CEOG Clinical and Experimental Obstetrics & Gynecology

A comparison in vitamin D receptor expression during oral menopausal hormone therapy and vaginal estrogen therapy

T.H. Kim[1], H.H. Lee[1], J.M. Kim[2], A. Lee[3], J. Park[4], Y. Kim[3]

[1] Department of Obstetrics and Gynecology, [2] Department of Urology, Soonchunhyang University College of Medicine, Bucheon
[3] Department of Interdisciplinary Program in Biomedical Science, Soonchunhyang University Graduate School, Asan
[4] Laboratory of Immunology and Infectious Diseases, Graduate School of Medical Science and Engineering, Korea Advanced Institute Science and Technology, Daejeon (Republic of Korea)

Summary

Objective: The aim of this study was to examine vitamin D receptor (VDR) expression in vaginal cell and vaginal maturation index (MI) of menopausal women after hormone replacement therapy (HRT). *Materials and Methods:* The authors enrolled 60 healthy postmenopausal women at one year after diagnosis of menopause who had no history of HRT. The subjects were randomized into two groups in an open label fashion. The oral HRT group was treated with one tablet (two mg drospirenone, one mg estradiol hemihydrate per tablet) per day for four weeks, and the vaginal estrogen therapy (ET) group used one vaginal suppository (500 μg estriol per tablet) every day over four weeks. The authors evaluated clinical characteristics, Kupperman index, and vaginal pH before and after treatment. Vaginal epithelial cells were obtained by vaginal swab and prepared to determine the vaginal MI or VDR expression using immunocytochemistry (ICC) and immunofluorescence, followed by confocal microscopy before and after treatment. *Results:* The authors confirmed cytoplasmic expression of VDR in vaginal epithelial cells and that VDR expression was increased in the vaginal epithelium after both oral HRT and vaginal ET, although VDR expression after oral HRT was more effective in increasing VDR than vaginal ET. *Conclusion:* VDR expression in the vagina is affected by different types of HRT.

Key words: Intravaginal; Estradiol; Estriol; Hormone replacement therapy; Receptors; Calcitriol.

Introduction

As the human life span increases, more than half of a woman's life is spent after menopause, which leads to estrogen deficiency and menopausal symptoms, such as hot flashes, night sweats, insomnia, and vaginal atrophy, which can be relieved with hormone replacement therapy (HRT) [1]. Non-hormonal therapy, such as moisturizers or vaginal lubricants, can also be used in addition to HRT to treat vaginal atrophy [2]. The prevalence of vaginal atrophy in postmenopausal women is estimated to be 10-40%, although the exact prevalence is difficult to determine because postmenopausal women accept this benign phenomenon as part of the aging process and are reluctant to discuss it with healthcare providers [3]. Asian women are more reticent than Western women when it comes to these complaints and complain less about vaginal atrophy and sexual dysfunction [4]. A survey of vaginal health views and attitudes in women from the United States revealed that 80% of women reported that vaginal atrophy negatively affected their lives and 75% reported a negative effect on sexual function [5]. Moreover, the diagnosis or management of vaginal atrophy is not a focus of healthcare providers, who may not be confident in their knowledge of the condition or find the consultation inconvenient [6]. Vaginal atrophy should be managed to increase the quality of life after menopause. Applying estrogen vaginal tablets as a local method of E_3 therapy is effective to relieve symptoms such as vaginal atrophy, vulvar itching, dyspareunia from estrogen deficiency during perimenopause and postmenopause or surgical oophorectomy [2]. A study to evaluate the effectiveness of systemic estrogen therapy (ET) on the menopausal vagina reported a pronounced shift in the vaginal maturation index (MI) towards more mature values [7]. However, vaginal atrophy symptoms remained higher; thus, systemic ET is not adequate for relieving symptoms of vaginal atrophy [8]. Furthermore, sometimes patients and health providers do not want to prescribe hormone treatment in cases of vaginal atrophy due to the side effect and lack of effectiveness. Thus, a non-hormonal therapy for vaginal therapy is necessary.

