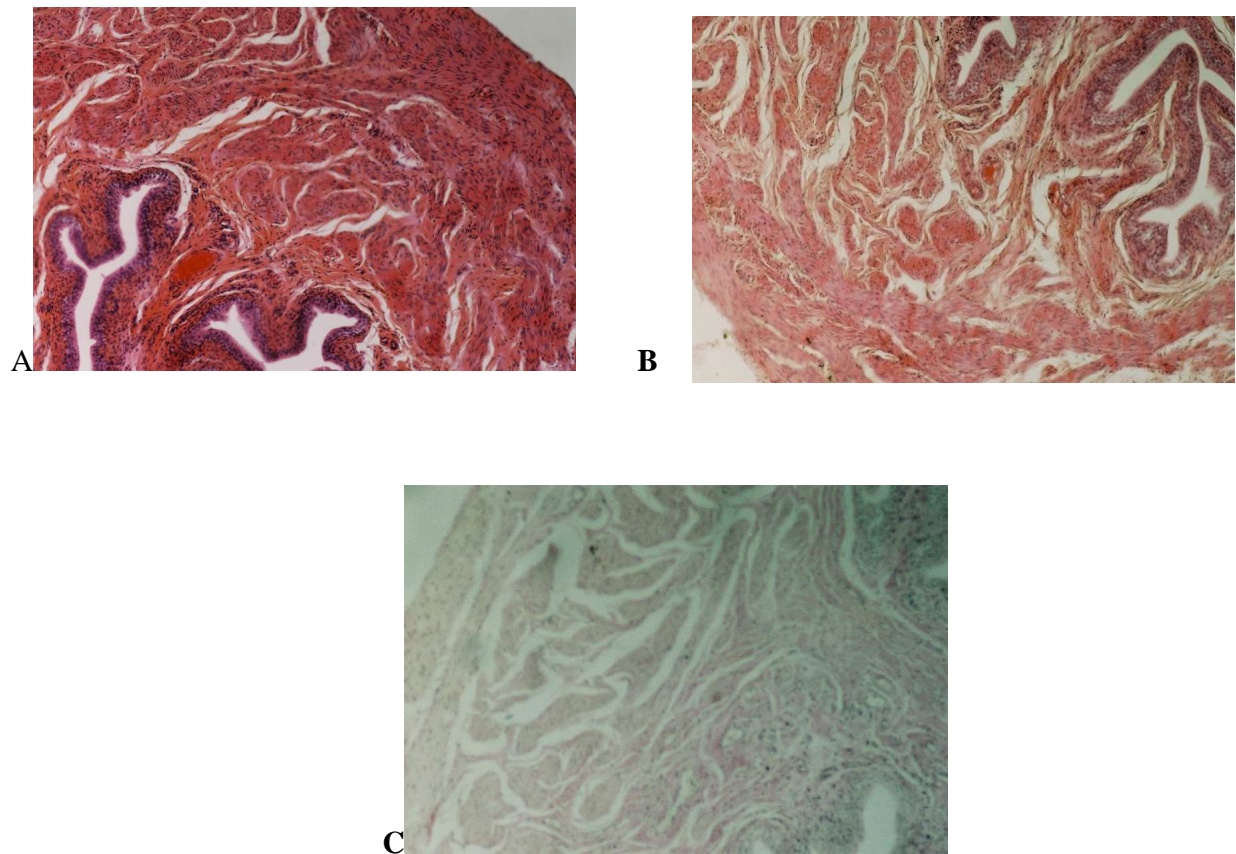
Figure 4.2.1. Histological section of the bladder stained with H&E. for sham operated group(A),ovx+ vehicle group (B) and ovx+soy isoflavone group(C). The final magnification is 100 x

Figure 4.2.2 Histological section of bladder stained with Van Giesson's stain for collagen fibres in sham operated group(A),ovx+ vehicle group (B) and ovx+soy isoflavone group(C). The final magnification is 100 x.

