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## Protective Effect of Defatted flaxseed on Urinary Bladder of Ovariectomized Albino Rats

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### ABSTRACT

**Background:** Postmenopausal period is associated with a high incidence of symptoms in the lower urinary tract. **Aim:** to investigate the beneficial effect of defatted flaxseed (FS), as a natural alternative to hormonal therapy, on the deterioration of urinary bladder in experimentally menopausal albino rats in view of the health risks associated with hormones. **Methods:** Thirty 3-month old non pregnant female albino rats were randomized into five equal groups: Group I (Sham-operated), Group II (Sham-operated with FS), Group III (ovariectomized), Group IV (ovariectomized + estrogen) and Group V (ovariectomized + FS). The experimental period lasted for 12 weeks. Animals' bladders were processed for light and scanning electron microscopic examination. In addition, quantitative assessments of collagen fiber content as well as estrogen receptor  $\alpha$  (ER- $\alpha$ ), estrogen receptor  $\beta$  (ER- $\beta$ ) and desmin immunoperoxidation were performed. **Results:** Our results revealed a significant protective effect of defatted flaxseed on the urinary bladder of ovariectomized rats. These beneficial effects of flaxseed were mainly due to its estrogenic action, upregulating ER- $\beta$  expression. **Conclusion:** Defatted flaxseed has promising effects on improvement of altered structure of the urinary bladder in ovariectomized rats. This may provide a potential therapeutic approach for lower urinary symptoms among menopausal women.

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## INTRODUCTION

The female genitals and lower urinary tracts share a common embryologic origin, arising from the urogenital sinus. Both are sensitive to the effects of female sex steroid hormones (Yang *et al.*, 2009). Females are exposed to periods of marked hormonal changes as during puberty, pregnancy and menopause. These hormonal changes induce significant variations in different body tissues including the lower urinary tract (Levin and Longhurst, 1996). Among these changes is the decrease in circulating estrogen after menopause. The urinary bladder is considered a target organ for the action of estrogen (Aikawa *et al.*, 2003). The cellular effects of oestrogen are mediated via its interaction with oestrogen receptors (ERs). Two distinct estrogen receptors, ER alpha and ER beta, encoded by separate genes, have been identified in rat, mouse and human (Cheskis *et al.*, 2007). Estrogen receptors (ERs) have been shown to be present in the vagina, urethra, bladder, and pelvic floor musculature. However, ER beta appears to be the predominant estrogen receptor type in the urinary bladder and urethral epithelium of both sexes (Taylor and Al-Azzawi, 2000).

Estrogen replacement therapy (ERT) has been used for many years to relieve urinary symptoms in postmenopausal women. Supplementary estrogen can improve bladder function which may be due to inhibition of collagen hyperplasia and increased smooth muscle density (Yang *et al.*, 2009). However, ERT is associated with a higher risk of hormone-related cancer (Anderson *et al.*, 2003) and other unfavorable adverse events (Morabito *et al.*, 2002). Consequently, it is necessary to develop safer and natural alternatives to ERT.

Phytoestrogens are naturally occurring plant compounds which were reported to have the ability to mimic the actions of oestrogen. They include the lignans, found in most plant foods but in the highest concentrations in flaxseed (Thompson, 2003) and the isoflavones, which are abundant in soy and soy products (Turner *et al.*, 2003). Flaxseed (*Linum usitatissimum*) is an oilseed increasingly used as an ingredient in food products because of its high alpha-linolenic acid and dietary fiber content, but recently also because of its secondary metabolites (Feng *et al.*, 2008). Important secondary metabolites are lignans, which are present in flaxseed in a higher concentration than in other edible sources (Milder *et al.*, 2005). The main lignan in flaxseed is

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secoisolariciresinol diglucoside (SDG), which is present in defatted flaxseed flour in concentrations up to 3% (w/w) (Johnsson *et al.*, 2000). It is metabolized into the more biologically active mammalian lignans enterodiol (END) and enterolactone (ENL) by microbiota in the colon (Jacobs *et al.*, 1999).

The aim of this study was to investigate the beneficial effect of defatted flaxseed on the urinary bladder morphology in experimentally menopausal albino rat and whether hormonal therapy could be replaced with natural compounds with similar effects.

## MATERIALS AND METHODS

### *Experimental substances:*

- 17alpha ethinylestradiol (E2) (Kahira pharmaceutical and Chem. Ind. Company. Cairo-Egypt), in the form of 50 µg tablets. The tablets were crushed in powder form, dissolved in distilled water and given by a gastric tube in a dose of 25 µg /Kg body weight/day (Elbostany *et al.*, 2013).
- Anti-Desmin (DE-R-11) mouse monoclonal antibody (Ready to use for immunohistochemical staining). It was supplied by Neomarkers Laboratories and Lab Vision Corporation.
- Anti ER alpha (ER-alpha) rabbit polyclonal antibody (Ready to use for immunohistochemical staining). It was supplied by Neomarkers Laboratories and Lab Vision Corporation.
- Anti ER beta (ER-beta) rabbit polyclonal antibody (Ready to use for immunohistochemical staining). It was supplied by Neomarkers Laboratories and Lab Vision Corporation.

### *Preparation of defatted flaxseed (lipid extraction):*

This preparation was done at Food Technology Research Institute, Dokky, Egypt. Flaxseed was purchased from the local market, washed and dried. The seed oil content was determined using the Soxhlet extraction. Flaxseeds were defatted by petroleum ether 40-60°C using Soxhlet apparatus. Residual solvent was evaporated from defatted flaxseed using hot air oven (rotavapor) at 40 °C. Lipid content was expressed as g/100 g of seed fresh weight (Elbostany *et al.*, 2013).

### *Animals:*

Thirty Sprague-Dawley female albino rats (3 months old, weighing 180–200 g) were obtained and housed in the animal house of Faculty of Medicine, Menoufia University, Egypt. Animals of each group were housed in separate hygienic cages at the room temperature. The cages were provided with white paper bedding to allow complete collection of any remaining food. The animals received a standard diet for rodents and allowed free access to water. The food was introduced daily and any remaining food was removed, weighed and recorded. The animals were treated humanely and care was taken to ease suffering. This study has been approved by the ethics committee on animal research, Faculty of Medicine, Menoufia University, Egypt.

### *Experimental design:*

Rats were randomly divided into five equal groups as the following description:  
Group I (Sham-operated): were fed on BD for 12 weeks and served as controls. Group II (Sham-operated + FS) were sham-operated and kept for 4 weeks after which, were fed on 10 g defatted flaxseed per 100g standard diet for 8 weeks. The other three groups were ovariectomized. Rats of Group III (Ovx) were fed on standard diet for 12 weeks. One month after ovariectomy, rats of Group IV (Ovx+ E) received 17alpha ethinylestradiol (E2) (25 µg /Kg body weight/day) orally by gastric tube, in addition to standard diet, for 8 weeks. Group V (Ovx+ FS): were fed on 10 g defatted flaxseed per 100g standard diet for 8 weeks.

### *Ovariectomy:*

Rats were placed in supine position under light ether anesthesia. Bilateral ovariectomy was performed through a midline incision, after shaving and disinfection of the anterior abdominal wall by povidone iodine (Betadine). The abdomen was explored for identification of the bicornuate uterus and then along the two uterine horns towards the ovaries, followed by ligation of the ovarian pedicle and vessels using vicryl 30 (synthetic, absorbable, polyglyctan) sutures (Ethicon Company, America) and then excision of the ovaries was performed (Waynforth, 1980). Aqueous penicillin (Epico pharmaceutical company, Egypt) in powder form was dissolved in distilled water and poured intraperitoneally at the time of the operation before closing the incision. Closure of the wound was done using the same suture material. Garamycin cream was applied locally as a wound dressing for 5 days post-operative to reduce the risk of wound infection (Kalu *et al.*, 1989). Sham-operated group underwent a similar surgical incision exposing the ovaries and replacing them in the same position, just to be exposed to the same stress of the operation. The same antiseptic precautions and the same way of wound closure were followed as for the ovariectomized rats (Behari and Behari, 2009).

