EFFECTS OF GENISTEIN, ESTROGEN AND PROGESTERONE THERAPIES ON BLADDER MORPHOLOGY AND M2, M3 RECEPTOR EXPRESSIONS IN OOPHORECTOMIZED RATS

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ABSTRACT

Aims: Investigating the effects of estrogen, estrogen/progesterone combination and genistein therapy on the expression of M2 and M3 receptors located on bladder walls and comparing the morphological and degenerative changes exerted on bladder walls.

Materials and methods: A total of 50 adult Sprague-Dawley female rats were randomly divided into five groups. Rats other than the sham group were ovariectomized. OVX group (control group) received water, OVX+G group received 10 mg/kg genistein, OVX+E group received 0.014 mg/kg 17-ß estradiol, OVX+E+P group received 0.014 mg/kg 17-ß estradiol plus 0.028 mg/kg drospirenone per day.

Results: When compared with the sham group, in the OVX group higher collagen fibre (CF): smooth muscle (SM) ratio, relatively increased fibrosis, oedema, space between detrusor smooth muscle fascicles, cytoplasmic vacuoles, and total M2, and M3 expression were observed. Relative to the OVX group, decreased CF: SM ratio and fibrosis in the OVX+G, OVX+E, and OVX+E+P groups, decreased oedema, spaces between detrusor muscle fascicles and cytoplasmic vacuoles in the OVX+G group and lesser total M2, and M3 expression in the OVX+G, OVX+E and OVX+E+P groups were observed.

Conclusion: Genistein therapy regresses unfavourable morphological changes effecting postmenopausal bladder and increases in M2 and M3 receptor expression more effectively than estrogen and estrogen/progesterone combination. Besides, genistein therapy almost completely regresses degenerative changes; however, estrogen and estrogen/progesterone combination therapies do not improve these degenerative changes except for fibrosis. We think that genistein will favourably contribute both to the conduction of more comprehensive studies in the future concerning its use in postmenopausal urinary incontinence where estrogen and estrogen/progesterone combination therapies do not provide any improvement and etiopathogenesis of urinary incontinence.

Key words: Urinary incontinence, genistein, estrogen, menopause, oophorectomized rat, muscarinic receptor.

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Introduction

Urinary incontinence (UI) is a frequently seen health problem in postmenopausal women, which is concealed from the public(1). Traditionally, it is divided in three main classes as stress urinary incontinence (SUI), urge urinary incontinence (UUI) and mixed urinary incontinence (MUI).

In the case of physical stress (coughing and heavy weight lifting) involuntary leakage of urine without any desire of passing urine before, is called SUI. UUI is an urgent and irresistible desire to pass urine, which results in involuntary leakage of urine.

MUI is a combination of these two conditions in various proportions(2). UUI develops on the ground of nervous dysregulation [so-called overactive bladder (OAB) or unstable bladder], while SUI develops as a consequence of inadequate closure mechanism of the urethral sphincter which cannot resist increased intra-abdominal pressure(3).

Menopause is associated with MUI in around 50%, SUI in 34% and UUI in 15% of cases(4). During menopause, SUI alleviates, while UUI and MUI deteriorate(2).

Although cause of postmenopausal UI is not known precisely, hormonal changes are thought to play a role(5). For this reason, hormonal replacement therapy (HRT) consisting of mostly estrogen (E) and less frequently estrogen/progesterone (E+P) combinations have been tried in the treatment of UI with diverse outcomes demonstrating its better or worse(2,11) effectiveness in the treatment of UI.
However, postmenopausal E therapy per se has been reported to be associated with risks of endometrial, ovarian and breast cancer and also addition of prostagen to HRT partially prevents proliferation associated with estrogen(12).

Genistein (G) is an important soy-derived isoflavone and phytoestrogen, which resembles structurally to 17b-estradiol (E2). Effects of E2 in the urethra and urinary bladder are exerted via both estrogen receptor (ER) subtypes as the presence of both the ERα and ERβ has been demonstrated in the urinary tract(13). G have estrogenic or anti-estrogenic activity. G has a protective role against endometrium cancer, breast cancer and some other types of cancers(14).