The muscularis thickness and muscle nuclei density are considerably higher in the sham vehicle treated animals (A) compared to groups B and C. There is higher vacuolation between the muscle fibres in B and C compared to A. The amount of collagen and connective tissue of the lamina propria layer are visibly higher in the ovariectomised isoflavone treated and the ovariectomised vehicle treated animals (B and C) compared to A. There are few large congested blood vessels in A compared to smaller several congested blood vessels in B and C.

4.2.2 Urethra

Figure 4.2.3 below shows the histomorphology of the urethra in sham vehicle treated rats (A),

ovariectomised vehicle treated rats (B) and ovariectomised isoflavone treated rats (C) .
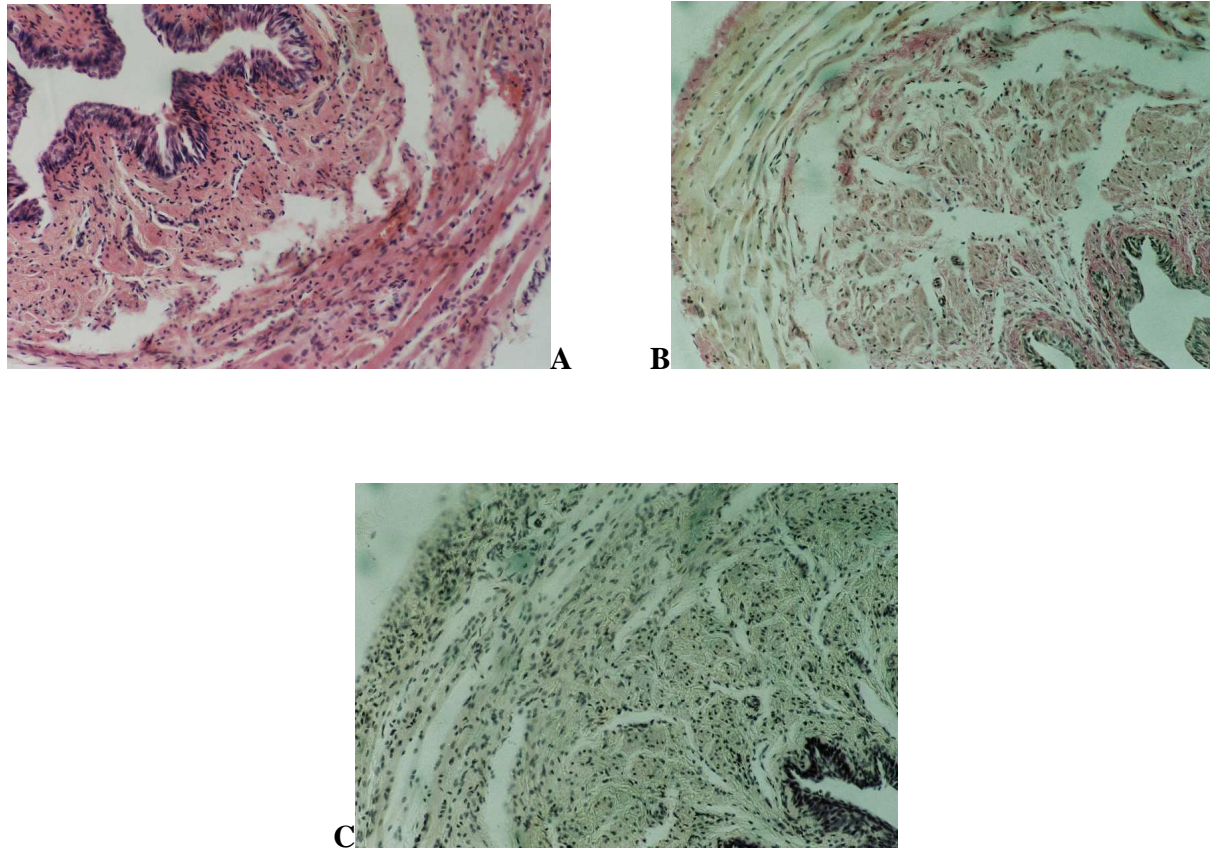
Figure 4.2.3 Histological section of the urethra stained with H&E. for sham operated group(A),ovx+ vehicle (B) and ovx+soy isoflavone (C) . Final magnification is 200x

Muscularis layer in C is thicker compared to B and C. Muscle nuclei density is high in picture C

compared to A and B. High vacuolation between muscle fibres was observed in groups B and C

compared to A.