After 12 weeks, all animals were anaesthetized with Phenobarbital sodium and were sacrificed by cervical dislocation. The bladder was removed through a lower midline abdominal incision. Using a sharp stainless steel razor blade, the bladder is divided longitudinally from the neck to the bladder fundus into equal ventral and

dorsal parts maintaining the ventral to dorsal orientation. Half of the ventral bladder was processed for light microscopy and saved in 10% neutral buffered formalin for 24h, dehydrated in ascending grades of ethanol, embedded in paraffin and processed to obtain paraffin blocks. The other half of the ventral part was prepared for scanning electron microscopy (SEM) by fixing in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer for 24 h. The specimens were rinsed twice in phosphate buffer for 15 min each, followed by the postfixative 1% osmium tetroxide in 0.1 mol/l phosphate buffer (pH 7.2) for 2 hours (Babinski, 2007).

#### *Histological and immunohistochemical studies:*

Paraffin sections were prepared with a thickness of 5 microns, mounted on glass slides and stained with H&E and Mallory's trichrome technique (Drury and Wallington, 1983). Immunohistochemical preparation was done for detection of desmin reaction in the smooth muscle cells, estrogen receptor alpha (ER-alpha) and estrogen receptor beta (ER- beta) using the labeled Avidin-biotin peroxidase complex technique (Bancroft and Cook, 1994). Formalin-fixed, paraffin-embedded sections of the urinary bladder were dewaxed and dehydrated with ethanol. Endogenous peroxidase activity was stopped by treating the sections with hydrogen peroxide. Sections were rinsed, treated with normal horse serum to reduce nonspecific background staining, rinsed, and then incubated with the Iry antibody. Sections were counterstained with hematoxylin, examined with an Olympus light microscope (BX51TF; Olympus, Tokyo, Japan) and photographed.

#### *Quantitative Morphometric assessment:*

Data were obtained using "Leica Qwin-C500" image analyzer computer system (England). The following parameters were measured:

- 1) The area percent of connective tissue in Mallory's trichrome stained sections.
- 2) The area percent of positive desmin immunoreactivity in smooth muscle fibers.
- 3) The percent of ER-alpha and ER-beta positive cells in urothelium in immunostained sections. The number of positive cells was counted and the data were presented as percentage of the total number of cells.

For each parameter, 10 non overlapping fields for every specimen, at magnification X 400, were examined.

#### *Statistical analysis:*

The data obtained were presented as mean  $\pm$  SD. Data analysis was performed using GraphPad Prism version 4.03 for Windows (GraphPad software Inc., San Diego, California, USA). The obtained data were analyzed through the use of the analysis of variance (ANOVA) and the differences among groups were determined by Newman-Keuls multiple comparison test as post test. The results were considered statistically significant and nonsignificant when the P values were  $<0.05$  and more than 0.05 respectively (Dawson and Trapp, 2001).

#### *Ultrastructural studies (SEM):*

After postfixation, specimens were rinsed with two changes of buffer for 15 min each to remove unbound osmium tetroxide out of the tissue. Dehydration was then carried out in graded series of ethanol, for 15min each. All the specimens were then dried with a Baltec 030 critical point dryer (Natick, Massachusetts, USA) and coated with gold using Baltec 030 sputter coater. Examination was carried out using Philips XL30 SEM (Amsterdam, Netherlands) under a high tension of 25 kV (Hayat, 1981; Bozzola and Russell, 1999). SEM processing and examination were carried out at electron microscopy unit, Faculty of Medicine Tanta University.

#### *Results:*

There was no significant difference between sham operated and flaxseed treated rats in all the outcomes of this study; therefore, these two groups were pooled in one group (control).

#### *Gross observations:*

There were no deaths in any of the groups. No abnormal signs were detected in the animals.

#### *Light microscopic results:*

Hematoxylin and Eosin (H&E) stained sections in the urinary bladder of control rats showed different layers. The mucosa was formed of transitional epithelium (urothelium) and underlying lamina propria of connective tissue (C.T.). The muscularis propria was formed of smooth muscle bundles that appeared intact. The adventitia was formed of loose C.T. The transitional epithelial cells were arranged in order on indistinct basement membrane; the basal cells, intermediate cells, that appeared polygonal, & the superficial large cells. Lamina propria was formed of C.T. in the form of superficial dense layer and deep loose layer. The muscularis propria showed intact, well distributed smooth muscle bundles separated by C.T. Mallory's Trichrome stained sections showed compact well-arranged smooth muscle bundles with collagen fibers in-between (Fig.1 a,b,c &d).

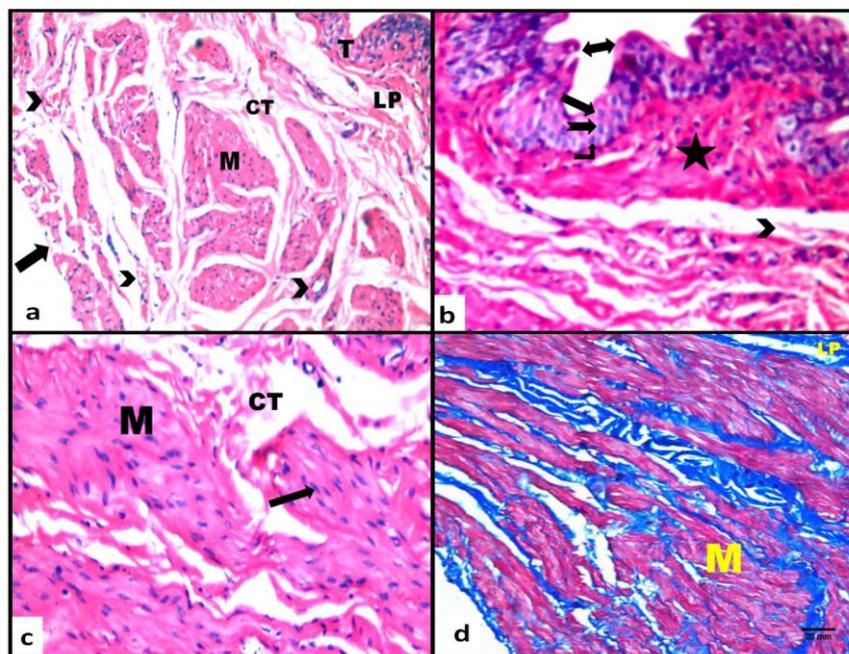
H&E stained sections in the urinary bladder of Group III (Ovx) showed that the transitional epithelium exhibited exfoliation and shedding in some areas of the bladder mucosa. Some transitional epithelial cells showed vacuolization and pyknotic nuclei in addition to widening of intercellular spaces. Lamina propria showed marked mononuclear cellular infiltration. Invagination of the urothelium was observed in the form of nests. At higher magnification, epithelium showed detachment and disruption. The remaining cells exhibited cytoplasmic vacuolation & pyknotic nuclei. In addition, bundles of smooth muscle fibers appeared widely separated and cytoplasm appeared pale with marked vacuolation. In Mallory's trichrome stained sections, smooth muscle bundles appeared less compact with widening of the spaces in-between and significant increased content of collagen fibers ( $P < 0.001$ ) as compared to control (Fig.2 a-f) & histogram (1).

Sections in the urinary bladder of Group IV (Ovx+ E) revealed a well-organized general architecture similar to that seen in Group I, however; some nuclei were still pyknotic. Smooth muscle cells appeared more or less normal. Mallory's trichrome stained sections showed a significant decrease ( $P < 0.001$ ) in collagen fibers when compared to Group III (Fig.3 a-d) & histogram (1).

In Group V (Ovx+ FS) H&E stained sections showed restoration of all layers and smooth muscle fibers appeared compact. However at higher magnification, details of transitional epithelium were detected in the form of cellular vacuolation and wide intercellular spaces. Lamina propria showed dilated congested blood vessels. The smooth muscle fibers appeared more or less normal; however, some showed cytoplasmic vacuolation. The collagen fibers stained with Mallory's trichrome were significantly decreased ( $P < 0.001$ ) in lamina propria and between muscle bundles, when compared to group III (Fig.3 e-h) & histogram (1).