Administration of estradiol (E_2) increases expression of the estrogen receptor (ER), which maintains bone strength and bone health, along with vitamin D [9]. E_2-mediated enhancement of vitamin D receptor (VDR) expression suggests synergy between E_2 and vitamin D_3 in the bone and vitamin D therapy has been shown to be effective in the prevention and treatment of postmenopausal osteoporosis [10-12]. Vitamin D also has other roles on the immune, en-

Revised manuscript accepted for publication May 4, 2016

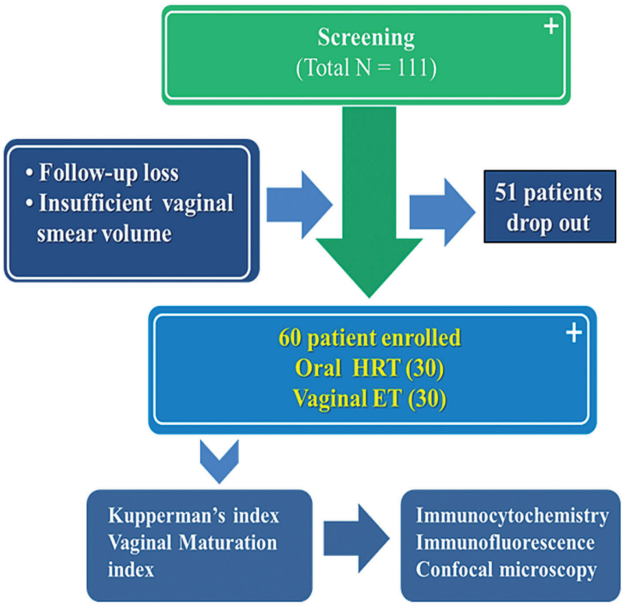

Figure 1. — Study flow chart. There were 111 subjects enrolled in this study. Of these, 60 were ultimately analyzed after losing 51 to follow-up.

docrine, and cardiovascular systems. Vitamin D stimulates E_2 biosynthesis in the ovaries and in cells pretreated with non-calcemic analogues; the expression of ERα protein is increased [11]. Moreover, VDR is a known estrogen responsive gene in cancer studies [13]. Thus, E_2, vitamin D, and the VDR expression are correlated in multiple human organs. Although high levels of VDR expression have been reported in many human tissues, the expression of VDR, or the effects of HT on VDR expression in the human vagina, has not been explored [14]. It is known that 1,25-dihydroxyvitamin D_3 regulates 1,25-dihydroxyvitamin D_3 receptor expression in rat vaginal epithelium [15].

The present authors hypothesized that vitamin D has an important role in the human vagina and that VDR expression may be altered by HT for vaginal atrophy. Thus, this study aimed to examine VDR expression in the human vagina, the regulation of VDR by HRT in the human vagina, and the differential effects of either oral HRT or vaginal ET.

Materials and Methods

This study was approved by the Institutional Review Board (SCHBC_IRB_2012-76). Informed consent was obtained from all participating women. The authors recruited 111 subjects from June 21, 2012 to January 9, 2014; however, because of loss to follow-up, 51 patients were excluded due to insufficient medical records or survey replies (Kupperman index) or inappropriate specimen quality. Therefore, 60 subjects were enrolled in the current study (Figure 1). The inclusion criteria were women who were diagnosed as menopausal at least one year prior with one of the presenting symptoms of vaginal atrophy, such as genital dryness, itching, burning, dyspareunia, burning leucorrhea, vulvar pruritis, feeling of pressure, yellow discharge, and no specific medical history indicating HRT. The exclusion criteria were women who had previously taken vitamin D supplements, other vitamins, and herbal remedies within three months of enrollment, women with neoplastic, metabolic, and infectious diseases, those with a uterus larger than that consistent with 12-weeks gestation, any abnormality on bimanual pelvic examination, concomitant use of any hormonal drug, body mass index (BMI) >30 kg/m^2, cigarette smoker, endometrial thickness > five mm or the presence of any endometrial abnormalities on transvaginal ultrasound. No subjects had a present or past history of venous thrombosis, cerebrovascular or cardiovascular disease, and women were excluded if they had any condition that could alter the pharmacokinetics or hypersensitivity to drospirenone or estradiol.