4.2.3 Ureter

Figure 4.2.4 shows the histomorphology of the ureter in sham vehicle treated rats (A),

ovariectomised vehicle treated rats (B) and ovariectomised isoflavone treated rats (C) .

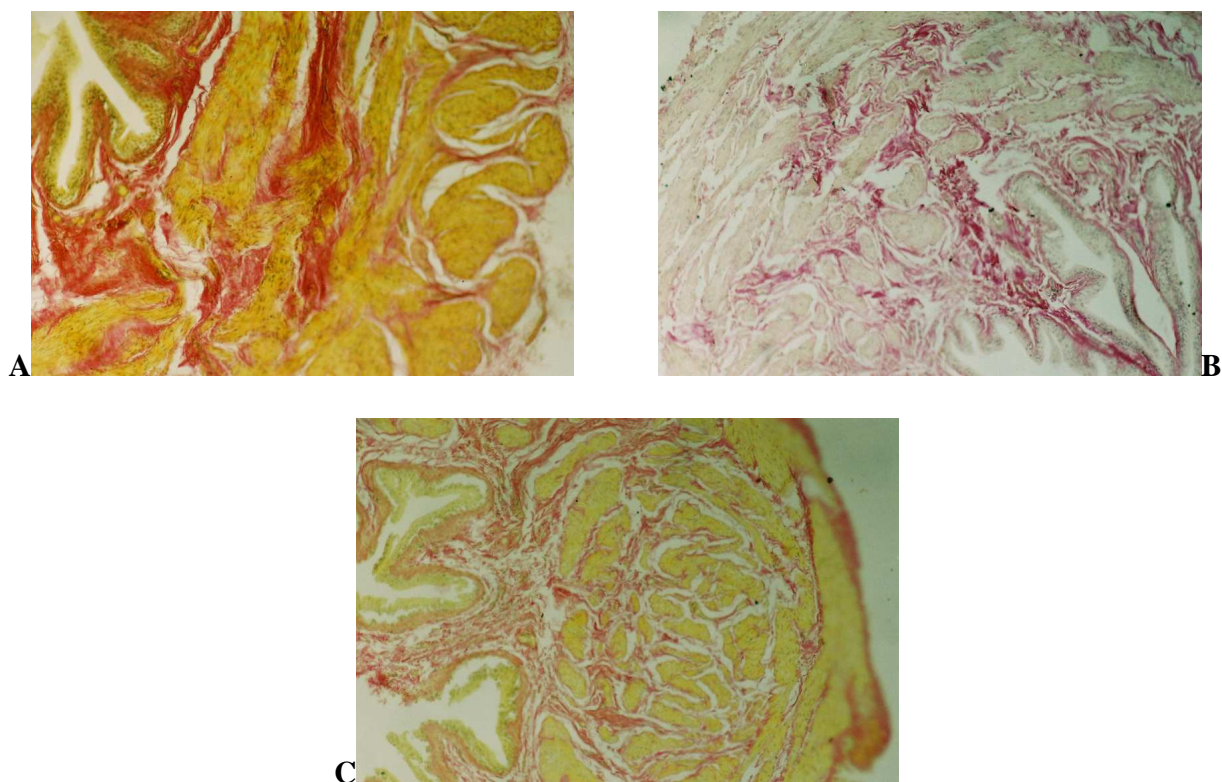