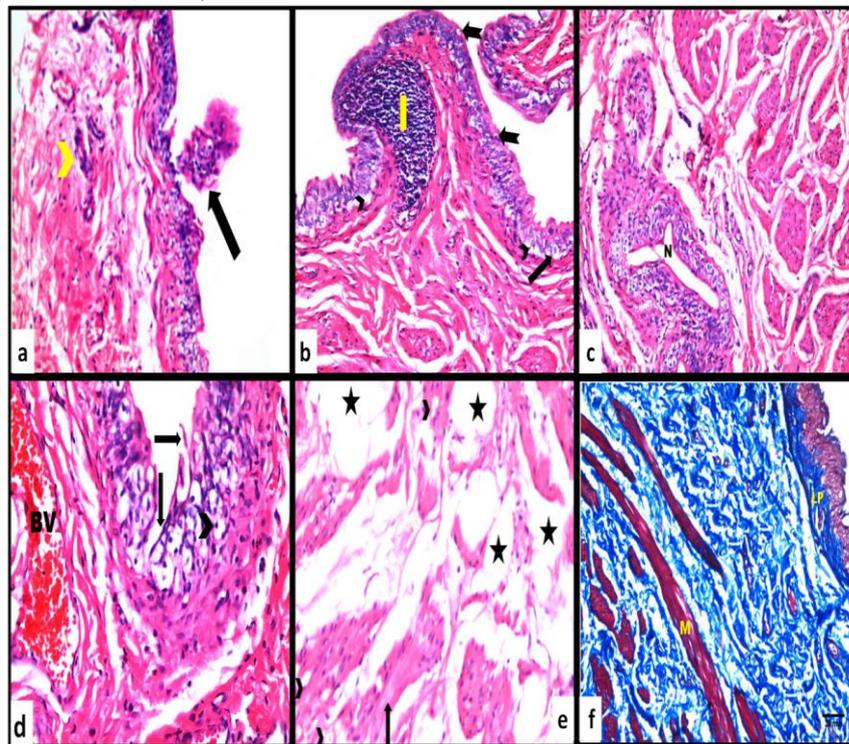
As regards the immunostained sections for intermediate filaments of desmin, the control rats showed positive reaction in the smooth muscle cells. In group II (Ovx), there was significant decrease ( $P < 0.001$ ) in the area percentage of desmin immunoreactivity, compared to control group, due to decreased muscle bulk. In Groups IV (Ovx+ E) and V (Ovx+ FS), the immunostained sections did not record a significant difference from that of control group but showed a highly significant increase ( $P < 0.01$ ) when compared to group III (Fig.4 a, b, c & d) & histogram (1).

Immunostained sections for ER-alpha in control rats showed that, the transitional epithelial lining cells exhibited positive reaction in some of their nuclei. In Group III (Ovx), the mean percent of ER-alpha positive cells was significantly upregulated ( $P < 0.001$ ) compared to control. administration of oestrogen and defatted flaxseed in groups IV and V respectively showed a non- significant decrease in ER-alpha expression compared to group III (Ovx) (Fig.5 a, b, c & d.) & histogram (2).



**Fig. 1:** Light microscopic pictures of urinary bladder from control rats showing (a) transitional epithelium (T), underlying lamina propria (LP), smooth muscle bundles (M) of muscularis propria, connective tissue (CT) between muscle bundles, blood vessels (arrow head) and loose connective tissue (arrow) of adventia (H&E, x200). (b) superficial epithelial cells (arrow), intermediate polygonal cells (notched arrow) and basal cells (bent-up arrow), plasma membrane of the luminal surface of the superficial cells (double headed arrow), superficial dense (star) and deep loose (arrow head) layers of lamina propria (H&E, x400). (c) smooth muscle bundles (M) of muscularis propria with homogeneously oesinophilic cytoplasm and oblong nuclei (arrow) and separated by connective tissue (H&E x400). (d) compact well-

arranged smooth muscle bundles (M) with collagen fibers (blue) in-between and in lamina propria (LP) (Mallory's trichrome, x200).



**Fig. 2:** Light microscopic pictures of urinary bladder from ovariectomized rats showing (a) exfoliation and shedding of the transitional epithelium (arrow) & mononuclear infiltration in the lamina propria (arrow head) (H&E, x200). (b) vacuolization and ballooning of epithelial cells (arrow), some cells with small pyknotic nuclei (notched arrow), widening of intercellular spaces (arrow head) & marked leucocytic infiltration (I) in the lamina propria (H&E, x200). (c) an invaginated nest of urothelium (N) in the lamina propria (H&E, x200). (d) detachment and disruption of the epithelium (arrows), cells with small pyknotic nuclei (arrow head) & a dilated congested blood vessel (BV) in the lamina propria (H&E, x400). (e) smooth muscle bundles with wide spaces in-between (star). Pale cytoplasm (arrow) with marked vacuolation (arrow head) (H&E, x400). (f) apparent increase in collagen deposition (blue) in lamina propria (LP) and between the widely separated muscle bundles (M) (Mallory's Trichrome x200).

In ovariectomised rats, there was significant downregulation of ER-beta immunostaining ( $P < 0.001$ ) in urothelial cells compared to control. This downregulation was significantly increased ( $P < 0.01$ ), compared to group III (Ovx), to become insignificant from control (Fig.6 a, b, c & d,) & histogram (2).

#### Electron microscopic results:

Scanning electron microscopy of urinary bladder surface epithelium of control group showed normal picture of epithelial surface in the form of flat, large cells without necrosis or proliferation. Group III (Ovx) exhibited multifocal urothelial necrosis and exfoliation. In some areas, the surface epithelium exhibited prominent pleomorphism and piling up of cells indicative of epithelial hyperplasia. In groups IV and V, the picture was more or less similar to that of control. In group V, however, piling up of cells was still observed in some areas (fig.7 a-e).

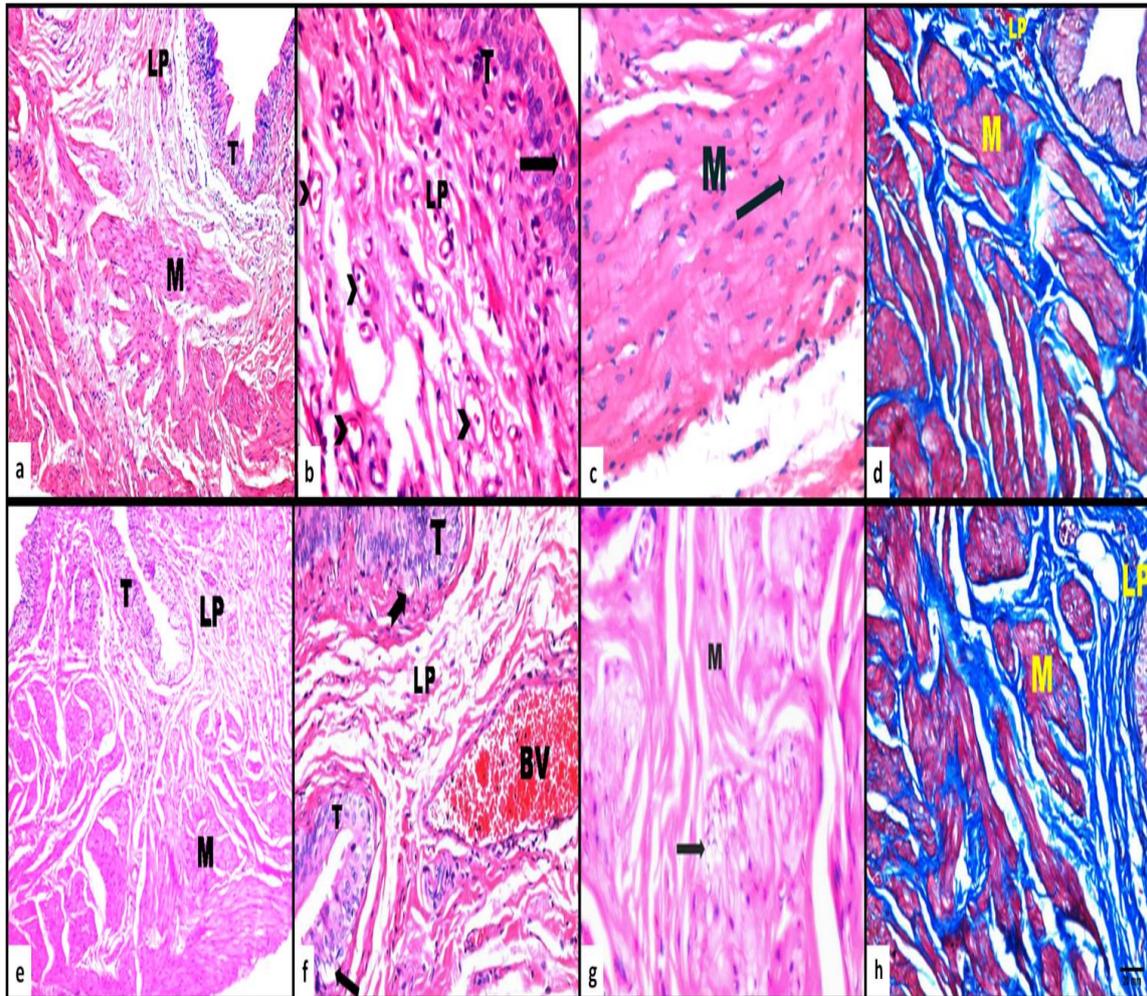
#### Discussion:

Postmenopausal estrogen deprivation has been suggested as the major risk factor for lower urinary tract dysfunction including stress incontinence, overactive bladder and recurrent urinary tract infections as well as urogenital atrophy. These symptoms associated with hypoestrogenism could have enormous effects on individuals and health-care providers in terms of impact of quality of life (Valentini *et al.*, 2011). Several clinical studies suggested that estrogen replacement therapy is associated with beneficial effects on the lower urinary tract in postmenopausal period (Aikawa *et al.*, 2003). On the other hand, hormone replacement therapy increases the risk for endometrial and breast cancer compared to other therapies (Beral *et al.*, 2005).

In our experiment, defatted flaxseed was chosen as a protective agent with estrogenic action. Secoisolariciresinol diglycoside (SDG), the most abundant lignan in flaxseed, is metabolized by colonic bacteria into the more biologically active mammalian lignans, enterodiol (ED) and enterolactone (EL) (Simbalista *et al.*,

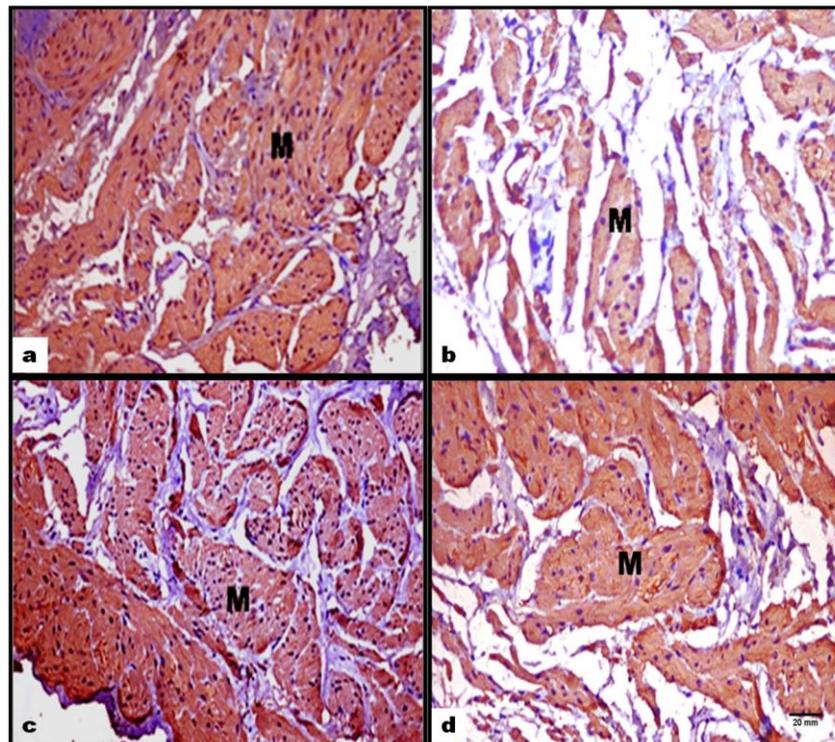
2010). Because the mammalian lignans share structural similarity to the steroid hormone 17 $\beta$ -estradiol, they can elicit estrogenic action in hormone-sensitive tissues and have the potential to modulate hormone-related diseases (Penttinen-Damdimopoulou *et al.*, 2009). Lignans were reported to exhibit protective effects against hormone-related types of cancer like breast cancer (Boccardo *et al.*, 2004). Furthermore, flaxseed extract was found to prevent bone loss resulting from oestrogen deficiency in aged female rats (Elbostany *et al.*, 2013).

Therefore, this study was performed to investigate whether defatted flaxseed has a protective effect on changes in the urinary bladder morphology associated with estrogen deprivation in rats. The study also targeted elucidating the possible mechanism of estrogen action via localization of ER- $\alpha$ , ER- $\beta$  in the urinary bladder transitional epithelium by immunohistochemical means.



**Fig. 3:** Light microscopic pictures of urinary bladder from group IV (Ovx+ E): a,b,c,&d and group V (Ovx+ FS): e,f,g&h treated rats showing (a) a well organized architecture of all bladder layers with restoration of transitional epithelium (T), lamina propria (L) & smooth muscle bundles (M) which appear compact and well distributed (H&E x200). (b) restoration of transitional epithelium (T) with epithelial cells lined in order, Some pyknotic nuclei (arrow) & a rich network of blood capillaries (arrow head) in the lamina propria (LP) (H&E x400).(c) normal appearance of smooth muscle fibers (M) with homogeneously eosinophilic cytoplasm and oblong nuclei (arrow) (H&E x400).(d) closely compact smooth muscle bundles (M) with less collagen fibers in-between and in lamina propria (LP) when compared to Group III (Ovx) (Mallory's trichrome x200). (e) nearly normal transitional epithelium (T), lamina propria (L) & closely compact smooth muscle bundles (M) (H&E x200). (f) transitional epithelium (T) with some vacuolated cells (notched arrow), widening of the intercellular spaces in a few areas (arrow) & a few dilated congested blood vessels (BV) in the lamina propria (LP) (H&E x400). (g) compact smooth muscle bundles (M) with cytoplasmic vacuolations in a few bundles (arrow) (H&E, x400). (h) compact smooth muscle bundles (M) with less collagen fibers (blue) in-between and in lamina propria (LP)

(Mallory's trichrome x200).

