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Restorative effects of myricetin and hyaluronic acid on vaginal epithelial atrophy in ovariectomized rats

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ABSTRACT

Objectives: Postmenopausal women often suffer from genitourinary syndrome (GUS), which is vulva-vaginal atrophy. The aim of this study is to evaluate the effect of hyaluronic acid and myricetin on GUS caused by ovariectomy.

Materials and Methods: Twenty-eight Wistar Albino female adult rats were randomly divided into 4 equal groups. Vaginal smear and pH were collected from all groups before the procedures. To the vaginal walls, for Sham group (Group 1, n=7): The abdomen was opened and closed, then 0.9% saline/0.5 cc/subcutaneously, for ovariectomy group (Group 2, n=7): Two weeks after ovariectomy, 0.9% saline/0.5 cc/subcutaneously, for ovariectomy + myricetin group (Group 3, n=7): Two weeks after ovariectomy myricetin 5 mg/0.5 cc/subcutaneously, for ovariectomy + hyaluronic acid group (Group 4, n=7): 2 weeks after ovariectomy hyaluronic acid 5 mg/0.5 cc/subcutaneously was applied. After 2 weeks of the injection, vaginal smear and pH were checked again in all rats. Vaginal smears were stained with May-Grunwald Giemsa and Pap. Meisel's vaginal maturation index was calculated. Vaginectomy was performed. hematoxylin eosin and caspase-3 immunostaining was performed and scored. Biochemically tissue SOD, AOPP, and TSH were measured.

Results: Vaginal Maturation Index and pH were significantly increased in group 3 and 4 compared to group 2. Vaginal epithelial thickness was increased in Group 3 and 4 compared to group 2. The vaginal epithelial thickness of group 3 increased more than group 4. No significant change in biochemical parameters was observed between groups.

Conclusion: We believe that myricetin will be a promising option for non-hormonal treatment methods in women with GUS symptoms.

Keywords: Hyaluronic acid; menopause; myricetin; ovariectomy; vaginal maturation index

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INTRODUCTION

Genitourinary syndrome (GUS), replacing the old terminology of vulva-vaginal atrophy, is defined as the sum of symptoms and signs associated with a decrease in estrogen and other sex steroids, including changes in the genitourinary organs. About half of postmenopausal women are affected by GUS.[1] Common symptoms according to the degree of atrophy include vaginal dryness (75% in postmenopausal women), painful sexual intercourse (dyspareunia) (38%), and vaginal itching-discharge (15%).[2] Differential diagnoses of GUS may include infections (bacterial vaginosis, trichomoniasis, candidiasis), contact irritants, foreign bodies, and sexual trauma.[3] Treatment methods used in GUS are hormone replacement therapy, ospemiphene which is one of the selective estrogen receptor modulators, laser applications and topical estrogen creams. Intravaginal oxytocin gel and dehydroepiandrosterone applications are being researched.[4,5] Due to possible side-effect profile of hormonal methods, interest in non-hormonal methods has increased recently.[6] Among the non-hormonal methods in the treatment of GUS, vitamins, phytoestrogens and hyaluronic acid (HA) are promising.[7]

HA is involved in important biological functions such as regulation of cell adhesion and motility, cell differentiation, proliferation and tissue repair in most connective tissues.[8] The cross-linked form (filler) is used for hydrodynamic volume change of the extracellular matrix to reduce the clinical effects of aging and volumize the skin.[9] The free, non-cross-linked form absorbs water and stimulates the dermal tissue biologically and this form is used in the treatment of atrophy due to its fibroblast cell activation property.[10]

Myricetin is a flavonoid derived from plant source.[11] Myricetin has been shown to have a therapeutic effect on many diseases, including different types of tumors, inflammatory diseases, atherosclerosis, thrombosis, cerebral ischemia, diabetes, Alzheimer's disease, and pathogenic microbial infections.[12] Myricetin has been shown to have anti-inflammatory, analgesic, antitumor, and antibacterial effects.[13-17]

In our study, it is predicted that myricetin can be used as a non-hormonal treatment option such as HA in women with GUS complaints due to its anti-inflammatory properties.