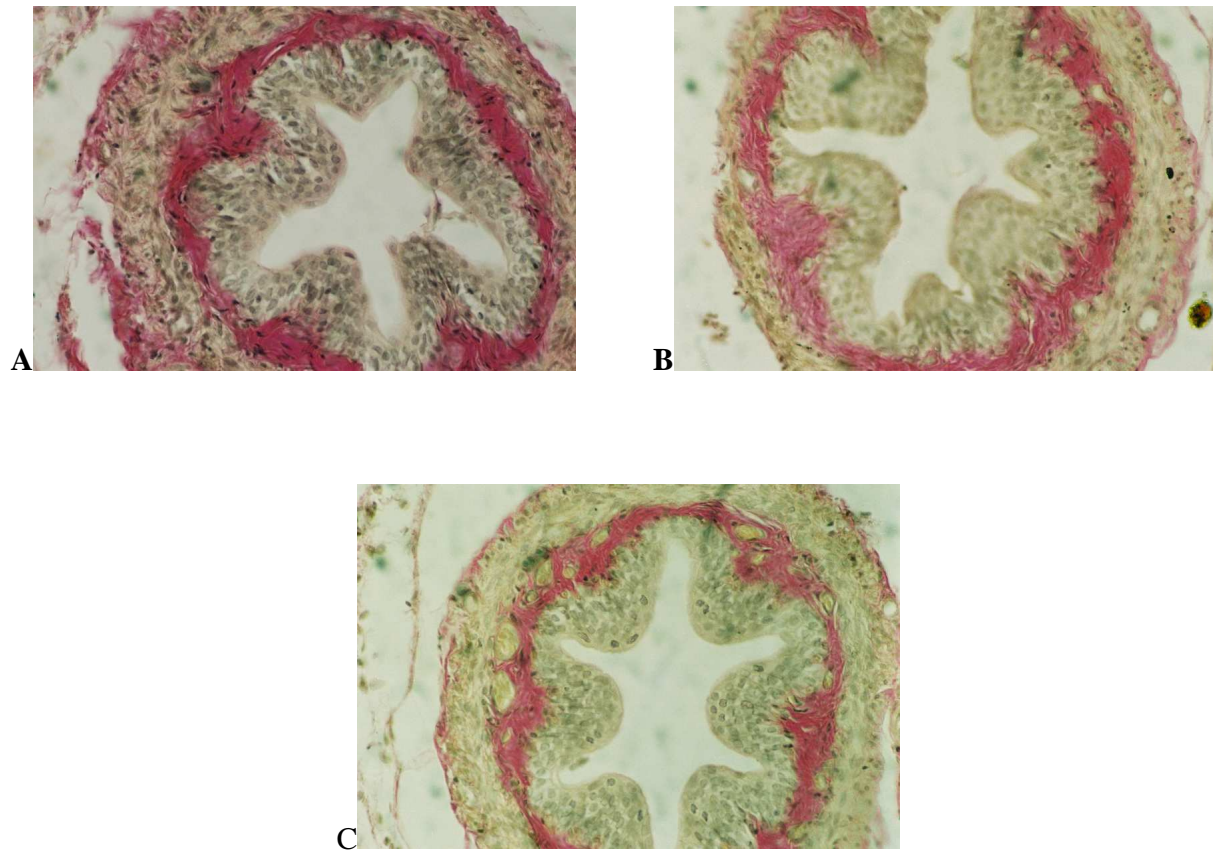**Figure 4.2.4 .**Histological section of ureter stained with Van Giesson's stain in sham operated group (A), ovx+ vehicle group (B) an ovx+soy isoflavone group (C). The final magnification is 400 x.