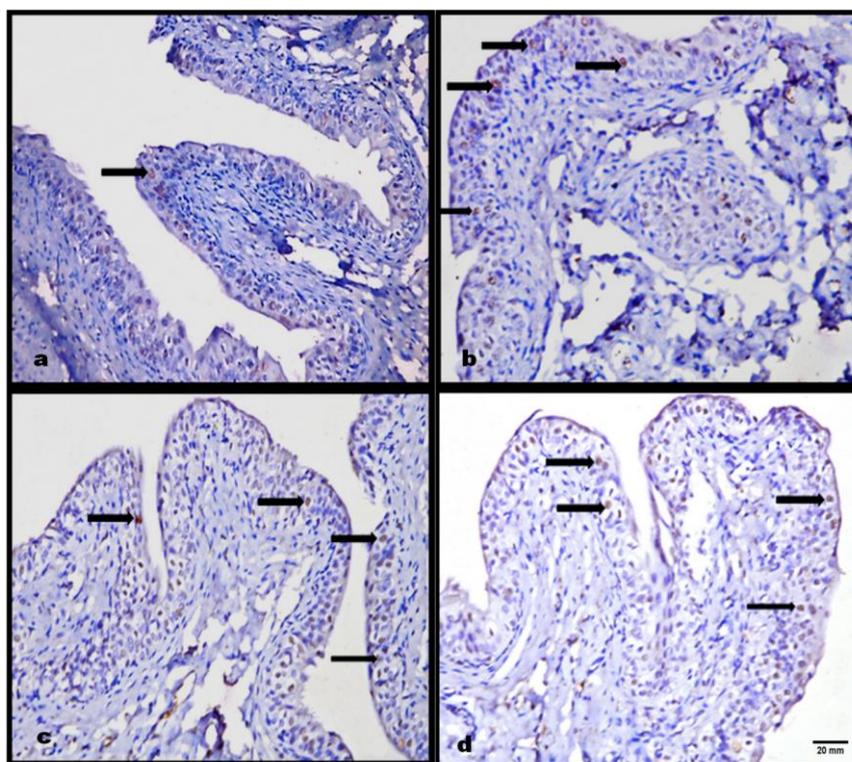


**Fig. 4:** Light microscopic pictures of urinary bladder sections immunostained for desmin from: (a) sham operated rats showing positive desmin immunostaining in smooth muscle bundles (M). (b) ovariectomized rats showing apparent decrease of desmin immunostaining in smooth muscle bundles (M) which appear thinner and widely separated. (d) group IV (Ovx+ E) showing increased desmin immunostaining as compared to ovariectomized rats. (e) group V (Ovx+ FS) showing a pattern more or less similar to that of control (desmin immunostaining, x200)

In this experimental study, adult female albino rats aged 3 months old were chosen to be in the reproductive period of life. According to Suckow *et al.* (2005) female rats reach sexual maturity at 2 months of age, their reproductive system is fully functioning and their maximum fertility is reached at between 3-10 months of age.

Marked epithelial changes were demonstrated in the current study on examining urinary bladder Hx & E stained sections of the Ovx group, ranging from disruption, exfoliation of some parts of the epithelium to complete epithelial shedding in other areas of bladder mucosa. Some of the remaining epithelial cells exhibited cytoplasmic vacuolation, while other epithelial cells showed pyknotic nuclei. Parekh *et al.* (2004) reported similar findings of bladder mucosal atrophy after Ovx. They postulated that chronic Ovx resulted in decreased level of circulating estrogen with significant decrease in the blood flow and increase in tissue hypoxia with consequent mucosal atrophy. In the current work, the presence of large cytoplasmic vacuoles and widening of intercellular spaces in some epithelial cells after Ovx might be related to bladder epithelial permeability or barrier function disorders which cause osmotic and trans-epithelial bulk water flow and appearance of these vacuoles (Cayan *et al.*, 2006).

In the current work, the marked mononuclear cellular infiltration observed in the lamina propria of connective tissue under the epithelium in the Ovx group, is probably resulting from estrogen deficiency and the consequent decrease in blood flow and hypoxia occurring to the urinary bladder. Hypoxia is a well-known stimulant for angiogenesis. Hypoxia inducible factor (HIF-1 $\alpha$ ) is an important mediator of vascular endothelial growth factor (VEGF) expression (Kazi *et al.*, 2005). This might lead to congestion & might explain the mononuclear cellular infiltration found in lamina propria of the Ovx group. This finding was also observed by Cayan *et al.* (2006) who reported marked increase in mast cell and leucocytic infiltration in cases of Ovx as well as in cases of Ovx with chronic cystitis, resulting in significant decrease in the mean maximal bladder capacity and compliance. Similar findings were reported by Tanidir *et al.* (2011) who reported significant histological changes in Ovx rats in the form of epithelial damage and inflammatory cell infiltration when compared to the control group. Further, parts of the urothelium were invaginated and formed nests. It was recorded by other investigators that the urothelium could proliferate into buds (nests of von Brunn), which grow down into the connective tissue beneath the epithelium in the lamina propria. It was considered a common chronic reactive inflammatory disorder (Grignon and Sakr, 1995).



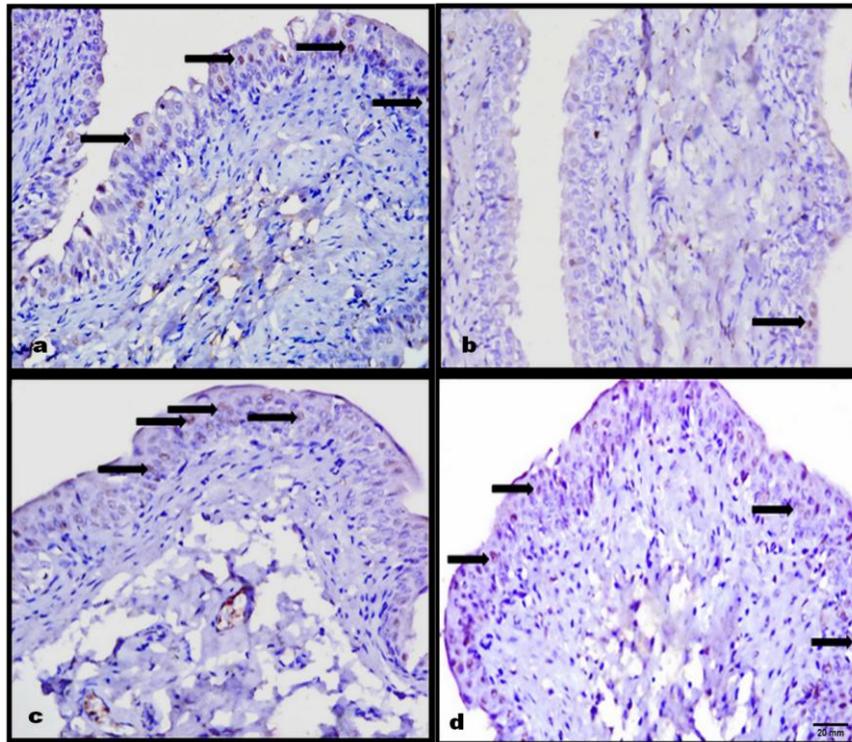
**Fig. 5:** Light microscopic pictures of urinary bladder sections immunostained for ER- $\alpha$  from: (a) sham operated rats showing positive ER- $\alpha$  Immunostaining in some nuclei of transitional epithelial cells (arrow). (b) ovariectomized rats showing apparent increase of positive ER- $\alpha$  Immunostaining in transitional epithelial cells when compared to Group I (arrows). (d) group IV (Ovx+ E) & (e) group V (Ovx+ FS) showing a pattern more or less similar to that of ovariectomized rats (ER- $\alpha$  immunostaining, x400 )

In the current work, sections in the urinary bladder of Group IV (Ovx+ E) revealed a well-organized general architecture similar to that seen in Group I however; some nuclei were still pyknotic and in Group V (Ovx+ FS) sections showed restoration of all layers however at higher magnification, details of transitional epithelium were detected in the form of cellular vacuolation and wide intercellular spaces. Lamina propria showed dilated congested blood vessels. These morphological alternations clearly indicate that estrogen or defatted flaxseed supplementations after Ovx resulted in epithelial growth and restoration. The presence of some pyknotic nuclei and cytoplasmic vacuolation may be a remaining atrophic symptom that did not completely improve with estrogen or defatted flaxseed supplementations.

Despite the obvious change in the thickness of the epithelium in the examined sections of different groups, it was not considered one of the morphometric items for measurement. This was based on the fact that the thickness of the urothelium of the bladder will not only vary according to the degree of distension and anatomical location but also according to the plane on which the tissue is cut (Mills, 2007).

