Original Article

The Differences of Pain Receptor and Pain-Related Neurotransmitters in the Vagina of Pre- and Post-Menopausal Women

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Objective: Post-menopausal vaginal pain and burning sensation are not solely relieved by improving vaginal dryness. Stimulation of pain receptor (nociceptor) may be a part of the post-menopausal vaginal pain mechanism. The authors primarily evaluated the level of substance P receptor in pre- and post-menopausal women and secondarily studied the level of calcitonin gene-related peptide (CGRP) receptor and nociceptor-activating substances in pre- and post-menopausal women. The association between vaginal pain score and the change in nociceptor and nociceptor-activating substances was analyzed.

Materials and Methods: A cross-sectional study was conducted in 122 pre- and post-menopausal women that underwent total abdominal hysterectomy. Vaginal specimens were obtained and stained for substance P receptor, substance P, CGRP receptor and CGRP, which were used as nociceptive parameters in the present study. Vaginal stromal cells were counted for pain-related protein expressions. Mean pain-related protein expressions in vaginal stromal cells were compared between pre- and post-menopausal women.

Results: Fifty-eight pre-menopausal women and 33 post-menopausal women were included for analysis. Mean substance P receptor, substance P, CGRP receptor and CGRP in post-menopausal women were higher than in pre-menopausal women (47.69, 42.32, 71.31 and 60.73 cells, respectively for post-menopausal women, and 22.03, 21.54, 41.45 and 35.80 cells, respectively for pre-menopausal women). These differences were under the major influence of hormonal status rather than age. The changes in mean pain-related protein expression in vaginal stromal cell after menopause were highest in the first two years. No difference in mean pain-related protein expression in vaginal stromal cell was observed between pain and no pain groups.

Conclusion: In the present study, post-menopausal women were found to have higher mean pain-related protein expressions in vaginal stromal cells than pre-menopausal women.

Keywords: Post-menopausal women, Vaginal pain, Vaginal dryness, Vaginal burning sensation, Vaginal nerve, Substance P, CGRP, Calcitonin gene-related peptide

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Post-menopausal women associated with higher mean pain-related protein expressions in vaginal stromal cells than in pre-menopausal women.

After menopause, the prevalence of urogenital atrophy was reported in up to 34%[1] with the complaints of vaginal dryness, soreness, and dyspareunia as high as 27%[2]. Interestingly, almost half suffered from vaginal pain with moderate to severe intensity.

Sex steroid hormones are essential for the maintenance of vaginal anatomy and function including vaginal epithelium, blood vessel, smooth muscle, mucous production, connective tissue, nerve, and neurotransmitter. The decline of estrogen after menopause causes structural and morphological changes in the vagina that can be relieved with estrogen treatment. Post-menopausal vaginal pain and burning sensation are believed to be caused by the lack of estrogen leading to vaginal dryness. Hence, estrogen is traditionally used to treat these bothersome symptoms. Nevertheless, estrogen was

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found to improve vaginal atrophy symptoms in only 80% to 90% of affected women[3-5], which leads to the need to rethink the mechanism of post-menopausal vaginal pain and burning sensation.

Generally, perceived pain results from tissue injuries that stimulate pain receptors (nociceptor) and causes a cascade of pain response. Pain response can be categorized into four steps, transduction, transmission, modulation, and perception. Transduction, the initial step of pain response, is the process that nociceptors are stimulated by any stimuli from tissue injuries. For visceral pain, which presents in upper part of vagina, pain stimuli are caused by stretching, tissue swelling, or oxygen deprivation. There are many factors that can affect the transduction process such as axon density, receptor potential, free-nerve-ending density, number of pain-related neurotransmitters, and amount of nociceptor-activating substances. Substance P and calcitonin gene-related peptide (CGRP) are in the group of nociceptor-activating substances[6,7].