MATERIALS AND METHODS

A total of 28 adult, virgin, 10-12 weeks old female Wistar albino rats were allocated in this study. The animals (200-250 g) were under conditions of 22±1 °C, 12-hours of day/night cycles and 50-60% humidity. The Muğla Sıtkı Koçman University Ethics Committee for Animal Research approved the animal protocol for this study (approval no: 06/22). Animal research investigations were guided in line with the Committee for Human Care. The rats were given water and standard rat feed. After being randomly divided into 4 groups, daily vaginal smears were taken from all groups. The rats having at least two documented 4-5 day menstrual cycles were included to the study. Vaginal pH and vaginal smears were checked for all experimental animals before the operation.

Surgical Procedure

Anesthesia was induced by intraperitoneal 10 mg/kg (Rompun, Bayer, Leverkusen, Germany) xylazine hydrochloride and 50 mg/kg (Ketalar, Eczacıbaşı, İstanbul, Türkiye) ketamine administration. After shaving and cleaning the surgical sites with antiseptic solution (Baticon, Drogsan, Türkiye), 2.5 cm midline vertical incisions were made in the periumbilical region. A surgical menopause model was created by performing ovariectomy. During the ovariectomy, the ovary was taken out of the abdomen by using the uterus catch hook. By pulling the tubas which are located at the end of the uterine horns are pulled. Then both ovarian arteries were ligated respectively. Then, the ovaries were excised by cutting. After the removal of the ovaries the abdomen was closed.

Groups

Ovariectomy was not performed on the rats in the Sham group (Group 1). The abdomen of the rats in the sham group was opened and closed, smears were taken and cytology was examined 4 weeks later. During this time, 0.5 cc 0.9% saline (subcutaneous) was applied to the vaginal walls. In groups 2, 3 and 4 ovariectomies were performed. 4 weeks after the ovariectomy procedures, vaginal cytology was also evaluated by taking vaginal pH and smear in all groups that underwent ovariectomy and the rats that developed atrophy according to cytology results were included in the study. 0.9% saline (0.5 cc/subcutaneous) was injected to the vaginal walls of the rats in ovariectomy + saline group (Group 2). Myricetin (25 mg/kg/subcutaneous) was injected to the vaginal walls of the rats in the ovariectomy + myricetin group (Group 3). HA (25 mg/kg/subcutaneous) was injected to the vaginal walls of the rats in the ovariectomy + HA group (Group 4). Vaginal pH and vaginal smears were checked again in all rats 4 weeks after the vaginal injections of 0.9% saline, myricetin and HA. Vaginectomy was performed in all rats which were then sacrified by injecting high-dose anesthetics.

Vaginal Smear and Vaginal Maturation Index

Two smear samples were taken from each rat. One of the samples was fixed by the air drying method and the other was fixed by

the alcohol fixation method. The air dried samples were stained with May-Grunwald Giemsa stain. The smears which were fixed with alcohol were stained using the Pap staining method.

For vaginal maturation index; Parabasal (P), Intermediate (I) and Superficial (S) cell counts are performed. It is multiplied by 0, 0.5 and 1.0 respectively. The sum of the three values gives the Meisel's vaginal maturation index (VMI). These procedures were performed by the gynecology and obstetrics physician.

Histopathological Analysis

Vaginal tissues were placed in 10% neutral formalin for fixation and prepared for routine paraffin embedding. Paraffin blocks were cut at 5 µm thick, mounted on slides, stained with hematoxylin-eosin (H-E). Vaginal changes including structure of lamina propria and blood vessels in the lamina propria were evaluated and vaginal epithelial thickness was measured in 5 different areas for each vaginal tissues under a light microscope.[18] All sections were examined with a Nikon Eclipse 80i light microscope and Nikon image analysis system (Digital Sight-L2, Ver=450.1032.3220.100531).

Immunohistological Analysis

For analysis by immunohistochemistry, the sections were placed on slides covered with polylysine. After rehydration, samples were heated in citrate buffer (pH 7.6) and microwave oven for 20 minutes. After waiting for 20 minutes at room temperature, the sections were rinsed with phosphate-buffered saline (PBS), then placed in 0.3% H_2O_2 for 7 minutes and rinsed with PBS. Sections were incubated with a primary rabbit-polyclonal anti-Caspase-3 (ab13847; Abcam) for 2 hours. They were washed in PBS. The biotinylated goat was incubated with an anti-polyvalent for 10 minutes. They were incubated with streptavidin peroxidase for 10 minutes at room temperature. They were performed using chromogen + substrate for 15 minutes, and slides were counterstained with Mayer hematoxylin for 1 minutes, rinsed in tap water, and then dehydrated. Anti-caspase-3 antibody was used following the manufacturer's instructions. Brownish granules in the cytoplasm were recognized as positive staining for Caspase-3. All of the sections were examined with a Nikon Eclipse 80i light microscope and a Nikon Image Analysis system (Digital Sight-L2, Ver=450.1032.3220.100531).