The bladder is rich in M2 and M3 muscarinic receptors, which mostly consist of M2 subtypes. M2 and M3 receptors can induce in vitro bladder contractions and they can mediate reflexive bladder contractions in vivo. Activation of M3 receptor is thought to induce direct contraction of detrusor, while activation of M2 receptor exerts an indirect effect on bladder and contracts it via reversing sympathetic activity-mediated relaxation. Muscarinic receptors generally increase in number during menopause(13,16), while E(17-20) or E+P therapy(13,16,20) usually decreases muscarinic receptors and spontaneous detrusor contractions.

Still, increase in the number of M2 and M3 muscarinic receptors(21) in OAB which is more frequently seen in especially postmenopausal patients, OAB symptoms induced by deficiency in ovarian hormones(22), and treatment of OAB mainly using anti-muscarinics which are thought to decrease detrusor contractions(23) all demonstrate the association between especially OAB and muscarinic receptors during menopause. Although only a few studies measured muscarinic receptors or analyzed their density so far, muscarinic receptor response has been evaluated more frequently.

Different from other studies, in our study, the effects of not only estrogen, but also estrogen+progesterone and especially genistein therapy which has not been investigated so far, on the expression of M2 and M3 receptors located on bladder walls with its advantage of protective effect in contrast to negative effects of estrogen during postmenopausal period have been analysed and these three treatment modalities have been compared as for morphological and degenerative changes exerted on bladder walls with the aim of contributing to the etiopathogenesis and treatment of urinary incontinence during post-menopausal period.

Materials and methods

Animals, care and nutrition
This study was designed in accordance with the “Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals” after the approval of the local ethics committee for care and use of laboratory animals in Dicle University and was carried out at the Dicle University Health Sciences Practice and Research Center (DUSAM).

A total of 50 adult Sprague-Dawley female rats, weighing 250-300 g, were housed in individual cages in the animal laboratory of our university. They were maintained in a 12 hours light/12 hours dark cycle at room temperature (25±3°C), with a standard pellet diet and water provided ad libitum. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary to produce reliable scientific data.

Experimental treatment
The rats were randomly divided into five groups and each group included ten rats as described below:

Group 1 (Sham group). The surgical procedure for the sham-operated rats, was the same except that the ovaries were not removed. Water was injected in each rat once daily for 8 weeks by orogastric catheter without any medication.

Group 2 (OVX; control group). Ovariectomized rats received water by orogastric catheter once daily for 8 weeks without any medication.

Group 3 (OVX+G). Ovariectomized rats received 10 mg/kg per day Genistein (Bonistein S®, DSM Nutritional Products, Istanbul, Turkey) by orogastric catheter for 8 weeks.

Group 4 (OVX+E). Ovariectomized rats received 0.014 mg/kg 17β estradiol (Estrofem®, Novo Nordisk) by orogastric catheter once daily for 8 weeks.

Group 5 (OVX+E+P). Ovariectomized rats received 0.014 mg/kg 17β estradiol plus 0.028 mg/kg drosperinone (Angelib®, Shering Alman) by orogastric catheter once daily for 8 weeks.

Anaesthesia was induced by ketamin hydrochloride (100 mg/kg i.p, Ketalar®, Pfizer) and Xylazine (1 mg/kg i.p, Rompun®, Bayer).

A midline abdominal incision was made at the pelvic level under general anaesthesia in all groups and as bilateral ovariectomy (OVX) was performed in all groups except the sham-operated group.
Musculo-peritoneum and fascia were sutured with 4/0 polyglactin (Ethicon®, Somerville, NJ, USA), while the skin incision was sutured with 3/0 a traumatic silk sutures. No antibiotics were administered before, during or after the intervention.

All treatments were initiated exactly one week after surgery. At the end of study, abdominal and thoracic cavities of all rats were explored after sacrifice by intra-cardiac blood aspiration. In ovariectomized rats, uterine atrophy was verified as indicative of successful removal of both ovaries. Bladders of rats were removed rapidly. Bladders were split into three pieces after total resection. The first specimen underwent histo-pathological examination under light microscopy after haematoxylin eosin (H&E) and Masson’s trichrome (MtCr) staining. The second specimen fixed with 2.5% glutaraldehyde for electron microscopy. The third pieces were used to prepare frozen sections to investigate the severity and extent of the M2 and M3 receptors of the bladder.