Substance P[6] and CGRP[7] can be found in many gynecologic organs such as ovary, fallopian tube, uterus, vagina, and vulva. Functions of substance P and CGRP are to activate nociceptors and to control blood supply of target organs, while substance P can also control smooth muscle function.

One of the previously known effects of estrogen on vaginal epithelium was its action over vaginal estrogen receptors. However, some recent data revealed more complex mechanisms on autonomic and sensory neuron via neuronal estrogen receptors[8-10].

The effect of estrogen on nerve density is still controversial in some animal studies[11-13]. A recent human study found that systemic and topical estrogen replacement therapy were associated with a decrease in nerve density[14]. However, the effect of estrogen on the amount of nociceptor and nociceptor-activating substances has never been studied.

Therefore, the primary objective of the present study was to evaluate the level of substance P receptor in vagina of pre- and post-menopausal women. The secondary objectives were to study the level of CGRP receptor and nociceptor-activating substances (substance P, CGRP) in vagina of pre- and post-menopausal women and to evaluate the association between vaginal pain score and the change in nociceptor and nociceptor-activating substances.

Materials and Methods

The present research was a cross-sectional descriptive study approved by the Research Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, IRB No.452/57. The study included all patients who underwent total abdominal hysterectomy at King Chulalongkorn Memorial Hospital, Bangkok, Thailand between December 10, 2014 and December 31, 2015. The exclusion criteria included patients with diagnosis of sexual arousal disorder, known cervical or vaginal cancer, urethritis, cystitis, genital tract infection, previous or current radiation of pelvic organs, and using medication that may affect pain-related neurotransmitter or pain receptor. All necessary information of the study were provided to the participants and written informed consent were obtained before performing any procedure. All participants were interviewed for their basic information, medical history, and vaginal pain score.

Sample sizes were calculated based on data obtained from the authors' pilot study, using $Z_{1-\alpha/2}$=1.96, d (acceptable error)=10% of mean. The sample sizes required for the present study were 53 participants for the pre-menopausal group and 27 participants for the post-menopausal group.

Specimens with a diameter of 0.5 cm at the anterior and posterior vaginal walls adjacent to cervix were collected intraoperatively after the uterus was removed. There was one missing data in the present study. The specimens were fixed in 10% formaldehyde solution before processing. After fixed in paraffin block, specimens were cut with microtome 0.4 micrometer thickness and incubated in 50°C overnight before immunohistochemistry staining with substance P (1:2,000, Merck), substance P receptor (1:5,000, Sigma-Aldrich), CGRP (1:100, Abnova) and CGRP receptor antibody (1:200, Sigma-Aldrich). Antigen retrieval process was done with standard antigen retrieval method-Heat Induced Epitope Retrieval (HIER) in buffer pH 6. HIER was performed by heating the TMA-slides immersed in retrieval buffer for four minutes at 125°C in the pressure boiler. After completed boiling, slides remained in the pressure boiler and were allowed to cool to 90°C. The total processing time was approximately 45 minutes. Subepithelial area were counted for the number of vaginal stromal cells and vessels were stained for substance P, substance P receptor, CGRP, and CGRP receptor with three high power fields for each specimen.

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). After testing for normal distribution with Kolmogorov-Smirnov test with a pre-menopausal group sample size of 60 cases,

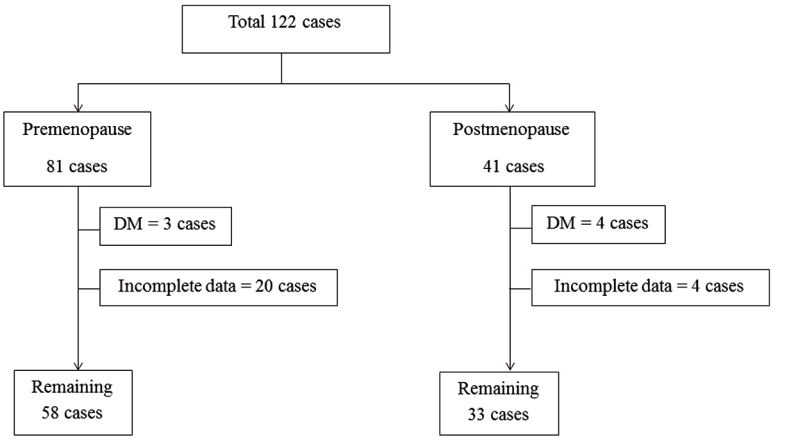

Figure 1. Participants inclusion and exclusion.

