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Original Article

Epithelial thinning in vaginal atrophy related to lowering of calcitonin gene-related protein, vascular endothelial growth factor, and nerve growth factor expressions in a menopausal rat model

[Adelgazamiento epitelial en la atrofia vaginal relacionado con la disminución de las expresiones de la proteína relacionada con el gen de la calcitonina, el factor de crecimiento endotelial vascular y el factor de crecimiento nervioso en un modelo de rata menopáusica]

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Abstract

Context: Vaginal atrophy has been observed as a common sexual problem in post-menopausal women. The targeted protein to counteract menopause problems related to vaginal epithelial thinning is currently a research problem that has not been fully investigated.

Aims: To explore the possible mechanism underlying vaginal atrophy in rat models.

Methods: Following three-week ovariectomy (OVX), Sprague-Dawley female rats were randomly divided into two groups and orally administered estradiol for two weeks in the treated group. In parallel with this, six rats with sham surgery were used as control. Marker-related vaginal atrophy, including calcitonin gene-related protein (CGRP), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF) in the vaginal wall, were compared using immunohistochemistry.

Results: OVX as a menopausal model significantly induced vaginal epithelial cell thinning and decreased the expression of CGRP, VEGF, and NGF compared with sham surgery animals ($p<0.05$). Estrogen replacement in OVX rats reversed the vaginal atrophic by recovering the protein expression CGRP, VEGF, and NGF ($p<0.05$).

Conclusions: Thus, it may be concluded that a possible mechanism underlying the OVX-induced vaginal atrophy may be related to the downregulation expression of CGRP, VEGF, and NGF in vaginal tissue.

Keywords: calcitonin gene-related protein; menopause; nerve growth factor; ovariectomy model; vaginal atrophy; vascular endothelial growth factor.

Resumen

Contexto: Se ha observado que la atrofia vaginal es un problema sexual común en las mujeres posmenopáusicas. La proteína dirigida a contrarrestar los problemas de la menopausia relacionados con el adelgazamiento del epitelio vaginal es actualmente un problema de investigación que no se ha investigado completamente.

Objetivos: Explorar el posible mecanismo subyacente a la atrofia vaginal en modelos de rata.

Métodos: Tras una ovariectomía (OVX) de tres semanas, se dividieron aleatoriamente ratas hembras Sprague-Dawley en dos grupos y se les administró estradiol por vía oral durante dos semanas en el grupo tratado. Paralelamente, se utilizaron como control seis ratas con cirugía simulada. Se compararon mediante inmunohistoquímica los marcadores de atrofia vaginal, como la proteína relacionada con el gen de la calcitonina (CGRP), el factor de crecimiento endotelial vascular (VEGF) y el factor de crecimiento nervioso (NGF) en la pared vaginal.

Resultados: La OVX como modelo menopáusico indujo significativamente el adelgazamiento de las células epiteliales vaginales y disminuyó la expresión de CGRP, VEGF y NGF en comparación con los animales sometidos a cirugía simulada ($p<0,05$). El reemplazo de estrógenos en las ratas OVX revirtió la atrofia vaginal recuperando la expresión de proteínas CGRP, VEGF y NGF ($p<0,05$).

Conclusiones: Por lo tanto, se puede concluir que un posible mecanismo subyacente a la atrofia vaginal inducida por la OVX puede estar relacionado con la disminución de la expresión de CGRP, VEGF y NGF en el tejido vaginal.

Palabras Clave: atrofia vaginal; factor de crecimiento endotelial vascular; factor de crecimiento nervioso; ; menopausia; modelo de ovariectomía; proteína relacionada con el gen de la calcitonina.

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Abbreviations: CGRP: calcitonin gene-related protein; E2: estradiol: VEGF: vascular endothelial growth factor; NGF: nerve growth factor; OVX: ovariectomy; TrkA: tropomyosin receptor kinase A.

INTRODUCTION

Female sexual dysfunction is a common problem in women. Laumann et al. (1999) reported that 43% of women undergo sexual problems, negatively impacting their quality of life (Gao et al., 2017). Lowering sexual arousal is the most common pattern encountered, be prone in post-menopausal women, and the exact mechanisms behind it are not entirely understood (Perez-Herrezuelo et al., 2020; Tsai et al., 2011). Vaginal lubrication is one indicator of the genital sexual arousal response. It ensures the experience is pleasurable and preventable, reducing friction or irritation to the vaginal wall (Geller et al., 2021). When a woman is not sufficiently lubricated, it can cause pain during sexual intercourse and also damage the vaginal lining (Edwards and Panay, 2016; Handy and Meston, 2021).