**Histo-pathological examination**

The bladder tissue was removed and fixed in 10% formaldehyde solution at room temperature. Serial cross-sections of 5 µm were prepared from the tissue. The sections were mounted and stained with haematoxylineosin and Masson’s trichrome. All slides were examined under light microscopy (Olympus BX50, Olympus, Japan) for evaluation in terms of epithelial thickness, smooth muscle thickness, connective tissue thickness, collagen fibre (CF): smooth muscle (SM) ratio, total bladder wall thickness, inflammation, fibrosis, oedema, spaces between the detrusor muscle fascicles and cytoplasmic vacuoles. Inflammation, fibrosis, oedema, spaces between the detrusor muscle fascicles and cytoplasmic vacuoles were scored subjectively as 0, 1 and 2. The pathologists were also blinded for allocation information.

**Immunohistochemistry**

The bladder tissue was removed, frozen in powdered dry ice, and stored at -70°C. After blocking with 2% normal goat serum for one hour, the sections were incubated for 12 to 15 hours at 4°C with a mouse monoclonal antibody against the M2 (Anti-Muscarinic Acetylcholine Receptor 2 antibody [B8E5] (ab90805) 50µg at 0.100 mg/ml, Abcam Inc., Cambridge, MA, USA) subtype receptor. Endogenous peroxidase was blocked for 30 minutes with 0.1% H₂O₂ and 100% methanol. The sections were then washed and incubated for one hour with biotinylated goat anti-rabbit IgG, followed by incubation for 20 minutes with avidin-biotin-horseradish peroxidase complex. The chromogen was 3,39-diaminobenzidine tetra-hydrochloride. Slides were counterstained with Mayer’s haematoxylin. Simultaneous control experiments with the omission of either primary or secondary antibody gave negative results.

**Scoring analysis of immune-reactivity**

M2 and M3 slides were evaluated with Nikon Eclipse 80i light microscope (Nikon, Melville, NY, USA) by an expert pathologist. At least five fields for each tissue were randomly selected and calculated based on average score. The results were scored according to epithelial and smooth muscle cells, depending on the severity and extent of staining. The severity of staining was assigned semi-quantitatively on a 4-point scale from 0 to 3 (0=no staining, 1=weak, 2=moderate, 3=strong).

Then, each severity score was added by their extent score from 1 to 3 (1=less than one third of the area stained, 2=more than one third but less than two thirds stained, 3=more than two thirds stained). The maximum score of immune-reactivity was six for each compartment.

**Transmission electron microscopy**

The tissue samples were immediately placed in 2.5% glutaraldehyde, buffered for 4 h, then fixed in OsO₄ for 2 h, dehydrated in graded ethanols, and embedded in araldite. Semi-thin 1µm-thick sections were cut and stained with methylene blue-azure II for light microscopic examination. Thin sections of 70 nm were stained with lead citrate-uranyl acetate, and examined and photographed under a Zeiss BRA120 electron microscope.

**Statistical analysis**

Data were analyzed using the Statistical Package for Social Sciences 18.0 for Windows (SPSS Inc., Chicago, IL). Kolmogorov-Smirnov test was used to evaluate the distribution pattern of the data. Kruskal-Wallis test was used for comparison of five groups.

We used post hoc Mann-Whitney U tests to determine which of the five groups differed from each other.
All differences associated with a chance probability of 0.05 or less were considered statistically significant. Means and standard deviations were used to describe numerical variables.

## Results

Sham, OVX, OVX+G, OVX+E and OVX+E+P groups were compared among themselves as for morphological changes on the bladder wall (Table 1).

### Table 1: The parameters of morphological changes in the bladder wall in five groups (mean ± standard deviation).
P values denoting the outcomes of comparison of the parameters of morphological changes in the bladder wall in five groups.

Relative to the Sham group in the OVX+G and OVX+E+P groups epithelial thickness decreased significantly (p=0.01, p=0.04, respectively). When compared with the Sham group, significantly increased connective tissue thickness was observed in the OVX and OVX+E groups (for both p=0.000). However relative to the OVX group, connective tissue layer was thinner in OVX+G, OVX+E and OVX+E+P groups (p=0.000, p=0.005, p=0.000, respectively).