Table 1. Characteristics of participants in premenopausal and postmenopausal groups

Variables	Premenopause (n=58); n (%)	Postmenopause (n=33); n (%)	p-value
Age (year); mean±SD	45.03±5.54	57.52±9.7	<0.001*
Parity; mean±SD	0.81±0.09	1.12±1.39	0.07
BMI (kg/m^2); mean±SD	24.31±4.42	24.35±3.61	0.94
Past SI	38 (65.5)	20 (60.6)	0.51
Burning	0 (0.0)	0 (0.0)	
Dull-aching	7 (12.1)	0 (0.0)	0.006*
Dyspareunia	1 (1.7)	0 (0.0)	0.29
Endometriosis			<0.001*
Minimal	11 (18.9)	0 (0.0)	
Moderate	5 (8.6)	2 (6.1)	
Severe	16 (27.6)	1 (3.0)	
Myoma uteri	40 (68.9)	14 (42.4)	<0.001*

SD=standard deviation; BMI=body mass index; Past SI=past history of having sexual intercourse

* p<0.05

and Shapiro-Wilk test with a post-menopausal group sample size of 30 cases, mean and standard deviation were calculated for each group. One-way analysis of variance (ANOVA) was used to compare mean between groups. Differences were considered statistically significant if p-value was less than or equal to 0.05. Pearson's correlation was used for correlation analysis. Subgroup analysis was done in women with difference of years after menopause and presence or absence of endometriosis or myoma uteri. Pain score was evaluated with visual analog scale (VAS).

Results

One hundred twenty-two cases were included in the present study, 81 cases in pre-menopausal group and 41 cases in post-menopausal group. After exclusion due to Diabetes Mellitus and incomplete data, 58 cases in pre-menopausal group and 33 cases in post-menopausal group were included for the analysis (Figure 1). Demographic data is shown in Table 1. There was no statistically significant difference between groups in parity, body mass index (BMI), history of sexual intercourse, burning sensation, and dyspareunia pain score. Age was found to be higher in post-menopausal group while dull-aching pain score, presence of endometriosis and myoma uteri were higher in pre-menopausal group.

There was no statistically significant difference between the number of vaginal stromal cells in anterior and posterior vaginal wall specimen