Postmenopausal women were randomized into one of the following two groups in an open label fashion: oral HRT group was treated with one tablet (two mg drospirenone, one mg estradiol hemihydrate per tablet) per day for four weeks, and the vaginal tablet therapy group used one vaginal suppository (500 μg estriol per tablet) every day over four weeks. The authors questioned referred to Kupperman index before HRT and after one month of HRT [16].

Vagina swab sampling was performed prior to and one month after HRT. After informed consent was obtained, vaginal epithelial cells were collected using a brush device in the upper third of the vagina. After rotating the brushes against the vaginal epithelium, they were rinsed and rotated as quickly as possible in preservCyt solution to release cellular material. A vaginal pH test was performed at the same time as vaginal swab sampling using pH paper.

Each sample was pipetted into a chamber, placed on a cytospin three-chamber and centrifuged through a horizontal tube to a strip of filter paper and onto poly-L-lysine-coated slides. The strip of filter paper was placed between the plastic chamber and the glass slide to absorb the supernatant fluid. The cells traveled through the hole in the filter paper and were deposited in a seven-mm circular area on the slide. All specimens were centrifuged at 300 *g* for five minutes. After the slides were dried, immunocytochemistry (ICC) and immunofluorescence were performed.

After cytospin preparation, all slides were rehydrated through graded ethanol and washed in phosphate-buffered saline (PBS; pH 7.4) three times for five minutes. Endogenous peroxidases were blocked by treating the slides with 0.3% hydrogen peroxide for 30 minutes, followed by washing three times with PBS. The slides were then incubated in a humidified chamber with blocking buffer (5% normal goat serum + 0.5% Tween-20 in PBS) for one hour at room temperature. A rabbit anti-VDR polyclonal primary antibody (ab3508, 1:2000 dilution) was applied to a moist chamber overnight at 4°C. After three additional rinsing steps with PBS for five minutes, a universal anti-rabbit/mouse IgG biotinylated antibody was added for one hour, and the samples were treated with the Vectastain avidin–biotin complex kit for 30 minutes. After being washed with PBS, the slides were developed using diaminobenzidine (DAB) for one minute at room temperature. Counterstaining was performed with Mayer's hematoxylin. All experiments were performed with control staining without the primary antibody to ensure that negative controls remained unstained.

The authors calculated the vaginal MI to compare patient groups. Slides prepared for ICC were used to calculate the MI by counting a minimum of 100 squamous cells. The index represents the relative proportion between parabasal, intermediate, and superficial cells (range 0-100). (Vaginal maturation index (VMI)= 0.2× percent parabasal cells + 0.6 × percent intermediate cells +

Table 1. — *Demographic data.*

	Oral HRT $n = 30$	Vaginal ET $n = 30$	Total $n = 60$	*p*-value
Age (years)	54.5 ± 3.0	54.9 ± 2.8	54.7 ± 2.9	0.602
Height (cm)	156.9 ± 5.6	155.6 ± 4.8	155.8 ± 5.2	0.827
Weight (kg)	56. 3 ± 7.9	58.1 ± 7.9	57.2 ± 7.9	0.378
BMI (kg/m^2)	23.2 ± 3.7	23.9 ± 3.0	23.6 ± 3.3	0.410
Menopause period (years)	5.1 ± 3.2	5.3 ± 3.4	5.2 ± 3.3	0.837
Obstetric history				
Term	2.0 ± 0.8	1.9 ± 0.6	1.9 ± 0.7	0.629
Preterm	0.3 ± 0.8	0 ± 0	0.1 ± 0.6	0.048*
Abortion	1.6 ± 1.7	1.7 ± 1.4	1.6 ± 1.6	0.690
Living baby	1.9 ± 0.7	1.9 ± 0.7	1.9 ± 0.7	0.999

BMI: body mass index; ET: estriol therapy; HRT: hormone replacement therapy.