<table>
<thead>
<tr>
<th>Group Parameters</th>
<th>Sham (n=10)</th>
<th>OVX (n=10)</th>
<th>OVX+G (n=10)</th>
<th>OVX+E (n=10)</th>
<th>OVX+E/P (n=10)</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial thickness (µm)</td>
<td>41.19±5.05</td>
<td>37.83±5.78</td>
<td>33.62±6.31</td>
<td>38.77±8.03</td>
<td>34.17±8.33</td>
<td>*0.01, †0.041, ‡0.001, †0.005, ❀0.000, ♣0.000, NS</td>
</tr>
<tr>
<td>Connective tissue thickness (µm)</td>
<td>242.82±45.73</td>
<td>2106.29±124.69</td>
<td>291.75±101.04</td>
<td>512.83±129.12</td>
<td>297.89±69.86</td>
<td>*0.000, †0.001, ‡0.003, ¶0.005, ❀0.000, NS</td>
</tr>
<tr>
<td>Smooth muscle thickness (µm)</td>
<td>696.71±17.94</td>
<td>565.99±103.16</td>
<td>1050.85±165.46</td>
<td>644.02±143.75</td>
<td>372.16±81.35</td>
<td>*0.000, †0.001, ‡0.002, ¶0.000, ¶0.002, NS</td>
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<tr>
<td>CF:SM ratio</td>
<td>0.35±0.07</td>
<td>1.27±0.24</td>
<td>0.28±0.11</td>
<td>0.82±0.23</td>
<td>0.83±0.26</td>
<td>*0.000, †0.001, ‡0.002, ¶0.000, NS</td>
</tr>
<tr>
<td>Total bladder wall thickness (µm)</td>
<td>1216.45±198.21</td>
<td>840.58±97.76</td>
<td>1353.73±101.71</td>
<td>1019.69±111.01</td>
<td>1705.20±100.74</td>
<td>0.017, 0.000, 0.001, 0.000, 0.002, 0.003, NS</td>
</tr>
</tbody>
</table>

(Abbreviations: CF=collagen fibre; SM=smooth muscle; µm=micrometer). P values: *Sham versus OVX, †Sham versus OVX+G, ‡Sham versus OVX+E, ¶Sham versus OVX+E/P, ◆OVX versus OVX+G, ◇OVX versus OVX+E, *OVX versus OVX+E/P, †OVX+G versus OVX+E/P, ‡OVX+E versus OVX+E/P; NS=Not significant between groups.

### Table 2: The parameters of inflammatory and degenerative changes in the bladder wall in five groups (Mean ± standard deviation).
P values denoting the outcomes of comparison of the parameters of inflammatory and degenerative changes in the bladder wall in five groups.

Compared to the Sham group, smooth muscle thickness was significantly decreased in the OVX and OVX+E+P groups (p=0.002, p=0.000, respectively); however, in the OVX+G group it was thicker (p=0.000). When compared with the OVX+G groups and thicker in the OVX+E+P group (p=0.001, p=0.013, p=0.000 respectively). When compared with the OVX group, it was thicker in the OVX+E+P group (p=0.032, p=0.018, p=0.047). In the OVX, OVX+E and OVX+E+P groups these ratios were lower than those observed in the OVX group (p=0.000, p=0.001, p=0.002, respectively).

Any significant difference was not detected among groups with respect to inflammation. When compared with the Sham group, fibrosis was more frequently observed in the OVX and OVX+E groups (p=0.000, p=0.044, respectively), while it
was seen less often in the OVX+G, OVX+E and OVX+E+P groups relative to the OVX group (p=0.001, p=0.048, p=0.003, respectively). When compared with the Sham group, oedema was observed more frequently in the OVX, OVX+E and OVX+E+P groups (p=0.024, p=0.032, p=0.032, respectively), however it was seen less often in the OVX+G group compared to the OVX group (p=0.012).

When compared with the Sham group, spaces between the detrusor muscle fascicles were wider in the OVX, OVX+E and OVX+E+P groups (p=0.005, p=0.018, P=0.044, respectively) and they were decreased in the OVX+G group relative to the OVX group (p=0.012). In comparison with the Sham group, number of cytoplasmic vacuoles were more numerous in the OVX, OVX+E and OVX+E+P groups (p=0.018, p=0.022, p=0.006, respectively), while they were fewer in the OVX+G group relative to the OVX group (p=0.018).