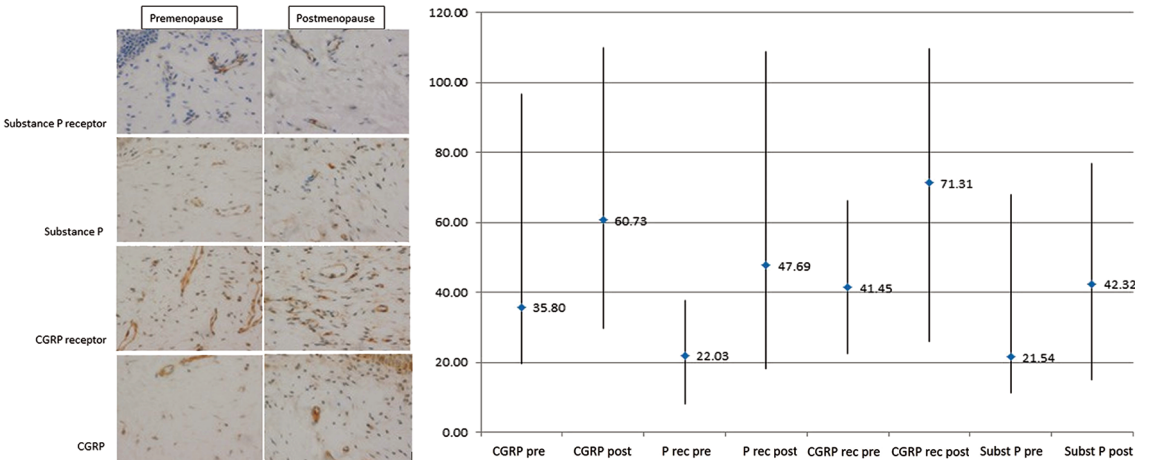

Figure 2. Left: Immunohistological staining of substance P receptor, substance P, CGRP receptor, and CGRP in vaginal subepithelial (stromal) area in pre and postmenopausal women. Right: Mean, minimum and maximum value of pain-related protein expression in vaginal stromal cells in pre- and post-menopause.

CGRP=calcitonin gene-related peptide; P Rec=substance P receptor; CGRP rec=calcitonin gene-related peptide receptor; Subst P=substance P; Pre=pre-menopause, Post=postmenopause

($p>0.05$). The mean number of vaginal stromal cells that were stained for substance P receptor, substance P, CGRP receptor, and CGRP was higher in post-menopausal group as shown in Figure 2. One-way ANOVA was used to compare the mean pain-related protein expression in vaginal stromal cells between groups. Post-menopausal group was associated with higher pain-related protein expression in the vaginal stromal cells ($p<0.05$). The mean pain-related protein expressions in the vaginal stromal vessels were higher in the pre-menopausal group, however, when the pain-related protein expressions in the vaginal stromal vessel were compared, statistical significance could not be reached. There was no statistically significant difference between anterior and posterior vaginal vessels expression (data not shown).

To evaluate the influence of age over the outcome, the authors stratified women by age into 5-year groups in pre-menopausal and into 1-year groups in post-menopausal. Weak association was found between age and the pain-related protein expression in vaginal stromal cells both in the pre-menopausal and the post-menopausal group (Figure 3) (r^2 for substance P receptor, substance P, CGRP receptor and CGRP in the pre-menopausal group were 0.041, 0.003, 0.0906 and 0.0824, respectively, r^2 for substance P receptor, substance P, CGRP receptor, and CGRP in the post-menopausal group were 0.1097, 0.0556, 0.1141, and 0.0647, respectively). In the post-menopausal group, the mean pain-related protein expression in vaginal stromal cells were highest in the first two years after menopause, then declined gradually and rose after year 16 (Figure 4).

Subgroup analysis was done in the pre-menopausal women with pelvic endometriosis and myoma uteri. For the pre-menopausal women with endometriosis, there were decreases in mean pain-related protein expression in vaginal stromal cells compared with women without endometriosis (Table 2). After women with pelvic endometriosis were excluded, the mean results of pain-related protein expression in the vaginal stromal cells in the pre-menopausal group were unchanged. Pain-related protein expressions in the pre-menopausal women with myoma uteri were not different from women without myoma uteri (data not shown).

Eight patients reported increased pain score and all were in the pre-menopausal group. Seven patients had dull-aching pain with visual analog score that ranged from 2 to 5 and one patient had dyspareunia with the score of 5. None of the patients reported a burning pain score. Women who reported a pain score had lower BMI and fewer endometriosis than those who did not. Mean pain-related protein expression in vaginal stromal cells was not different between women with and without vaginal pain (Table 3). After exclusion of women with pelvic endometriosis, participants who reported vaginal pain had significant lower substance P receptor ($p=0.04$) and CGRP expression ($p=0.004$) and non-significant lower substance P and CGRP receptor ($p=0.12$ and 0.28, respectively) (Figure 5).