Thus, it is clear that estrogen and defatted flaxseed have profound effects on the female urinary bladder especially at periods of hormonal changes. Such estrogenic effects are probably mediated through estrogen receptors as indicated by upregulation of ER-beta expression. ER-alpha and ER-beta were seen mainly in epithelial cells however, ER-beta appeared to be the predominant estrogen receptor type. In this study, ovariectomy resulted in a significant increase in ER-alpha and decrease in ER-beta. Moreover, estrogen or defatted flaxseed supplementation caused a significant increase in ER-beta towards control levels with no significant effect on ER-alpha expression. This was agreed by Yang *et al.* (2009) who stated that, with estrogen deficiency ER-alpha was upregulated and ER-beta down regulated. They added that after estradiol augmentation, ER-alpha expression had a trend to change to Sham levels but there was no statistical difference when compared to Ovx. ER-beta increased significantly compared to Ovx group but did not reach the levels of the Sham group. The mechanism by which ER-alpha was upregulated in Ovx group was not clearly understood. It is well established that estrogen receptors mediate proliferation of epithelia, including human urothelial cells probably through stimulation of nerve growth factor (NGF) synthesis (Bai *et al.*, 2000). Such down regulation of ER-beta in the Ovx group probably explains the atrophic changes induced in the urothelium, in the form of exfoliation and shedding of epithelial cells, as detected by light and scanning electron microscopy, in some parts

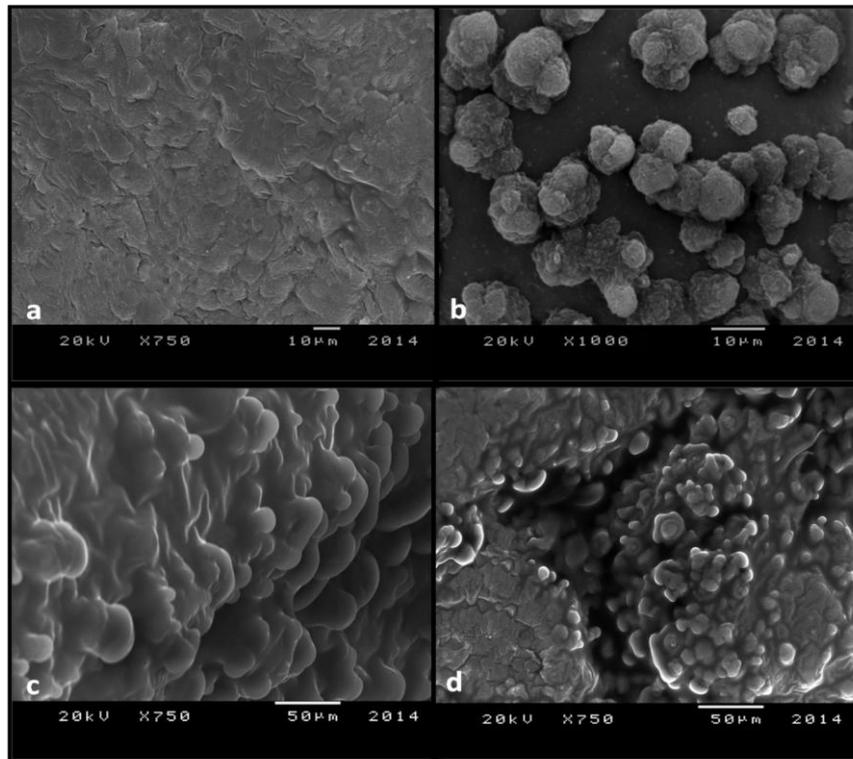
of the bladder mucosa. In groups IV and V, the surface epithelium exhibited prominent pleomorphism and piling up of cells indicative of increased epithelial thickness (hyperplasia). Then, the up-regulation of ER-beta in the groups treated with estrogen or defatted flaxseed clearly indicates that ER-beta expressed by urothelial cells mediate estrogen-induced cell proliferation in these cells, causing restoration of the lining transitional epithelium which appeared well-formed. These findings suggest that ER-beta isoform plays an important role in modulating the effect of estrogen and consequently, further improvement of the urinary bladder (Teng *et al.*, 2008).



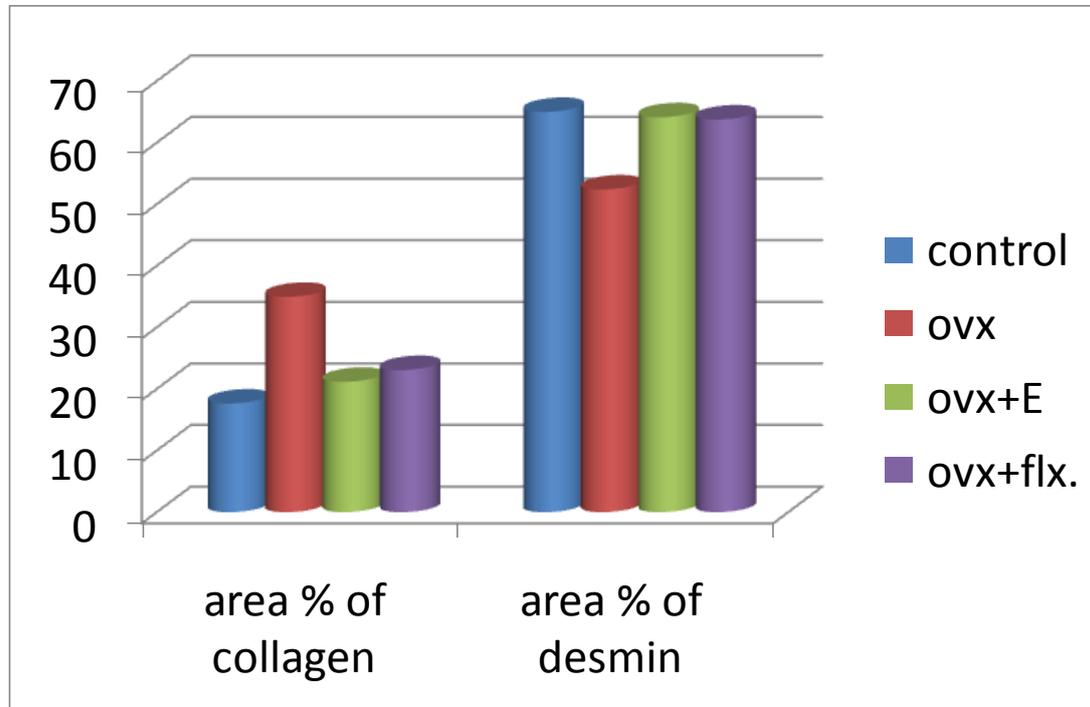
**Fig. 6:** Light microscopic pictures of urinary bladder sections immunostained for ER-  $\beta$  from: (a) sham operated rats showing positive ER- $\beta$  immunostaining in many nuclei of the lining transitional epithelial cells (arrows). (b) ovariectomized rats showing a few cells of transitional epithelium expressing positive immunostaining in their nuclei (arrows). (d) group IV (Ovx+ E) & (e) group V (Ovx+ FS) showing a pattern more or less similar to that of control (arrows) (ER- $\beta$  immunostaining x400 ).

On examining the musculosa of the Ovx group, the smooth muscle bundles appeared disrupted, widely separated with marked cytoplasmic vacuolation and increased content of connective tissue in-between. These changes could be explained by a drop in the blood flow caused by estrogen deficiency with subsequent reduction of oxygen supply and hypoxia resulting in an ischemic environment within the detrusor muscle compartment of the urinary bladder with consequent smooth muscle atrophy (Aikawa *et al.*, 2003). Our findings agree with those of Galvin *et al.* (2004) who hypothesized that smooth muscle cells respond to the presence of hypoxia through significant up regulation of survival factors, (HIF 1 alpha) and (VEGF) in a time dependent manner. Hypoxia doesn't induce cell death, but significantly reduces the rate of proliferation overtime, associated with an increase in the cell cycle inhibition. Azadzo *et al.* (1996) reported that such ischemic insult to detrusor smooth muscle causes activation of sensory nerves with subsequent detrusor overactivity, which may be related to instability symptoms such as urgency, frequency and incontinence ( the components of overactive bladder syndrome). So ovarian hormonal production is an important factor for mediating the integrity of adult detrusor as well as its innervations in female mammals.