**Figure 1:** Demonstration of inflammatory and degenerative changes on the bladder wall in five groups using Masson’s trichrome staining. **A-Sham group:** Normal distribution of muscle fibres within muscular layers and normal transitional epithelial. **B-OVX group:** Relative to the Sham group, increase in the fibrosis, oedema and the number of polygonal cells and cytoplasmic vacuoles, dilatation of spaces between detrusor muscle fascicles. **C-OVX+E group:** Compared with the Sham group marked oedema of the sub-epithelial connective tissue, widening of the spaces between detrusor muscle fascicles, increase in the number of cytoplasmic vacuoles and necrotic cells on the surface epithelium, fibrosis, patchy areas of desquamation. **D-OVX+E/P group:** When compared with the Sham group, increase in the number of desquamative cells on the surface epithelium and cytoplasmic vacuoles, marked oedema and dilatation of the spaces between detrusor muscle fascicles. **E-OVX+G group:** Relative to the OVX group, enlargement of the muscular layer, decreased fibrosis, lesser number of cytoplasmic vacuoles, decreased oedema, regular intermuscular space without any difference between the Sham group.

**Figure 2:** Comparison of the bladder wall at the ultrastructural level in five groups using Transmission Electron Microscopy. **A-Sham group:** Normal appearance of the bladder detrusor muscle Bar 2µm. **B-OVX group:** Cytoplasmic vacuoles and small spaces were seen on the periphery of the muscle cells. Dilatation of the spaces between detrusor muscle fascicles, marked increase in the number of fibres together with collagen synthesis and oedema Bar 2µm. **C-OVX+E group:** Dilatation of the spaces between detrusor muscle fascicles and fibrotic cells were observed. Increase in collagen tissue near gap junction connections Bar 2µm. **D-OVX+E/P group:** Hypertrophic muscle cell nuclei, dilatation of the spaces between detrusor muscle fascicles. Mild oedema and rarefied collagen fibre density Bar 2µm. **E-OVX+G group:** Oval muscle cell nuclei, a few number of cytoplasmic vacuoles, prominent peri-nuclear Golgi complexes, nearly normal appearing spaces between detrusor muscle fascicles and distinct gap junction connections were observed.

**Figure 3:** Immunohistochemical expression of M2 receptor on the bladder wall in five groups. **A-Sham group:** M2 expression (arrow) in the epithelial and muscle tissue. **B-OVX group:** When compared with the Sham group, intense (arrow) M2 expression in the muscle cells. **C-OVX+E group:** Moderate (arrow) degrees of M2 expression in the epithelial and muscle cells similar to those seen in the Sham group. **D-OVX+E/P group:** Moderate degrees of M2 expression in the basal layer and muscle layer in the vicinity of the epithelium similar to those seen in the Sham group. **E-OVX+G group:** Mild degrees of M2 expression in the epithelial and muscular layers which are less intense than observed in the Sham group.
Discussion

During the postmenopausal period urinary incontinence especially OAB is an important health problem which affects millions of people from social, psychological, professional, physical and sexual perspectives\(^{(1, 24)}\). Although its precise cause is not known, the role of hormonal changes in its mechanism has been presumed\(^{(5)}\). In some studies, beneficial effects of estrogen have been demonstrated in the treatment of UI\(^{(6-8)}\) and UUI\(^{(9, 10)}\). Still, addition of progesterone to the UI treatment has been demonstrated in various studies as beneficial\(^{(15)}\) or futile\(^{(6)}\) attempts. On the contrary, some studies have asserted that systemic estrogen increased severity of UI\(^{(2, 11)}\) and UUI\(^{(11)}\) and recommended use of vaginal estrogen in place of systemic estrogen in the treatment of UI and UUI\(^{(2, 11)}\).

In our study, HRT has been evaluated with respect to morphological changes on the bladder wall. Besides, oophorectomy did not change thickness of the epithelial layer of the bladder wall, on the contrary it increased connective tissue thickness, CF:SM ratio and decreased smooth muscle and total bladder wall thickness.