IHC evaluation: The semiquantitative H-Score was calculated by counting the positively stained cells in 5 randomly selected areas for each group [H-Score: ∑Pi (i+1) (Pi: % number of positively stained cells; i: staining intensity].[19]

Biochemical Analysis

Determination of SOD activity

For super oxide dismutase (SOD) activity, 150 µL of supernatant was taken, 150 µL of equal volume of chloroform/ethanol (3:5, V/V) was added to it and vortexed for 10 seconds.[20] The extraction mixture was centrifuged at 12000 xRCF (xg) (10519 RPM) for 30 min at +4 °C. After phase separation, enzyme activity and protein determination were made in the clear upper part. Reagent mixture was prepared by mixing 0.45 mg/mL Stock Xanthine, 0.6 mM EDTA, 150 µM NBT, 400 mM Na_2CO_3 and 1 mg/mL BSA. Then, samples were prepared with the reagent mixture and incubated for 20 min at room temperature (25 °C) in the dark. 0.8 mM $CuCl_2$ was added at the end of the incubation. The results were immediately read at a wavelength of 560 nm in a Multiskan Go microplate reader of Thermo Fischer company. Results were calculated according to the SOD activity inhibition rate. Enzyme activity was calculated considering that 50% inhibition provides 1U activity. SOD enzyme activity was divided by protein amounts and the results were given as U/mg protein.

Determination of advanced oxidation protein products (AOPP)

The spectrophotometric method described by Witko-Sarsat et al.[21] was used to measure AOPP levels.[19] The supernatant obtained after tissue homogenization was diluted with potassium phosphate pH=7.4 buffer at the appropriate rate. Diluted samples were vortexed by adding 1.16 M KI and acetic acid, and spectrophotometric measurements were made against blank at 340 nm absorbance. 0-100 µM Chloramine T standards were run as a sample. The results were calculated from the standard curve and given as µmol/g protein.

Determination of total sulfhydryl (TSH)

The spectrophotometric method described by Taylan and Resmi[22] was used to determine the total sulphhydryl content. The supernatant obtained after tissue homogenization was dissolved with an equal volume of 1:1 (v/v) 6% SDS. Afterwards, 4 mg/mL DTNB was added to the samples and incubated for 15 minutes at room temperature in the dark. At the end of the incubation, spectrophotometric measurements were made against the blank at a wavelength of 412 nm in the Multiskan Go cuvette reader of Thermo Fischer company. Standards of 0-500 µM reduced glutathione (GSH) were run as samples. The results were calculated from the standard curve and given as µmol/mg protein.

Determination of protein

The amount of protein in the samples was measured according to the Lowry method (ref). 2 mL of the reagent mixture (0.1 N NaOH in 0.5 mL 2% Na_2CO_3, 0.5 mL 2% Na-K tartrate, 16.5 mL 1% $CuSO_4$) was added to the appropriately diluted supernatant and vortexed, and incubated for 15 min at room temperature in the dark. At the end of the incubation, 200 µL of Folin ciocalteu's phenol was added and mixed and incubated in the dark for 30 min at room temperature. Samples were read in a microplate reader (Multiskan Go of Thermo Fischer) at a wavelength of 750 nm. The results were calculated on the standard curve prepared with bovine serum albumin and given as mg/mL.[23]

Statistical Analysis

One-Way ANOVA test was used to analyze quantitative variables. *P*-value <0.05 was considered significant. Multivariate test was used to compare pre- and post-treatment variables and because it contained repetitive variables. *P*-value <0.05 was considered significant. All data were analyzed by SPSS Statistics for Windows, version 22.0 software (Chicago, IL, USA).