There were no notable differences in the muscularis thickness of all three groups. There was

however observable vacuolation between the muscle fibres in both B and C. Lamina propria

thickness is greater in group a compared groups B and C. There were a higher number of blood

vessels observed in the lamina propria of B and C compared to A.

CHAPTER 5

DISCUSSION

Hypoestrogenism causes harmful consequences which might drastically reduce the quality of life for affected individuals. Ovariectomy is a method which has effectively led to hypoestrogenism and its sequel. In this study, ovariectomy resulted in smooth muscle depletion and atrophy in conjunction with an increase in the lamina propria thickness. Blood vessel numbers remained unchanged in the bladder and ureter after ovariectomy whilst in the urethra the numbers were reduced. Epithelium layers in the ureter and urethra were not affected by ovariectomy whilst bladder epithelium was reduced.

In this study, isoflavone treatment reversed the effects of ovariectomy on the overall morphology of the ureter, bladder and urethra to some extent. The result is in agreement with other research done elsewhere (Falconer *et al*., 1998; Sartori *et al*., 2001). The connective tissue was shown to respond by lowering collagen concentrations in the detrusor and urethra of estrogen treated ovariectomised female rats (Falconer *et al*., 1998). The current study observed an increase in collagen fibres and connective tissue following ovariectomy and vehicle treatment as well as ovariectomy and isoflavone treatment in the ureter, urethra and bladder. However, treatment with soy isoflavone elicited a slight reduction in the connective tissue and collagen content of the ureter and urethra. The result from the current study agrees in part with the observation by Santos *et al*. (2010) who also observed an increase in urethral collagen content in late treatment of ovariectomised rats with soy isoflavones. However, early isoflavone treatment resulted in marked reversal of the effects of ovariectomy in the urethra. The observed increase in collagen synthesis is thought to result in the formation of a more rigid extracellular matrix which is less

pliable mechanically (Falconer *et al*., 1998). This then influences and offsets the relationship between the flexibility and stability of the lamina propria in the excretory tubes and bladder. The bladder may become less able to comply with stretch and contraction forces thus leading to incontinence.

The current study is in agreement with the findings by Santos *et al*. (2010) above. The responses obtained in this study are similar to the late treatment group. Failure by soy isoflavones to markedly reverse the effects of ovariectomy could be explained by the fact that not all individuals can metabolise daidzen to a more potent form, equol. About 33% of humans studied in western population can produce eqoul (Setchell *et al*., 2002). Equol, was detected in rat urine suggesting that also a small proportion of rats are able to convert daidzen to equol. Another explanation could be that the isoflavone levels did not rise to effective concentrations in serum due to loss in digestion and assimilation processes. Serum levels of isoflavones were not evaluated so knowledge of the actual effective concentrations was not available. The weak estrogenic activity of isoflavones is largely compensated for by higher serum concentrations. It therefore follows that if high concentrations are not achieved, no effects can be observed. Elia and Bergman (1993) postulated from their study that vaginally applied estrogen had better results than oral treatment of stress urinary incontinence. The weak estrogenic effects of soy isoflavones could have been less compensated for by the oral route of administration.

A study of the same nature conducted in rabbits analysed the influence of varying estrogen levels on the bladder collagen fibre content. Ovariectomy, which is a state of hypoestrogenism did not elicit any changes in the collagen tissue amounts (Persson *et al*., 1996). This differs from findings in this study where ovariectomy increased the collagen content of the urinary bladder,

urethra and ureter. It has been suggested that the effects of estrogens in the lower urinary tract may be influenced by several factors. These include modifications of the hormonal receptor response due to low serum estrogen (Endo *et al*., 2000). Variation in estrogenic action and responses can also be influenced by the organ, species or individual being studied (Endo *et al*., 2000).

Urethral blood vessel counts were significantly increased (p=0.02) by isoflavone treatment in this study. This finding is in agreement with that obtained by Endo *et* al. (2000) where estrogen therapy resulted in an increase in bladder and periurethral blood vessels. Inorder to achieve urinary continence, urethral pressure should exceed vesical pressure. Urethral blood vessels and connective tissue and muscle contribute to maintenance of the high urethral pressure. Periurethral vessels contribution to total urethral pressure is about 33 %(Rud, 1980).

Furthermore, the methods that were used had limitations that could have affected the analysis. Manual analysis was used whereas other studies used automated modern technology. Modern technology involves the use of a microscope connected to a computer screen where both morphometry and morphological analysis can be recorded automatically.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Anatomical evidence from this study has shown that soy isoflavones can elicit morphological changes to some extent in response to hypoestrogenism of post menopause and post spay. This information is going to contribute to the pool of evidence in the validation of current conflicting findings.

It is recommended that more research in this area should be performed so as to help patients make the best decision. Studies should also incorporate the histopathology if any caused by soy in the urinary tract. This might also assist in establishing the benefits as well as the side effects involved with use of soy for hypoestrogenic urinary incontinence.

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