Figure 4: Immunohistochemical expression of M3 receptor on the bladder wall in five groups. \(\text{A-Sham group: M3 expression in the epithelial and muscle tissue (arrow). B-OVX group: Increase in the M3 expression in the epithelial, basal membrane and muscle tissue when compared with the Sham group. C-OVX+E group: Decreased M3 expression in the vicinity of the intermuscular spaces relative to OVX group. D. OVX+E/P group: M3 expression on the periphery of the muscle cell is decreased when compared with the OVX group. E-OVX+G group: M3 expression in the muscle tissue is decreased relative to the OVX group.}\)

Connective tissue thickness decreased in all three treatment regimens, more prominently during G and E+P therapies. Smooth muscle thickness was regained during G therapy; on the contrary, E+P decreased smooth muscle thickness more severely.

Table 3: M2 and M3 expression in the bladder wall in five groups (Mean ± standard deviation). p values denoting the outcomes of comparison of M2 and M3 expression of the five groups.

<table>
<thead>
<tr>
<th>Group Parameters</th>
<th>Sham (n=10)</th>
<th>OVX (n=10)</th>
<th>OVX+G (n=10)</th>
<th>OVX+E (n=10)</th>
<th>OVX+E/P (n=10)</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2 severity (0-3)</td>
<td>1.90±1.07</td>
<td>2.00±1.70</td>
<td>1.60±0.63</td>
<td>1.80±0.03</td>
<td>1.80±0.79</td>
<td>0.014, 0.015, 0.029, 0.038, 0.042, 0.053, NS</td>
</tr>
<tr>
<td>M2 extent (1-3)</td>
<td>2.00±1.07</td>
<td>2.00±1.70</td>
<td>1.20±0.42</td>
<td>2.10±0.03</td>
<td>1.20±0.42</td>
<td>0.002, 0.005, 0.006, 0.007, 0.012, 0.042, 0.053, NS</td>
</tr>
<tr>
<td>M2 Total score (0-6)</td>
<td>3.90±1.10</td>
<td>5.40±0.70</td>
<td>2.80±0.18</td>
<td>3.90±0.88</td>
<td>3.00±0.94</td>
<td>0.002, 0.07, 0.04, 0.014, 0.022, 0.037, 0.053, NS</td>
</tr>
<tr>
<td>M3 severity (0-3)</td>
<td>1.90±0.57</td>
<td>2.70±0.48</td>
<td>1.80±0.42</td>
<td>2.00±0.82</td>
<td>1.80±0.79</td>
<td>0.011, 0.006, 0.017, 0.04, 0.04, 0.053, NS</td>
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<tr>
<td>M3 extent (1-3)</td>
<td>2.10±0.74</td>
<td>2.90±0.32</td>
<td>1.70±0.82</td>
<td>1.90±0.57</td>
<td>2.00±0.47</td>
<td>0.001, 0.001, 0.007, 0.002, 0.007, 0.012, 0.053, NS</td>
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<tr>
<td>M3 Total score (0-6)</td>
<td>4.00±1.05</td>
<td>5.60±0.52</td>
<td>3.50±1.08</td>
<td>3.90±1.20</td>
<td>3.80±0.92</td>
<td>0.001, 0.001, 0.007, 0.002, 0.007, 0.012, 0.053, NS</td>
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</table>

P values: *Sham versus OVX, ‡Sham versus OVX+G, †Sham versus OVX+E, ¶Sham versus OVX+E/P, €OVX versus OVX+G, ‡OVX versus OVX+E, #OVX versus OVX+E/P, ¶OVX+G versus OVX+E, #OVX+G versus OVX+E/P, ‡OVX+E versus OVX+E/P. NS not significant between groups.
Effects of genistein, estrogen and progesterone therapies on bladder morphology and m2, m3

when compared with oophorectomy. However CF:SM ratio was decreased during all three therapies, being more markedly during G therapy. Total bladder wall thickness was increased again in all treatment modalities.

However, bladder wall thickness increased maximally in E+P and minimally in E treatment arms. In studies where the effects of E deficiency and treatment on bladder functions and morphology in oophorectomized rats were analyzed, in the oophorectomized group increases in bladder collagen fibre area density and collagen fibre to smooth muscle area density ratio were reported. The changes were similar between OVX+E and non-oophorectomized groups.