During menopause, as a result of hormonal changes, the vaginal wall becomes thin out and loses coating resulting reduce the secretion of lubricant, leading to vaginal dryness, dyspareunia, and pain during sexual intercourse (Nappi et al., 2019; Naumova and Castelo-Branco, 2018; Palacios et al., 2018). The vaginal epithelium thinning and decreased vaginal secretion represent vaginal atrophy most closely related to lowering estrogen levels during menopause (Bleibel and Nguyen, 2022; Kasap et al., 2019). Several studies have shown that the decline in serum estradiol levels observed in post-menopausal women is followed by an increased level of plasma calcitonin gene-related peptide (CGRP). This neuropeptide is a potent vasodilator that contributes to the hot flush (Oliveira et al., 2019). Furthermore, vaginal atrophy due to low estrogen levels followed by lowering VEGF and elevating NGF or TrkA (a transmembrane tyrosine kinase, high-affinity NGF receptor) has been reported early (Shang et al., 2021; Yin et al., 2013).

Classical estrogen signaling in women's reproductive tract induces the formation of vaginal integrity by maintaining the thickness of the vaginal epithelium, which is covered by a layer of glycoprotein-containing mucus. Lack of estrogen (E2) leads to the deterioration of vaginal epithelium due to the absence of proper structural integrity of the vaginal lining (Li et al., 2018; Shen et al., 2013). The targeted protein to counteract menopause problems related to vaginal epithelial thinning is currently a research problem that has not been fully investigated. Although E2 supplementation is the current treatment for most post-menopausal symptoms in women, alternative non-hormonal treatment options are often desired (Naumova and Castelo-Branco, 2018; Pan et al., 2022).

The tested hypothesis is that VEGF, NGF, and CGRP changes showed a specific pattern representing the thickening of the vaginal epithelial cell structure. Therefore, this study aimed to generate similar models to understand better alternative targeted protein for post-menopausal women.

MATERIAL AND METHODS

Experimental animals and treatments

This study protocol was approved by the Ethics Committee of Brawijaya University of Medical Faculty No. 106-KEP-UB-2020. Eighteen-month-old female Sprague-Dawley rats (n = 18; weighing 300 g), procured from the pharmacology laboratories, Medical Faculty, Universitas Brawijaya, Indonesia, were housed under standard light (12 h light, 12 h dark) and given free access to food and water. The animals were anesthetized by ketamine hydrochloride and xylocaine as per the approved recommendations. Bilateral ovariectomy or sham surgery was performed by a standardized procedure. The fur was shaved off, and a dorsal midline skin incision was made on both sides to remove the ovaries, except in the sham group. The ovariectomized (OVX) animals were placed individually in separate cages and observed for twenty days before estradiol treatment.

This study was performed on three groups of rats (n = 6 each): (i) Sham-operated; (ii) OVX, and (iii) OVX-estradiol treatment. One group of the OVX rats was administered estrogen subcutaneously through implantation of silastic tubes (Dow Corning, Midland, Michigan) containing 0.1% 17β-estradiol in ethanol for two weeks. All other groups received the vehicle only. Subjects were excluded if any mortality or disease.

Tissue processing, embedding, and sectioning

Samples (vaginal tissue) were taken from 2/3 of the posterior part of the vagina, then cut with a thickness of ± 3 mm and 1 cm in diameter. The vaginal specimens obtained were then fixed with a 10% neutral buffer solution of formalin and left at room temperature for ± 48 h. The sample was fixated and immersed in 10% buffered formalin (Cat No. HT501128-4L, Sigma-Aldrich, Merck & Co., Inc - USA). After that, samples underwent tissue processing overnight (Leica TP1020, USA) before embedding in molten

paraffin wax (HistoCore Arcadia H - Heated Paraffin Embedding Station, Leica, USA). Sections were cut at 4 µm rotary microtome (RM2235, Leica, USA). Paraffin ribbons were flattened in a water bath at 40°C and collected onto polysilane microscope slides (Thermo Scientific) before drying at 60° C for 16 h (Sakura Heater, Tokyo, Japan).