RESULTS

Histopathological Findings

Vaginal tissues belonging to the Sham group were in normal histological structure (Figure 1A, B). Vaginal epithelial thickness was 119.71±5.28 µm in the sham group. In the histopathological examination of the ovariectomy group, it was observed that the vaginal epithelium was 1-2 rows. (Figure 1C, D). Vaginal epithelial thickness was 17.45±1.42 µm in the ovariectomy group. It was statistically significantly decreased in the ovariectomy group compared to the sham group (p=0.000). In the ovariectomy + myricetin group, the vaginal epithelium had a 2-3 layered appearance (Figure 1E, F). Vaginal epithelial thickness was 23.57±1.60 µm in the ovariectomy + myricetin group. When the ovariectomy group and the ovariectomy + myricetin group were compared, a statistically significant increase in epithelial thickness was observed in the myricetin treatment group (p=0.002). In the ovariectomy + HA group, the vaginal epithelium was 2-3 layered, similar to the ovariectomy + myricetin group (Figure 1G, H). Vaginal epithelial thickness was 21.33±1.02 µm in the ovariectomy + HA group. Compared with the ovariectomy group, the increase in epithelial thickness in the ovariectomy + HA group was statistically significant (p=0.012). There was no statistically significant difference between the ovariectomy + myricetin and ovariectomy + HA groups (p>0.05). Vaginal epithelial thickness was shown in Table 1.

Immunohistochemical Findings

Mild staining was detected with anti-Caspase-3 antibody in the sham group (Figure 2A). In the ovariectomy group, the intensity of the staining increased (Figure 2B). When the sham and ovariectomy groups were compared, a statistically significant increase was observed in the staining intensity with anti-Caspase-3 antibody (p=0.002). Staining intensity was decreased in the ovariectomy + myricetin group compared to the ovariectomy group (Figure 2C). When the ovariectomy and ovariectomy + myricetin groups were compared, a statistically significant decrease was observed (p=0.002). Staining intensity

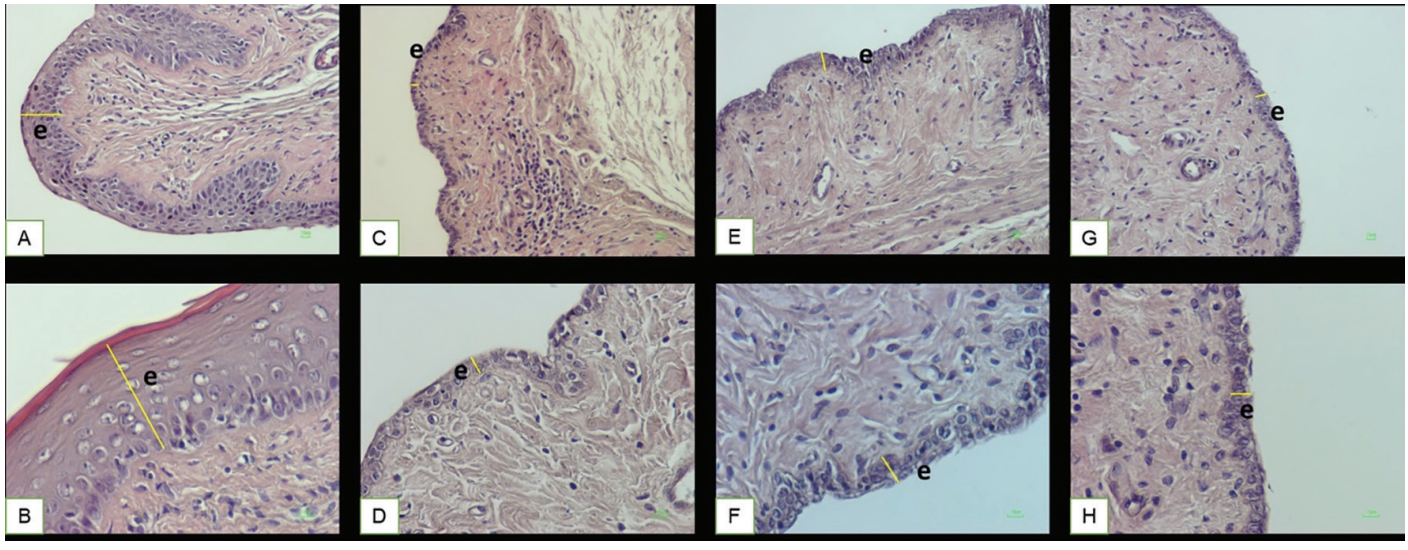

Figure 1. Vaginal epithelium (e) consist of 6-8 layered squamous cells. A. Group 1: Sham, H-E; X20. B. Group 1: Sham, H-E; X40. C. Group 2: Ovariectomy, H-E; X20. D. Group 2: Ovariectomy, H-E; X40. E. Group 3: Ovariectomy + Myricetin, H-E; X20. F. Group 3: Ovariectomy + Myricetin, H-E; X40. G. Group 4: Ovariectomy + Hyaluronic acid, H-E; X20. H. Group 4: Ovariectomy + Hyaluronic acid, H-E; X40