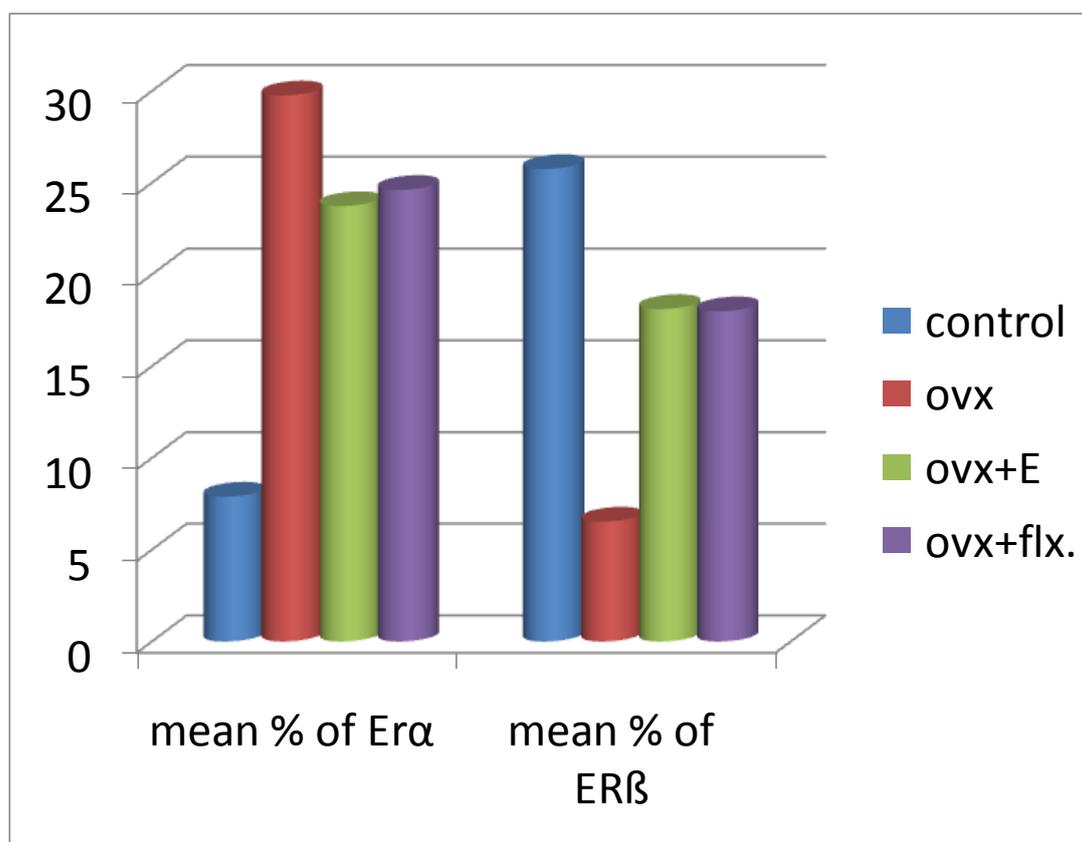
Following estrogen or defatted flaxseed supplementation, smooth muscle bundles appeared compact with decreased content of connective tissue. Estrogen supplementation induces its effect on smooth muscle by stimulating increased blood flow and angiogenesis. Estrogen is proposed to modulate angiogenesis directly via effects on endothelial cells (Losordo and Isner, 2001). Marked changes in smooth muscle bulk were further demonstrated and verified using desmin immunostained sections. Desmin-deficient mice showed structural changes with a partial disruption of the wall of the urinary bladder. The authors concluded that desmin in the bladder smooth muscle is not needed for growth but has a role in active force transmission and maintenance of wall structure (Scott *et al.*, 2008).



**Fig. 7:** Scanning electron microscopic pictures of urinary bladder from (a) sham operated rats showing normal urothelial surface in the form of large flat cells (X 750). (b) ovariectomized rats showing areas of exfoliation (arrows) in superficial urothelial cells (X 1000). (c) group IV (Ovx+ E) & (d) group V (Ovx+ FS) showing a nearly normal urothelial surface with areas of pilling up of cells (X 750).



**Histogram 1:** Comparison of the mean area % of collagen and mean area % of desmin positive immunoreactivity between the different studied groups.



**Histogram 2:** Comparison of the mean percent of ER- $\alpha$  positive cells and mean percent of ER- $\beta$  positive cells between the different studied groups.

The current results revealed that there was a highly significant increase in the area percentage of collagen fibers in the wall of the urinary bladder in OvX group. It was postulated that chronic inflammation could account for the increased collagen fiber deposition in ovariectomized rats (Zeidel, 1997). Xin *et al.* (2009) reported that in OvX, collagen fiber hyperplasia induced weaker bladder compliance. In addition, it may interfere with electrical transmission, increasing the bladder's instability & influencing detrusor muscle contractility. Thus, it could be suggested that increased collagen deposition in urinary bladder could certainly affect the functional properties of the lower urinary tract and thereby interfering with bladder contraction and again having a great contribution to urinary bladder dysfunction as in cases of females with urinary incontinence. In the estrogen or defatted flaxseed treated groups, smooth muscle density increased with consequent decrease in collagen fiber content. This might be a protective mechanism induced by estrogen as reported by Blacher *et al.* (2000) who reported that there is evidence that estrogen exerts its cardioprotective action against collagen formation by preventing the proliferation of fibroblasts and collagen deposition in the blood vessels of the heart and this action may be via the endothelial release of nitric oxide and estrogen might probably protect the bladder by the same mechanism of action.

Findings from this study revealed that defatted flaxseed may provide a dietary approach for maintaining normal bladder morphology after cessation of endogenous estrogen production. Such dietary intervention may act as a natural alternative to traditional hormone replacement therapy among postmenopausal women. Therefore, the current study recommends that the partially defatted flaxseed (cake), produced from oil extraction, can be reused and incorporated into human daily food as bread or another food product.

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#### REFERENCES

Aikawa, K., T. Sugino and S. Matsumoto, 2003. The effect of ovariectomy and estradiol on rabbit bladder

smooth muscle contraction and morphology. *J Urol.*, 170: 634-637.

Anderson, G.L., H.L. Judd, A.M. Kaunitz, D.H. Barad, S.A. Beresford, M. Pettinger and J. Liu, 2003. Effects of estrogen plus progestin on gynecologic cancers and associated diagnostic procedures: the Women's Health Initiative randomized trial. *JAMA*, 290: 1739-1748.

Azadzoï, K.M., M. Pontari, J. Viachiotis and M.B. Siroky, 1996. Bladder blood flow and oxygenation: Changes induced by filling, contraction and outlet obstruction. *J Urol*, 155: 1459-1465.

Babinski, M.A., W.S. Costa, F.J. Sampaio and L.E. Cardoso, 2007. Structural organization of fibrous connective tissue in the peri-acinar region of the transitional zone from normal human prostates as revealed by scanning electron microscopy. *BJU Int.*, 100: 940-944.

Bai, W., B. Oliveros-Saunders, Q. Wang, M.E. Acevedo-Duncan and S.V. Nicosia, 2000. Estrogen stimulation of ovarian surface epithelial cell proliferation. *In Vitro Cell Dev. Biol Anim.*, 36: 657-666.

Bancroft, J.D. and H.C. Cook, 1994. Immunocytochemistry. In: *Manual of Histological Techniques and Diagnostic Applications*. 1<sup>st</sup> ed., Churchill Livingstone, Edinburgh, London, Madrid, Melbourne, New York and Tokyo, pp: 263-325.

Behari, J. and J. Behari, 2009. Changes in bone histology due to capacitive electric field stimulation of ovariectomized rat. *Indian J Med Res.*, 130: 720-725.

Beral, V., D. Bull and G. Reeves, 2005. Million women Study collaborators. Endometrial cancer and hormone-replacement therapy in the Million Women Study. *Lancet*, 365: 1543-1551.