Table 2. — *Effects of hormone replacement therapy on the Kupperman index and vaginal pH.*

	Mean	†S.D.	*p*-value
*Kupperman index (before)	31.94	3.84	
*Kupperman index (after)	26.47	8.95	
Kupperman index (before) minus Kupperman index (after)	-5.47	9.99	<0.001‡
pH (before)	6.77	0.35	
pH (after)	5.22	0.61	
pH (before) minus pH (after)	1.55	0.53	<0.001‡

[Kupperman index (KI), 15 ≤ KI ≤ 20: mild, 21 ≤ KI ≤ 35: moderate, KI > 35: severe]. † S.D.: standard deviation. ‡ Paired *t*-test, $p < 0.05$.

1.0 percent superficial cells.)

After cytospin preparation, the slides were washed in phosphate-buffered saline (PBS; pH 7.4) three times for five minutes. The slides were covered with ice-cold 100% methanol using enough volume to cover completely to a depth of three- to five-mm and incubated in methanol for ten minutes at -20°C, followed by washing three times with PBS. The slides were then incubated in a humidified chamber with blocking buffer (5% normal goat serum + 0.3% Triton X-100 in PBS) for one hour at room temperature. Rabbit anti-VDR primary polyclonal antibody (ab3508, 1:2000 dilution) was applied in a moist chamber overnight at 4°C. After three additional rinsing steps with PBS for five minutes, anti-rabbit IgG H&L antibody (ab150079, 1:250) was added for one hour at room temperature in the dark. After being washed with PBS, slides were coverslipped with fluoroshield mounting medium and DAPI (ab104139). All experiments were performed with control staining without the primary antibody to ensure that the negative controls remained unstained.

The authors entered data into Excel (and performed statistical analyses using Statistical Package for the Social Sciences (SPSS) v12.0. Demographic data comparison between oral HRT and vaginal ET were analyzed by Student's *t*-test. Changes in the Kupperman index, vaginal pH, and vaginal MI were analyzed using a paired *t*-test. All results are expressed as means ± standard deviation (SD) unless otherwise stated. Differences were considered statistically significant at $p < 0.05$.

Results

There were no differences in age, BMI, number of children, or time since menopause between the groups. The only significant difference was in preterm delivery history between the groups (Table 1).

To show the effect of HRT on the vagina, the authors analyzed the Kupperman index, vaginal pH, and vaginal MI before HRT and after four weeks of HRT. Four weeks after HRT or vaginal ET, Kupperman index and vaginal pH decreased significantly (Table 2, $p < 0.001$). The authors also determined that the vaginal MI improved with both oral HRT and vaginal ET. In the oral HRT group, the decrease in parabasal cells was significant, while in the vaginal ET group, there was a significant increase in superficial cells (Table 3).

Figure 2 shows the effect of HRT on the vagina. Before treatment, few cells were found and those present were generally parabasal or basal cells. However, after four weeks of HRT, polygonally-shaped and distinctly flat superficial cells were predominantly seen. Thus, HRT induced proliferation, differentiation, and cornification of the vaginal epithelium.

Figure 3 shows the expression of VDR in vaginal epithelial cells. Before HRT, few cells were present and most of the vaginal epithelial cells are torn or amorphous. However, after both oral HRT and vaginal ET, vaginal epithelial cells increased and superficial cells dominated, compared to the distinct, intact shape of cells before HRT (Figures 3A, B, E, and F).

Immunohistochemistry (Figures 3A, B, E, and F) and immunofluorescence (Figures 3C, D, G, and H) showed a change in VDR expression on vaginal epithelial cells with both oral HRT and vaginal ET. Furthermore, VDR expression after oral HRT was more effective in increasing VDR than vaginal ET.