Hematoxylin and eosin (HE) staining

First, the tissues were stained with Harris' hematoxylin solution for 6 h at a temperature of 60-70°C and were then rinsed in tap water until the water was colorless. Next, 10% acetic acid and 85% ethanol in water were used to differentiate the tissue two times for 2 h and 10 h, and the tissues were rinsed with tap water. In the bluing step, we soaked the tissue in saturated lithium carbonate solution for 12 h and then rinsed it with tap water. Finally, staining was performed with eosin Y ethanol solution for 48 h.

Vaginal epithelial thickness measurement

Vaginal epithelial thickness presence or increase of papillae were observed using HE staining. Measurements were carried out using image-raster software by drawing a line perpendicular to the cross-section of the basalis of the vaginal mucosa, from the widest epithelial cells to the basement membrane (at 100× magnification of the microscope). The measurement results are presented in units of micrometers (mM).

CGRP, VEGF, and NGF measurement

Immunohistochemistry (IHC) staining was conducted using a 3.3'- diaminobenzidine stain kit (DAB) (Cat No. D7304-1SET, Sigma Aldrich, US). Antibody monoclonal for CGRP distribution (anti-CGRP, Santacruz Biotech Cat, No. sc-sc-393347), VEGF (anti-VEGF Santacruz Biotech Cat. No. sc-sc-7269), NGF-1 (Anti-NGF Santacruz Biotech Cat. No. sc-32300). The slides of HE and IHC staining were observed by Eromex Iscope light microscope with Sony A7 digital camera attached. The observation was done by calculating the cells observed in 20 fields of views with the magnification of 100×, containing approximately1500 cells.

Statistical analysis

Statistical analysis was performed using one-way variance analysis (ANOVA) followed by a *post hoc* test for ascertaining significant differences. Data were represented as mean ± standard deviation. Value with $p<0.05$ was considered significant.

RESULTS

Histological profile

To determine the effect OVX on the histological profile of the vaginal wall, the epithelial thickness of vaginal tissue in all animal groups was assessed. As shown in Fig. 1, OVX significantly ($p<0.05$) decreased the vaginal epithelial thickness as compared to control rats (sham-operated). The vaginal epithelial thickness was elevated following estrogen treatment of OVX rats ($p<0.05$) (Table 1). This present study demonstrated that OVX significantly induced vaginal epithelial thinning.

Effect of OVX on CGRP, VEGF, and NGF expression

The expression of CGRP, VEGF, and NGF in all animal groups was examined using the IHC method. Table 2 demonstrates CGRP, VEGF, and NGF expressions in rats' vaginal tissue. Ovariectomy, i.e., estrogen depletion, produced a decrease of approximately in protein expression of CGRP, respectively, compared to the control. Administration of estradiol to OVX rats produced an increase in CGRP expression.

Similarly, quantification of IHC data demonstrates a 57% decrease in the VEGF expression following bilateral ovariectomy compared to sham-operated rats. Treatment of OVX rats with estradiol significantly restored VEGF expression. Next, we investigated the effect of OVX on NGF expression in vaginal tissue. Once again, OVX reduced NGF expression (Fig. 2).

Table 1. Differences in the thickness of the appearance of epithelial cells in the observed groups.

Group	Vaginal epithelial thickness (µm)	p-value
Control	76.744 ± 13.98[a]	
Ovariectomy	17.636 ± 9.67[b]	0.036*
Ovarectomy + Estradiol	59.598 ± 20.07[a]	

Data are expressed as mean ± SD (n = 6). Treatments not sharing the same letters in the same column are significantly different by ANOVA followed by a Tukey's test ($p<0.05$).

Table 2. Expression of CGRP, VEGF, and NGF protein in vaginal tissue.

Protein expression	Animal group			p-value
	C	OVX	OVX + E2	
CGRP	3.50 ± 1.049[a]	0.97 ± 0.636[b]	6.03 ± 1.240[c]	0.006*
VEGF	7.19 ± 2.120[a]	2.33 ± 1.033[b]	9.14 ± 2.156[b]	0.018*
NGF	7.39 ± 1.925[a]	3.11 ± 1.186[b]	8.56 ± 1.822[b]	0.036*

All data representation mean ± SD protein staining intensity. Treatments not sharing the same letters in the same row are significantly different by ANOVA followed by a Tukey's test ($p<0.05$). CGRP: calcitonin gene-related protein; VEGF: vascular endothelial growth factor; NGF: Nerve Growth factor: C: control; OVX: ovariectomy; OVX + E2: Ovariectomy with estradiol treatment.