was decreased in the ovariectomy + HA group compared to the ovariectomy group (Figure 2D). When the ovariectomy and ovariectomy + hyaluronic groups were compared, a statistically significant decrease was observed (p=0.041). When the ovariectomy + myricetin groups were compared with the ovariectomy + hyaluronic groups, no statistically significant change was observed in the staining intensity with anti-Caspase-3 antibody (p>0.05). H-score values were shown in Table 2.

Vaginal Maturation Index

Vaginal maturation index value, when the samples taken were compared, the differences in the data were found to be statistically significant (p=0.000). Vaginal maturation index values were shown in Table 3.

Biochemical Findings

When the SOD value was compared among all four groups, 41.3±11.07 U/mg protein in the sham group, 42.51±10.64 U/mg protein in the ovariectomy group, 45.41±26.94 U/mg protein in the ovariectomy + myricetin group, while it was found to be 34.73±3.09 U/mg protein in the ovariectomy + HA group. No statistically significant difference was found between the groups (p=0.663). When the AOPP value was compared among all four groups, 22.22±7.18 µmol/g protein in the sham group, 20.34±11.22 µmol/g protein in the ovariectomy group, in the ovariectomy + myricetin group 21.87±14.78 µmol/g protein, while it was 17.42±2.67 µmol/g protein in the ovariectomy + HA group. No statistically significant difference was found between the data of the four experimental groups (p=0.793). TSH value was 119.76±46.92 µmol/mg in the sham group, 101.98±31.93 µmol/mg in the ovariectomy group, 98.40±35.01 µmol/mg in the ovariectomy + myricetin group and it was calculated as 122.47±46.01 µmol/mg in the ovariectomy + HA group. Biochemical values were shown in Table 4. No statistically significant difference was observed between the groups by means of TSH levels (p=0.650). When the experimental groups were compared, a statistically significant difference was found after injection compared to after ovariectomy in vaginal pH value (p=0.022). Vaginal pH values were shown in Table 5.