Table 3. — *Effects of hormone replacement therapy on the vaginal maturation index.*

	Oral HRT		*p-value*	Vaginal ET		*p-value*
	Baseline	1 month		Baseline	1 month	
Parabasal cell (%)	32.3 ± 29.1	3.5 ± 4.3*	0.045	30.6 ± 32.6	9.5 ± 11.6	0.139
Intermediate cell (%)	51.2 ± 21.2	66.6 ± 28.4	0.241	57.1 ± 26.4	60.4 ± 22.0	0.594
Superficial cell (%)	16.6 ± 15.1	19.9 ± 28.0	0.085	12.1 ± 11.6	30.0 ± 24.1†	0.010
Vaginal maturation index (%)	53.7 ± 16.5	70.5 ± 11.3*	0.009	52.6 ± 16.4	68.2 ± 12.2†	0.010

* Statistically different from the baseline values in the oral hormone replacement therapy group.
† Statistically different from the baseline values in the vaginal estriol therapy group.

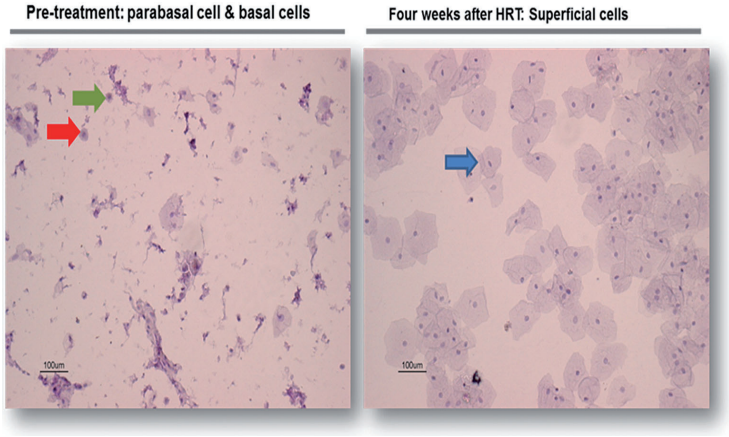

Figure 2. — Hematoxylin staining of vaginal epithelial cells. Before treatment, parabasal, and basal cell are primarily seen. After one month of hormone replacement therapy, a greater number of total cells are seen and superficial cells predominate (×200).

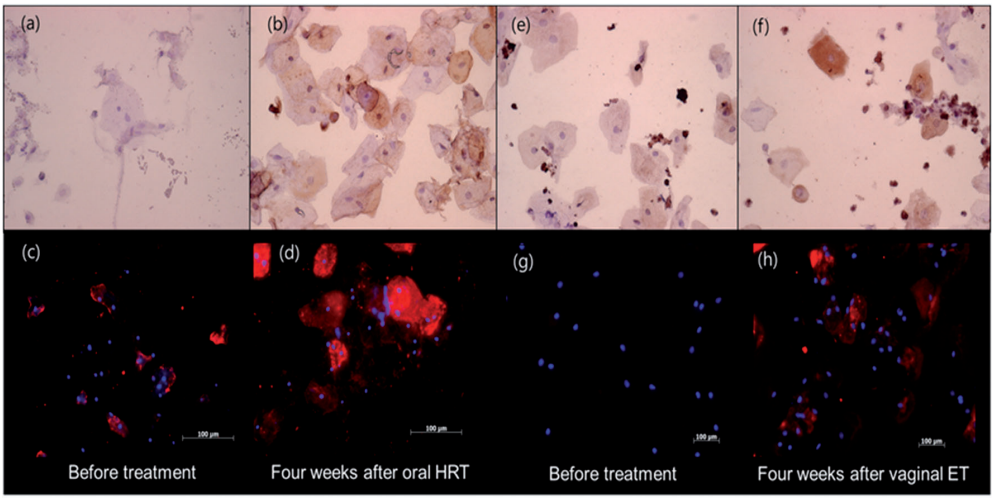

Figure 3. — Immunohistochemistry (A, B, E, and F) and immunofluorescence (C, D, G, and H) of vitamin D receptor expression in vaginal epithelial cells (×200). A, C, E, and G represent expression before treatment. B and D represent expression four weeks after oral HRT. F and H represent expression four weeks after vaginal ET. ET: estriol therapy; HRT: hormone replacement therapy.

Discussion

The aim of this study was to examine VDR expression in the human vagina, the regulation of VDR by HRT, and the differential effects of either oral HRT or vaginal ET. The authors confirmed VDR expression in human vaginal cells and that VDR expression was differentially increased, with oral HRT being more effective than vaginal ET.