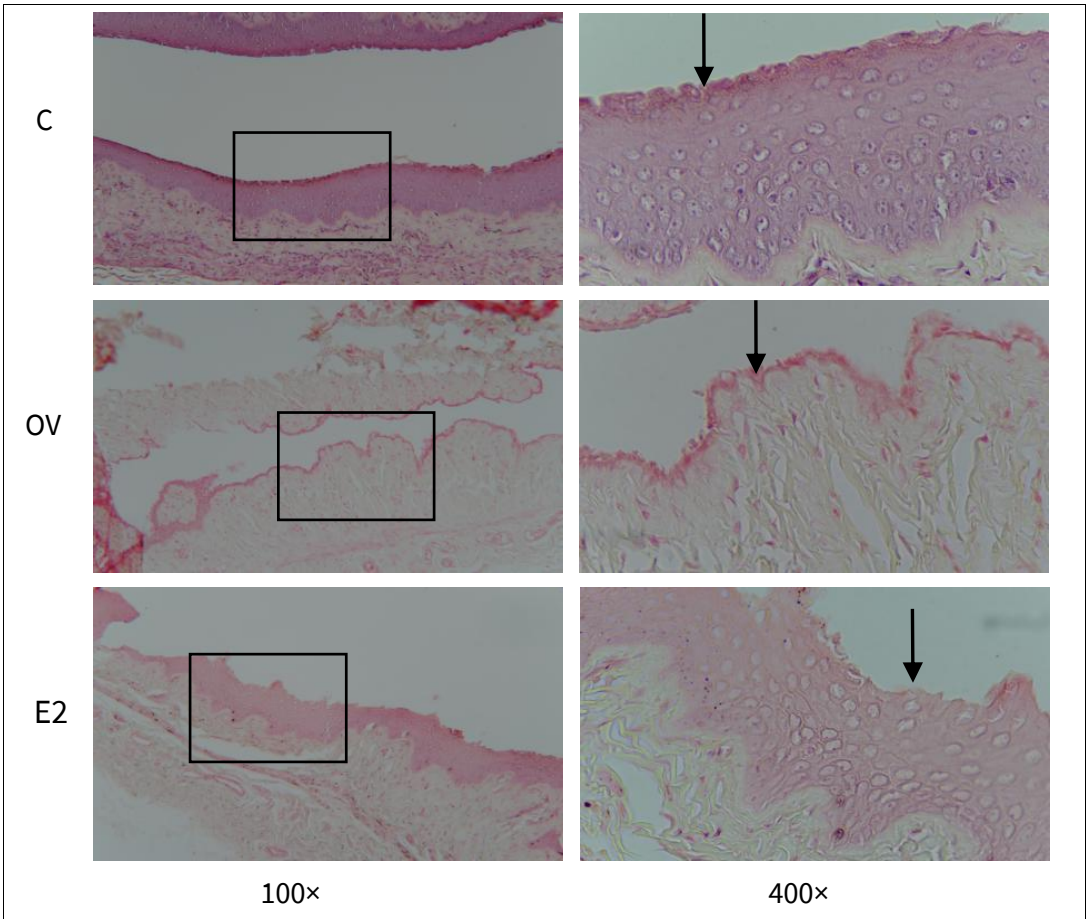

Figure 1. Hematoxylin and eosin stained section of the rat vaginal models.

Control tissue showed a gradual stratified epithelial layer. Removal of ovarium by OVX procedure in rat models showed the absence of vaginal epithelial components. Scale bar = 100 µm. OVX: ovariectomy; E2: estradiol.

DISCUSSION

Menopause is a phenomenon that inevitably occurs with clinical pathological changes, including dystrophic vaginal mucosa, which leads to vulvovaginal atrophy (VVA) (Dos Santos et al., 2021; Naumova and Castelo-Branco, 2018). We show here the protein profile, including CGRP, VEGF, and NGF of vaginal tissue after OVX and treated with physiological levels of either female sex hormones E2. We determined that vaginal epithelial thickness decreased after OVX, followed by a significant decrease in CGRP, VEGF, and NGF expression. The administration of estradiol, as one of the modalities of menopause therapy, showed the effect of improving vaginal epithelial cell thickness on rat models, followed by increased protein expression of CGRP, VEGF, and NGF. This shows that CGRP, VEGF, and NGF can be used as preclinical determinants of the human vaginal for the repair of the epithelial lining of the vaginal wall in menopause.