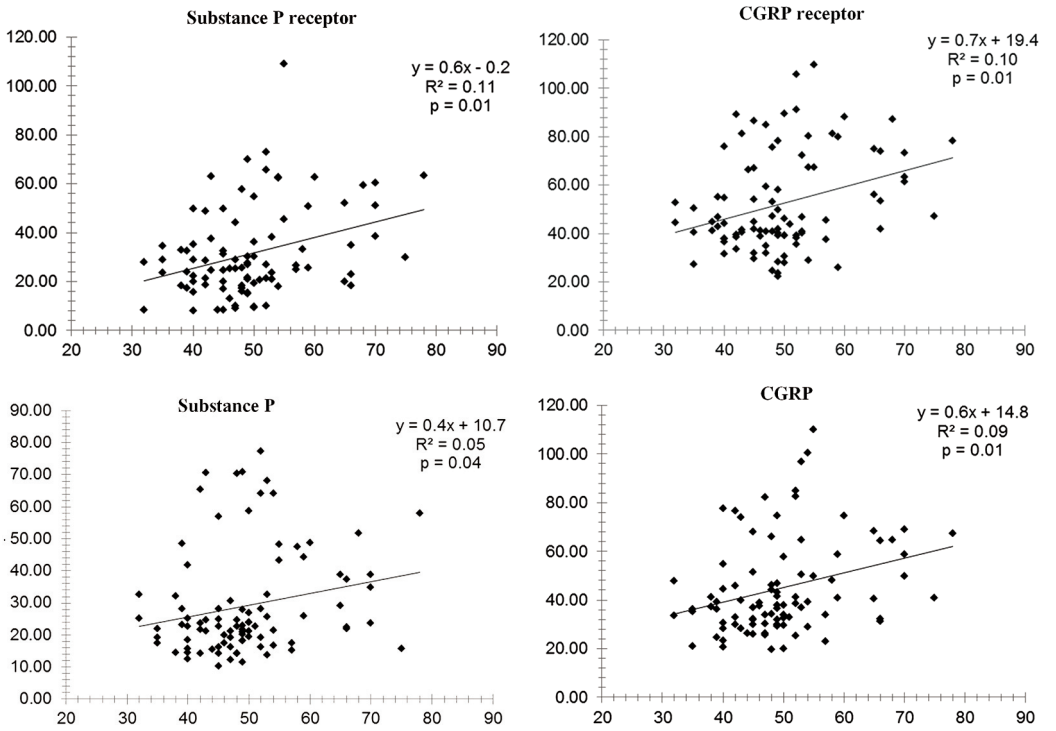

Figure 3. Regression analysis between age (X-axis,years) and pain-related protein expression (Y-axis, cells/high power field) in pre- and postmenopausal group. These plots demonstrate weak association between age and pain-related protein expression.

CGRP=calcitonin gene-related peptide

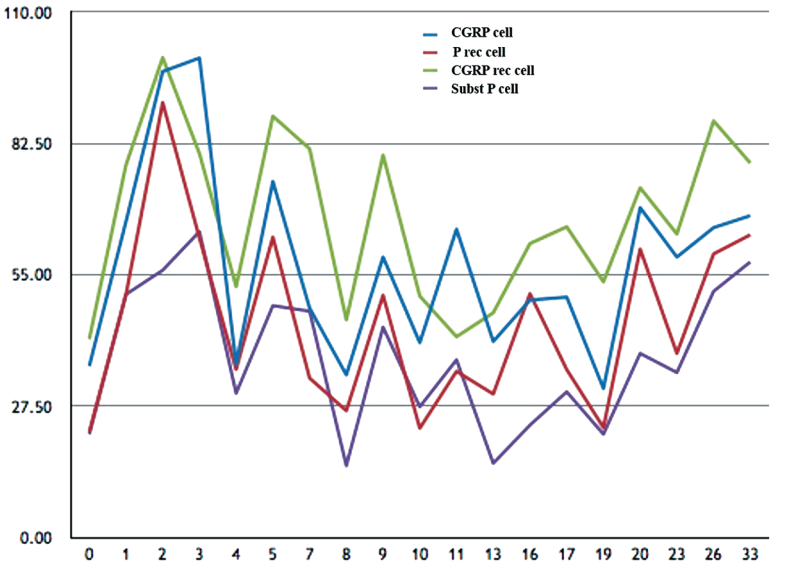

Figure 4. Mean pain-related protein expressions (Y-axis, cells/high power field) in menopausal women stratified by years after menopause (X-axis). The changes in pain-related protein expressions were highest in the first two years, then decline afterward.