The authors suspected a role for VDR in the human vagina that was influenced by HRT and differently affected by the mode of replacement. A literature search produced only five studies utilizing the keywords VDR, human, and vagina as of March 2014. Three of the articles studied the human vagina and VDR or an effect of vitamin D. One of these studies indicated that after 1,25-dihydroxycholecalciferol treatment, the vaginal MI, symptoms, and vaginal pH differed from control, which suggested that vitamin D analogues would be helpful for vaginal atrophy, although this study did not explore changes in VDR expression in human vagina [17]. In another study, raloxifene treatment combined with vitamin D improved the vaginal MI and pH, suggesting that vitamin D improves vaginal atrophy, although changes in VDR expression were again not confirmed [18]. Freedman *et al.* [19] reported that there were higher numbers of estrogenic vaginal cells after a high dose of 2,250,000 to 2,550,000 units of vitamin D in humans but did not report any information about VDR or other pathways.

The present authors' literature search did not discover any reports of VDR expression or VDR expression differences in the human vagina following HRT as of March 2014. However, data from this study support the use of HRT in improving the vaginal MI. Other reports have determined that VDR expression was increased in rat vaginal tissue when vitamin D was administered [15]. Since VDR expression is mediated by E_2, it is reasonable to hypothesize that vitamin D and E_2 act synergistically [12]. During the estrous cycle, the rat vaginal epithelium showed increased VDR in all cell types, except for the mucoid and cornified layers [20]. However, VDR expression was not observed in ovariectomized rats [20]. Since it is known that the VDR is strongly expressed in the rat vagina during the estrous cycle, this suggests that actions of the VDR may be accompanied by E_2.

VDR in the rat has a role in proliferation and differentiation of vaginal squamous epithelium in a manner similar to the E_2. Furthermore, VDR expression in endometrial cells varied with changes in the estrus cycle in several mouse models [21]. Thus, increased expression of VDR may be correlated with a rise in plasma E_2 concentrations. This study has several limitations. For example, the available methodology is limited and it remains difficult to obtain sufficient quantities from a vaginal smear sample for Western blot analysis to confirm changes in VDR during HRT or ET. However, in the present study, the authors did observe increased VDR expression by ICC and immunofluorescence confocal microscopy. This increase in VDR expression suggests that VDR function was also increased. Further studies are necessary to confirm increased expression of VDR by Western blot and increased VDR function by gene sequencing. The VDR is a member of a super family of nuclear receptors and mediates the actions of vitamin D. The VDR is a well-known mediator of remodeling and mineralization in normal bone with other activities in immune, neural, epithelial, and endocrine cells [22]. Membrane signaling can be correlated to non-genomic effects of the VDR, although there are no reports of a non-genomic pathway mediated by the VDR [22]. In addition, there are no reports of VDR in the vagina or how the VDR affects HT or vitamin D regimens. The present data suggest that VDR has a role in the human vagina during vaginal atrophy and after HR. This will be the basis for further work examining the role of vitamin D and VDR in the vagina.

Acknowledgments

This work was supported by the 2012-2014 International Menopause Society (IMS) Research Bursary Award. This research was also supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2014R1A1A3049819)

The authors thank Ms. Yesol Kim (Department of Interdisciplinary Program in Biomedical Science, Soonchunhyang University Graduate School, Asan, Republic of Korea), and Mr. Seung-Rae Yeom, RN (Department of Obstetrics and Gynecology, Soonchunhyang University Hospital, Republic of Korea), for assisting in data collection. Prof. Jong Min Kim, PhD (Dong-A University College of Medicine, Republic of Korea) interpreted pathologic findings.

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Corresponding Author:
H.H. LEE, M.D., Ph.D.
Department of Obstetrics and Gynecology
Soonchunhyang University Bucheon Hospital
170 Jomaru-ro, Wonmi-gu, Bucheon-si
Gyeonggi-do, 14584 (Republic of Korea)
e-mail: hhl22@schmc.ac.kr