Blacher, J., H. Dabire and J.P. Pomies, 2000. Long term cardiovascular effects of high "osteoprotective" dose levels of 17-beta estradiol in spontaneously hypertensive rats. *Cardiovas. Drug Ther.*, 14: 303-310.

Boccardo, F., G. Lunardi, P. Guglielmini, M. Parodi, R. Murialdo, G. Schettini and A. Rubagotti, 2004. Serum enterolactone levels and the risk of breast cancer in women with palpable cysts. *Eur J Cancer*, 40: 84-89.

Bozzola, J.J. and L.D. Russell, 1999. *Electron microscopy: Principles and techniques for biologists*. 2nd ed. Boston: Jones and Bartlett Publishers, pp: 48-202.

Cayan, S., B. Canpolat and F. Cayan, 2006. The effect of chronic inflammatory condition of the bladder and estrogen replacement therapy on bladder functions and histology in surgically menopause and chronic cystitis induced rats. *Neurourol Urodyn*, 25(2): 194-201.

Cheskis, B.J., J.G. Greger and L.P. Nagpal, S. Freedman, 2007. Signaling by estrogens. *J Cell Physiol.*, 213(3): 610-617.

Dawson, B. and R.G. Trapp, 2001. *Basic and clinical biostatistics*. 3rd ed. New York: Lange Medical Books-McGraw Hill, pp: 161-218.

Drury, R.A.B. and E.A. Wallington, 1983. *Carleton's histological technique*, 4<sup>th</sup> ed., London, Oxford university press, New York, Toronto, pp: 114-17 & 167-169.

Elbostany, A.N., H.A. Thabet and H.F. Ahmed, 2013. The effect of supplementation with flaxseed and its extract on bone health. *Nature and Science*, 11(5): 71-80.

Feng, J., Z. Shi and Z. Ye, 2008. Effects of metabolites of the lignans enterolactone and enterodiol on osteoblastic differentiation of MG-63 cells. *J Biol Pharm Bull*, 31: 1067-1070.

Galvin, D.J., R.W. Watson and A. Neill, 2004. Hypoxia inhibits human bladder smooth muscle cell proliferation: a potential mechanism of bladder dysfunction. *Neurourol. Urodyn*, 23(4): 342-348.

Grignon, D.J. and W. Sakr, 1995. Inflammatory and other conditions that can mimic carcinoma in the urinary bladder. *Pathol Annu.*, 30(Pt 1): 95-122.

Gustafsson, J.A., 1999. Estrogen receptor- $\beta$ , a new dimension in estrogen mechanism of action. *J. Endocrinol*, 163: 379-383.

Hayat, M.A., 1981. *Fixation for electron microscopy*. New York: Academic Press Inc., pp: 206-261.

Jacobs, E., S.E. Kulling and M. Metzler, 1999. Novel metabolites of the mammalian lignin enterolactone and enterodiol in human urine. *J Steroid Biochem Mol Biol.*, 68: 211-218.

Johnsson, P., A. Kamal-Eldin, L.N. Lundgren and P. Aman, 2000. HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. *Journal of agricultural and food chemistry*, 48 (11): 5216-5219.

Kalu, D.N., C.C. Liu, R.R. Hardin and B.W. Hollis, 1989. The aged rat model of ovarian hormone deficiency bone loss. *Endocrinol.*, 124: 7-16.

Kazi, A.A., J.M. Jones and R.D. Koos, 2005. Chromatin immunoprecipitation analysis of gene expression in the rat uterus in vivo: estrogen-induced recruitment of both estrogen receptor alpha and hypoxia-inducible factor 1 to the vascular endothelial growth factor promoter. *Molecular Endocrinol.*, 19: 2006-2019.

Levin, R.M. and P.A. Longhurst, 1996. Pharmacological basis of bladder and urethral function and dysfunction. In: *Female Urology*, 2<sup>nd</sup> ed. Edited by S.Raz. Philadelphia: W. B. Saunders Co., The Curtis Center, pp: 43-56.

Losordo, D.W. and J.M. Isner, 2001. Estrogen and angiogenesis: a review. *Arteriosclerosis Thrombosis and Vascular Biology*, 21: 6-14.

Milder, I.E., C.W. Arts, B. van de Putte, D.P. Venema and C.H. Hollman, 2005. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. *Br J Nutr.*, 93:

393-402.

Mills, S.E., 2007. Urinary bladder, ureter and renal pelvis. In: Chapter IX. Histology for pathologist. 3rd ed. Philadelphia: Lippincott Williams & Wilkins, pp: 234-246.

Morabito, N., A. Crisafulli, C. Vergara, A. Gaudio, A. Lasco, N. Frisina, *et al.*, 2002. Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: a randomized double-blind placebo-controlled study. *J Bone Miner Res.*, 17: 1904-1912.

Parekh, M., P. Chichester and R. Lobel, 2004. Effects of castration on female rabbit bladder physiology and morphology. *Urology*, 64(5): 1048-1051.

Penttinen-Damdimopoulou, P.E., K.A. Power, T.T. Hurmerinta, T. Nurmi, P.T. Van der Saag and S.I. Makela, 2009. Dietary sources of lignans and isoflavones modulate responses to estradiol in estrogen reporter mice. *J Mol Nutr Food Res.*, 53: 996-1006.

Scott, R.S., Z. Li, D. Paulin, B. Uvelius and J.V. Small, 2008. Role of desmin in active force transmission and maintenance of structure during growth of urinary bladder. *Am J Physiol Cell Physiol.*, 295: C324-C331.

Simbalista, R.L., A.V. Sauerbronn, J.M. Aldrighi and J.A. Areas, 2010. Consumption of a flaxseed-rich food is not more effective than a placebo in alleviating the climacteric symptoms of postmenopausal women. *J Nutr.*, 140: 293-297.

Suckow, M., S. Weisbroth and C. Franklin, 2005. The laboratory Rat, Second Edition (American College of Laboratory Animal Medicine), Toronto: Academic Press, pp: 540.

Tanidir, Y., T. Tarcan and F. Ercan, 2011. Exogenous testosterone and estrogen affect bladder tissue contractility and histomorphology differently in rat ovariectomy model. *J Sex Med.*, 8(6): 1626-1637.

Taylor, A.H. and F. Al-Azzawi, 2000. Immunolocalization of estrogen receptor beta in human tissues. *J Mol endocrinol*, 24: 145-155.

Teng, J., Z.Y. Wang, D.F. Jarrard and D.E. Bjorling, 2008. Roles of estrogen receptor alpha and beta in modulating urothelial cell proliferation. *Endocr. Relat. Cancer*, 15(1): 351-364.

Thompson, L.U., 2003. Flaxseed, lignans, and cancer. In: Thompson LU, Cunanne S, editors. Flaxseed in Human Nutrition. 2nd ed. Illinois: AOCS Press, pp: 194-222.

Turner, N.J., B.M. Thomson and I.C. Shaw, 2003. Bioactive isoflavones in functional foods: the importance of gut microflora on bioavailability. *Nutr Rev.*, 61: 204-213.

Valentini, F.A., G. Robain and B.G. Marti, 2011. Urodynamics in women from menopause to oldest age: what motive? What diagnosis? *Int Braz J Urol.*, 37(1): 100-107.

Waynforth, H.B., 1980. Experimental and surgical technique in the rat. 2<sup>nd</sup> edition, Academic press. New York, pp: 161-163.

Xin, Y., L. Ya-zhen, M. Zhuo, *et al.*, 2009. Effects of estrogen and tibolone on bladder histology and estrogen receptors in rats. *Chin Med J.*, 122(4): 381-385.

Yang, X., Z. Mao, P. Gu and M. Shang, 2009. Effects of estrogen and tibolone on bladder histology and estrogen receptors in rats. *Chin Med J.*, 122(4): 381-385.

Zeidel, M.L., 1997. Functional anatomy of the mammalian urinary bladder. In: Sips IG, McQueen CA, Gandolf AJ, editors. *Comprehensive toxicology*. 1st ed. New York: Pergamon, pp: 155-159.