CGRP=calcitonin gene-related peptide; P rec=substance P receptor; CGRP rec=calcitonin gene-related peptide receptor; Subst P=substance P

Discussion

The present study confirms the association of pain-related protein expression in vaginal stromal cells and menopausal status. Findings from the present

Table 2. Mean pain-related protein expression in vaginal stromal cells (demonstrated as cells/high power field) in women with and without endometriosis

Variables	Endometriosis (n=35); mean±SD	Without endometriosis (n=56); mean±SD	Mean difference
Substance P receptor	24.72±10.6	36.29±21.2	11.5±3.3
Substance P	42.69±15.0	59.18±22.5	16.49±3.7*
CGRP receptor	23.25±11.8	33.23±19.8	9.97±3.1*
CGRP	37.46±14.5	49.17±21.5	11.7±3.5*

SD=standard deviation; CGRP=calcitonin gene-related peptide

* p<0.05

Table 3. Characteristics of women who reported vaginal pain

No.	Age	Mens	SI	Burning	Aching	Dyspareunia	BMI	Endometriosis	SP rec	SP	CGRP rec	CGRP
1	40	Pre	Y	-	-	5	26	-	8.17	12.17	38	28.33
2	32	Pre	N	-	2	-	18.4	-	8.33	25	44.50	33.67
3	47	Pre	N	-	5	-	19	-	9	12	59.25	25.75
4	49	Pre	Y	-	3	-	23.9	Moderate	30.33	20.67	23.67	36.67
5	49	Pre	Y	-	2	-	20.8	-	27.67	21.33	39.33	29.83
6	47	Pre	N	-	3	-	26.2	-	29	19	41	26.33
7	45	Pre	Y	-	2	-	19.5	-	17.17	14	29.50	26
8	47	Pre	N	-	5	-	19	-	10.17	16	31.75	34
Pain	44.5	Pre	50%	-	2-5	5	21.6	12.5%	17.48	17.52	38.37	30.07
No pain	45.1	Pre	68%	-	0	0	24.7*	62%*	22.76	22.18	41.94	36.71

Mens=menstrual status (premenopause, postmenopause), SI=past history of having sexual intercourse; Y=yes; N=no; BMI=body mass index; SP rec=substance P receptor; SP=substance P; CGRP rec=calcitonin gene-related peptide receptor; CGRP=calcitonin gene-related peptide

* p<0.05

Women without vaginal pain

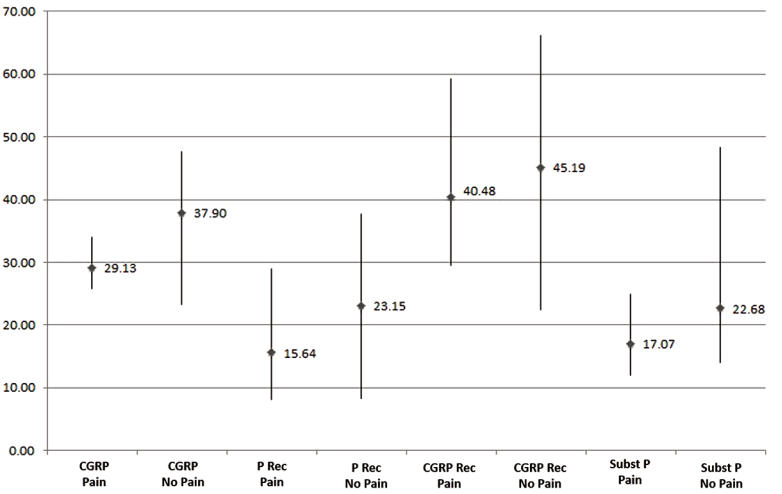

Figure 5. Mean, minimum, and maximum value of pain-related protein expressions in premenopausal women with and without vaginal pain after exclude endometriosis. Premenopausal women who reported vaginal pain had lower pain-related protein expressions compared with women without vaginal pain.