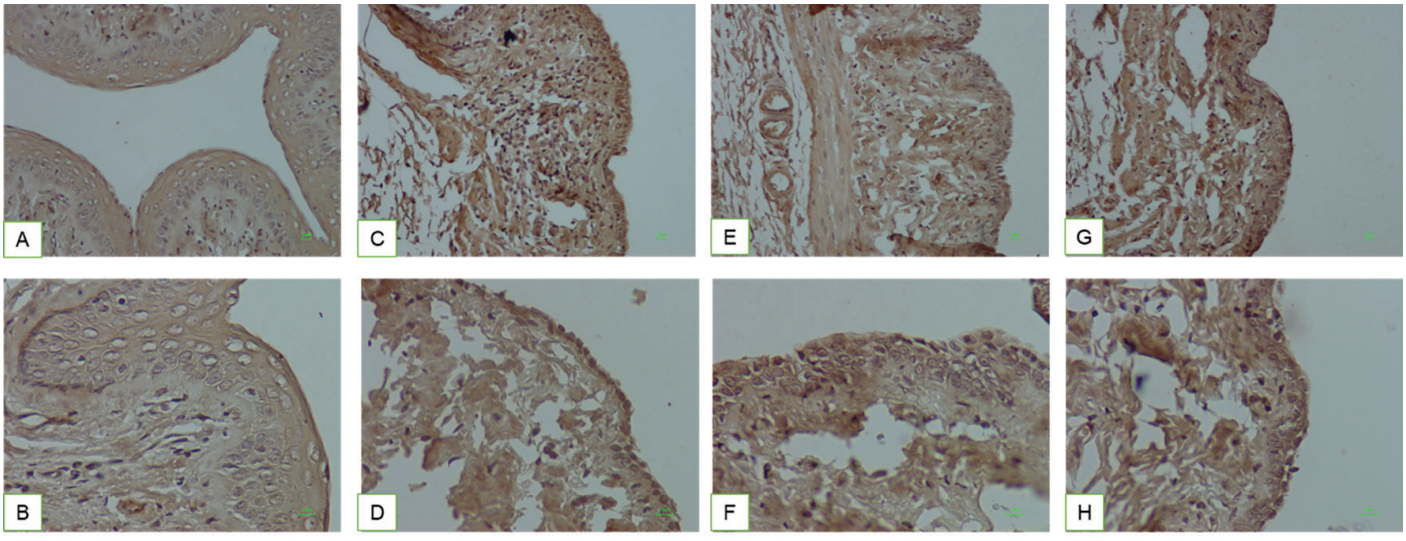

Figure 2. A. Group 1: Sham, anti-Caspase-3; X20. B. Group 1: Sham, anti-Caspase-3; X40. C. Group 2: Ovariectomy, anti-Caspase-3; X20. D. Group 2: Ovariectomy, anti-Caspase-3; X40. E. Group 3: Ovariectomy + Myricetin, anti-Caspase-3; X20. F. Group 3: Ovariectomy + Myricetin, anti-Caspase-3; X40. G. Group 4: Ovariectomy + Hyaluronic acid, anti-Caspase-3; X20. H. Group 4: Ovariectomy + Hyaluronic acid, anti-Caspase-3; X40

Table 1. Vaginal epithelial thickness (µm)

Groups	Vaginal epithelial thickness (µm)
Group 1: Sham	119.71±5.28
Group 2: Ovariectomy	17.45±1.42[a]
Group 3: Ovariectomy + myricetin	23.57±1.60[a,b]
Group 4: Ovariectomy + hyaluronic acid	21.33±1.02[a,c]

Data are expressed arithmetic mean ± SEM (n=7).
[a]=p=0.000 Group 1 vs. Groups 2, 3, 4; [b]=p=0.002 Group 3 vs. Group 2; [c]=p=0.012 Group 4 vs. Group 2

DISCUSSION

According to current literature, our study is the first to examine and compare the effects of both myricetin and HA in experimental rat menopause models. Our study also reports the improval of vaginal Ph, vaginal maturation index, vaginal epithelial thickness and vaginal epithelial nucleus morphological changes after myricetin application in rats which were confirmed to have vaginal atrophy after menopause model construction. Wang et al.[24] reported that HA injection reduced oxidative stress markers, nevertheless it did not make a difference in SOD levels in osteoarthritis synovial fluid. In our study, we also observed that both myricetin and HA did not create a significant difference in SOD levels in menopausal rat vaginas. This finding might be due to short interval between injections and vaginectomies. Almeida et al.[25], demonstrated that AOPP levels induced by myocardial infarction do not change with estradiol injection in female rats that underwent ovariectomy. In our study, we observed that myricetin and HA did not make a significant difference in AOPP levels in rat vaginas meanwhile the histopathological and immunohistochemial findigs are promising. Those irrevelant findings might also be due to short interval between injections and vaginectomies. Korkmaz et al.[26] reported that oxytocin which is being used for its anti-inflammatory effects, did not affect thiol levels in animals under chronic stress, but decreased serum levels in rats under acute stress. In despite of these results when TSH levels were analyzed, no statistically significant difference was observed in our study. This finding might be due to gradual settlement of atrophy after menopause model application in rats.

Nappi et al.[27] reported that an improvement in vaginal pH was observed after HA-based pessary treatment on vulvovaginal atrophy in postmenopausal women. In our study, our findings

Table 2. H-score values

Groups	H-score
Group 1: Sham	198.33±15.05
Group 2: Ovariectomy	328.67±15.25[a]
Group 3: Ovariectomy + myricetin	277.5±25.64[a,b]
Group 4: Ovariectomy + hyaluronic acid	293.33±33.4[2a,c]

Data are expressed arithmetic mean ± SEM (n=7).
[a]=*p*=0.002 Group 1 vs. Groups 2, 3, 4; [b]=*p*=0.002 Group 3 vs. Group 2;
[c]=*p*=0.041 Group 4 vs. Group 2

Table 3. Vaginal maturation index values

Groups	Before overectomy	After overectomy	After injection
Group 1: Sham	73.5±11.53	80.3±7.83	75.00±13.30
Group 2: Ovariectomy	81.78±7.59	2.25±0.64[a]	3.50±1.77[a]
Group 3: Ovariectomy + myricetin	78.21±7.59	3.71±3.08[a]	57.14±17.52[a]
Group 4: Ovariectomy + hyaluronic acid	78.57±9.33	5.64±2.79[a]	42.85±11.49[a]