The authors correlated these changes to the lack of inhibitory effects of E on collagen hyperplasia\(^7,15\) and indicated beneficial effects of E on bladder functions\(^7\). Urinary bladder instability is associated with collagen accumulation. A decrease in the urinary bladder compliance and detrusor instability develops due to collagen increase and consequently a change occurs in sensorial threshold regarding response to cholinergic stimulation. E inhibits accumulation of collagen within bladder wall\(^16\). Chen et al. reported that in the oophorectomized group, bladder wall thickness decreased significantly in the oophorectomized group, while E therapy increased bladder wall thickness with a hypertrophic impact on detrusor\(^25\).

Yu et al. reported that ovariectomy induced a thinner bladder wall. In the OVX group, smooth muscle and total bladder wall thickness decreased, while in the OVX+E and OVX+E+P groups, baseline thickness of these structures were regained. In an electron microscopic analysis performed in the OVX group smooth muscle compartments appeared mildly decreased and abundant collagen was deposited in the space between muscle fascicles\(^26\). Juan et al. confirmed that surgical menopause can increase bladder SM or mucosa cell apoptosis and a significantly greater collagen and CF:SM ratio.

These pathological ultra-structural changes were reflected by abnormal low bladder volume and decreased bladder compliance, which could be indicators of a loss of bladder wall elasticity. Ovary hormone deficiency can lead to OAB symptoms\(^22\).

Elbadawi et al. examined biopsy materials retrieved from the bladder walls of the patients with lower urinary tract symptoms (like UUI) under electron microscope, detected areas of collagenesis within markedly widened spaces between detrusor muscle fascicles and implicated this finding as a potential cause of voiding dysfunction\(^9\).

However, some studies asserted that thickness of the epithelial layer of the bladder wall and CF:SM ratio did not change with oophorectomy, estrogen or progesterone treatment\(^16\).

In our study, more effective reversal of the unfavourable changes on the bladder wall induced by oophorectomy was detected in the G treatment group when compared with E and E+P therapies.

This phenomenon together with consideration of the presumptive association between these changes and OAB symptoms might lead the way to further investigations on the use of genistein in the treatment of UI and especially UUI.

In our study HRT has been evaluated with respect to inflammatory and degenerative changes on bladder wall and we have observed that inflammation did not differ between groups and oophorectomy increased the severity of degenerative changes including fibrosis, oedema, spaces between the detrusor muscle fascicles and cytoplasmic vacuoles.

Besides, G therapy regressed all of these degenerative changes almost completely, while E and E+P therapy did not improve these changes excluding fibrosis. Similarly, development of degenerative changes within the detrusor musculature of oophorectomized rats has been also reported\(^7,15\). Chen et al. showed wider spaces between the detrusor muscle fascicles and degeneration of detrusor muscle cells were seen in the bladder walls of the OVX group, OVX+E and OVX+E+P group.

A lot of cytoplasmic vacuoles was seen in the group treated with E. Higher dose of E may destroy bladder structure. In addition, progesterone can compromise the effect of E\(^25\). Yu et al. performed electron microscopic analyses and indicated impairment of detrusor musculature as an outcome of oophorectomy with markedly widened spaces between detrusor muscle fascicles, in addition to irregular and disorganized cellular structure of muscle fibres. Besides, they reported that high dose E treatment had led to the formation of large number of caveolae between detrusor muscle fascicles\(^26\).

Bladder wall is damaged significantly following oophorectomy and unfortunately E and E+P therapies are not effective in the repair of these destructive changes. This finding might explain failure of E and E+P therapies in the treatment of UI and UUI in postmenopausal women. In our study, we observed a significant improvement in these degenerative changes with G therapy.
A previous study also reported increase in oxidative stress and interstitial fibrosis on the bladder wall following surgical menopause\(^{(22)}\). This might be explained by antioxidant and cell-protective effects of G\(^{(14)}\). However, we haven’t encounter any literature study on the effects of G on bladder wall muscle. Thanks to its favourable effects, genistein therapy might be beneficial in cases with urinary incontinence where estrogen and estrogen/progesterone treatments failed.

In this study we analyzed the effects of E, E+P and G therapies on M2 and M3 receptors and observed increased M2 and M3 receptor expressions during postmenopausal period. Besides, we noted that all three treatment modalities decreased M2 and M3 receptor expressions when compared with the untreated menopause group. However, G therapy even decreased M2 receptor expression below that of the group who did not go through menopause. In a study performed by Shapiro et al., the investigators reported a 45% decrease in the density of muscarinic receptors on the female rabbit bladder and urethra after 3 weeks of E therapy\(^{(17)}\).