CGRP=calcitonin gene-related peptide, P Rec=substance P receptor; CGRP Rec=calcitonin gene-related peptide receptor; Subst P=substance P

study show that post-menopausal status is associated with higher pain-related protein expressions in vaginal stromal cells. The increase in pain-related protein expressions were highest in the first two years after menopause, then declined gradually. The sample size after year 16 may be too small for any significant interpretation. After age stratification, weak association between age and pain-related protein expression was found. This may imply that the changes in pain-related protein expressions might not mainly result from the aging process both in pre- and post-menopausal groups.

The presence of previous pain exposure such as previous vaginal pain or pelvic endometriosis associated with lower pain-related protein expressions in vaginal stromal cells. The present study found that eight participants who reported vaginal pain were all in the pre-menopausal group. This may be explained by the desensitization theory[15,16], the prolong exposure to pain stimulation causes the decrease in pain-related neurotransmitters. However, the present study may not have the power to demonstrate significance on vaginal pain according to the small number of participants who report having vaginal pain.

The process of pain perception is dependent on many mechanisms such as neuron activity, other substances that can activate free-nerve endings, or number of vessels and its elasticity as was seen in migraine episode. The present study fails to demonstrate the association between vaginal pain score and the number of pain-related protein expressions. This may be due to the small numbers of women who reported vaginal pain.

Previous studies on the effect of estrogen on nerve density are still controversial[11-13]. In animal studies, estrogen was reported to increase, decrease, or have no change in vaginal nerve density. Human study from Griebling et al[14] found that treating post-menopausal women with systemic and topical hormonal therapy decreased vaginal nerve density and CGRP receptor. The present study is the first to evaluate pain-related protein expression in terms of pain-related neurotransmitters and receptors in vaginal stromal cells in pre- and post-menopausal women.

Nevertheless, the study may not have the power to demonstrate adequate association among the groups. The studied population was women who were indicated for hysterectomy, which may not represent the general population.

Results from the present study imply that menopausal status has effects on pain-related protein expressions. This may be a preliminary finding that may lead to the discovery of a different pain mechanism beyond pain caused by vaginal dryness in post-menopausal women. The present study may open a new chapter of treatment for women who do not respond to hormonal treatment or for those hormonal treatment is not applicable. Further studies should focus on the association between pain perception and the level of protein expressions in post-menopausal vaginal cells.

Conclusion

The present study was initiated to evaluate the differences of pain receptor and pain-related neurotransmitters in the vagina of pre- and post-menopausal women. Immunohistochemistry staining was used to evaluate the outcome. Post-menopausal vaginal stromal cells were associated with higher pain receptor and pain-related neurotransmitters. The present study demonstrates that menopausal status has effects on pain-related protein expression in vaginal stromal cells. However, the association between vaginal pain score and the number of pain-related protein expressions cannot be demonstrated. This may be due to the small numbers of women who reported vaginal pain. Nevertheless, pain perception and responses are influenced by variety of factors that are difficult to control. Large scale analytic and long-term studies are needed to confirm the difference and the association between pain-related protein expressions, menopausal status with pain perception.

What is already known on this topic?

Systemic and topical estrogen replacement therapy were associated with a decrease in nerve density in post-menopausal women.

What this study adds?

There was an increase in pain receptor and pain-related neurotransmitters in the vagina of post-menopausal compared to pre-menopausal women.

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Conflicts of interest

The authors declare no conflict of interest.

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