Data are expressed arithmetic mean ± SEM (n=7).
[a]=*p*=0.000 Group 1 vs. Groups 2, 3, 4

Table 4. Biochemistry values

Groups	SOD (U/mg)	TSH (µmol/mg)	AOPP (µmol/g)
Group 1: Sham	41.33±11.07	119.76±46.92	22.22±7.18
Group 2: Ovariectomy	42.5±10.6	101.98±31.9	20.3±11.2
Group 3: Ovariectomy + myricetin	45.41±26.94	98.4±35.01	21.87±14.78
Group 4: Ovariectomy + hyaluronic acid	34.73±3.09	122.47±46.01	17.42±2.67

Data are expressed arithmetic mean ± SEM (n=7).
SOD=super oxide dismutase; TSH=total sulfhydryl; AOPP=advanced oxidation protein products

Table 5. Vaginal pH values

Groups	Before overectomy	After overectomy	After injection
Group 1: Sham	5.02±0.66	5.35±0.59	5.22±0.44
Group 2: Ovariectomy	4.84±0.72	5.4±0.70	5.90±0.57[a]
Group 3: Ovariectomy + myricetin	4.60±0.56	5.50±0.60	5.90±0.57[a]
Group 4: Ovariectomy + hyaluronic acid	4.7±0.39	5.32±0.33	4.87±0.51[a]

Data are expressed arithmetic mean ± SEM (n=7).
[a]=*p*=0.022 Group 1 vs. Groups 2, 3, 4

are relevant and we found that HA caused a statistically significant improvement in vaginal pH.[27] Hersant et al.[28] reported that in postmenopausal women with a history of breast cancer, HA and platelet-enriched concentrate treatment caused an improvement in vaginal pH. In our study, findings are relevant with the literature and it was also reported that the effect of myricetin on pH was more effective than the HA application.

Doğanay et al.[29] reported that estrogen and vitamin E treatment increased vaginal maturation index on postmenopausal women in cervicovaginal smear preparations. In our study, an increase in the maturation index was observed in the cervicovaginal smear preparations of both HA-treated rats and myricetin-treated rats.[29] Tersigni et al.[30] reported that vaginal epithelial thickness measurements improved after the Isoflavones (Perilei Pausa®) application.

In the findings of this study, which was conducted with the treatment strengthening hypothesis by combining HA, the vaginal epithelial thickness measurements obtained after treatment were positive and were similar to the effects on epithelial thickness in the HA group in our study.[30]

Lima et al.[31] demonstrated that isoflavone gels could be effective on atrophic changes of the vagina by an increase in the thickness of the vaginal epithelium. Myricetin is also in the isoflavone class, and our findings on the thickness of the vaginal epithelium were similar with the findings of this study. The changes in the vaginal epithelium might be due to anti-oxidative and anti-apoptotic properties of isoflavones.[31] Liu et al.[32] reported that HA gel could be effective an increase on vaginal epithelial thickness in ovariectomized rats. In this study, Western blot analysis performed in rats treated with HA gel. In our study, we observed that myricetin increased epithelial thickness with anti-inflammatory and anti-apoptotic effects, and its effect on vaginal HA concentration is still a matter of curiosity. Through its anti-oxidative and anti-apoptotic effects, menopausal aging effects like changes in vaginal pH, vaginal maturation index, vaginal epithelial thickness, vaginal epithelial nucleus morphological changes were improved by myricetin.

CONCLUSION

In our study, by examining the effects of myricetin and HA, we detected that myricetin is as effective as HA in the treatment of GUS, and that it is an effective substance that can be used in cases where HA and estrogen are contraindicated.

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ETHICS

Ethics Committee Approval: The experiments were conducted at the Department of Experimental Animals Application and Research Center. The Muğla Sıtkı Koçman University Ethics Committee for Animal Research approved the animal protocol for this study (approval no: 06/22).

Informed Consent: Animal research investigations were guided in line with the Committee for Human Care.

Contributions

Surgical and Medical Practices: S.K.M., M.N.A.; Concept: S.K.M., B.K.; Design: S.K.M., B.K.; Data Collection or Processing: S.K.M., D.Ç., M.N.A.; Analysis or Interpretation: S.K.M., B.K., H.E., D.Ç., Ü.Ö.T.; Literature Search: S.K.M.; Writing: S.K.M., B.K., A.A.S.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

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