Batra and Andersson showed that after E treatment for four weeks, the muscarinic receptor concentration was reduced to approximately 10% of the control value in the ovariectomized rabbit\(^{(18)}\). Liang et al. indicated a decrease in the number of M2 receptors in the bladder of rats with E treatment after castration\(^{(19)}\). M3 receptor mRNAs in the detrusor muscle were significantly decreased in the estradiol-treated rats, while there were no differences noted for the M2 receptor mRNAs\(^{(20)}\). Ratz et al. studied the bladder of rabbits under the effects of estradiol and found a significant reduction in contractility mediate by muscarinic receptors. The inhibitory effect was also discovered to be affected by progesterone\(^{(20)}\).

Fleischmann et al. suggested not only that sex hormone deficiency may be associated with an increased muscarinic activity, but also hormone replacement with estrogen plus progesterone may be better in restoring the ‘normal’ contractility state than the estrogen-only treatment\(^{(15)}\). An in vitro study demonstrated that treatment with E+P decreases muscarinic activity in oophorectomized rats, whereas estrogen-only treatment does not\(^{(16)}\).

Contrary to these studies, some studies have reported that gene expressions of M2 and M3 receptors on the bladder of castrated virgin rats hadn’t changed at all\(^{(23)}\). While others indicated a decrease\(^{(28)}\) after menopause or increase\(^{(29,30)}\) in muscarinic receptor density after E therapy. Mukerji et al. also showed significantly increased M2 and M3 muscarinic receptor immune-reactivity in myofibroblast-like cells in bladder specimens from patients with idiopathic detrusor overactivity.

Furthermore, the increase in M2 and M3 immuno-staining in myofibroblasts significantly correlated with the urgency score\(^{(24)}\). In postmenopausal women, increase in the severity and the number of symptoms of OAB\(^{(22)}\), relatively higher number of M2 and M3 receptors in OAB and menopause, routine use of anti-muscarinics in the treatment despite their partial effectiveness\(^{(23)}\) demonstrate the role of M2 and M3 receptors, estrogen and progesterone in the etiopathogenesis of this disease.

Although numerous studies have demonstrated beneficial\(^{(8,10)}\) or unfavourable\(^{(11)}\) effects of HRT on UI, very few studies have investigated efficacy of G in the treatment of UI and UUI, and indicated its beneficial effects\(^{(10,22)}\). Manonai et al. demonstrated a significant increase of complaints regarding UUI and vaginal atrophy after a 12 week soy-free diet of 36 climacteric Thai women. A soy-rich diet however, in contrast could not relieve the pertained complaints\(^{(31)}\). However, we haven’t encountered any study in the literature which investigated the impact of G on M2 and M3 receptors on the bladder wall.

In our study, we have demonstrated that genistein exerts effects similar to those obtained with estrogen and estrogen+progesterone combination on M2 and M3 receptors on the bladder wall and lowers M2 receptor expression below the normal threshold. These outcomes of our study might contribute to the studies investigating the use of genistein in the treatment of urinary incontinence and especially OAB.

**Conclusion**

M2 and M3 receptor expression on the bladder wall increase during postmenopausal period.

Together with unfavourable morphological changes such as increased collagen connective tissue, degenerative changes occur on the bladder wall including fibrosis, oedema, spaces between the detrusor muscle fascicles and cytoplasmic vacuoles.

Genistein therapy regresses unfavourable morphological changes effecting postmenopausal bladder and increases in M2 and M3 receptor expression more effectively than estrogen and estrogen/progesterone combination.
Besides, genistein therapy almost completely regresses degenerative changes as fibrosis, oedema, spaces between the detrusor muscle fascicles and cytoplasmic vacuoles. However, estrogen and estrogen-progesterone therapies do not improve these degenerative changes except for fibrosis. In consideration of all these favourable effects, we think that genistein will favourably contribute both to the conduction of more comprehensive studies in the future concerning its use in postmenopausal urinary incontinence and especially in OAB where estrogen and estrogen-progesterone therapies do not provide any improvement and etiopathogenesis of urinary incontinence.

References


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