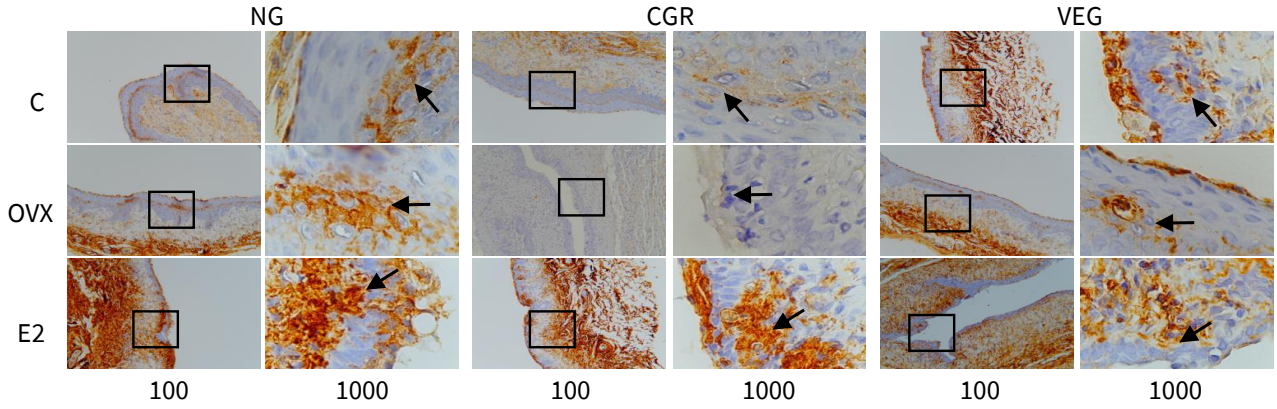

Figure 2. Representation of immunohistochemical staining results against NGF, CGRP, and VEGF in vaginal preparations (cross sections) with a thickness of 4 mm.

C: mice without OVX; OVX: group of old mice with OVX; and E2: group of old mice with OVX gave E2 preparation. Expression of NGF, CGRP, and VEGF, indicated by the presence of brown color in the cell nucleus (which is a visualization of the dye DAB/3,3'-diaminobenzidine).

Molecular understanding of the involvement of the protein in the vaginal epithelium microenvironment as a molecular target could enhance our knowledge of the vaginal pathological mechanism related to the alteration of the structural integrity of the epithelial vagina inherent in menopause (Isaza, 2019; Nakamura et al., 2012). Furthermore, it is well documented that the cell division and differentiation of vaginal epithelial cells are essential to replenish and maintain the protective epithelial lining of the vaginal tract to prevent the entry of pathogens (Li et al., 2018; Shafaat et al., 2022). When women undergo menopause at the age of 48 to 52 years, leading to a decline in estrogen levels, which modulates the thickness of the vaginal epithelium changes, lack of lubrication, and the vaginal tissue becomes vulnerable to physical damage, e.g., intercourse (Li et al., 2018, Karppinen et al., 2022). Although E2 supplementation is the current treatment for most post-menopausal symptoms in women, alternative non-hormonal treatment options are often desired (Li et al., 2018; Mueck et al., 2018; Pan et al., 2022).

Ovariectomy (OVX) was clearly found to induce significant vaginal atrophy and decrease the expression of various angiogenic factors, including VEGF (Yin et al., 2013). The changes in CGRP expression after OVX may underlie various changes related to vaginal atrophy, which were validated by thinning of vaginal epithelial cell structure as a result of decreased potent vasodilator CGRP and control of proliferation by VEGF. Our finding showed that E2 treatment alleviated the epithelial vaginal thickness, followed by elevation of CGRP, VEGF, and NGF. While the results in the previous study clearly showed that NGF level was down-regulated by E2 (Liu et al., 2018; Molnár, 2020; Shang et al., 2021), within our finding, NGF expression was up-regulated by E2 treatment. These changes suggest that E2 signaling within vaginal tissue plays an essential role in controlling vaginal epithelial growth and differentiation (Cora et al., 2015; Li et al., 2018; Winuthayanon et al., 2017).

In line with the previous study, the upregulation of NGF expression after sex steroid hormones administration was related to enhanced CGRP synthesis (Armayanti and Wulansari, 2020). Age-related decline in E2 level in humans leads vaginal epithelial regression followed by loss of defense against pathogenic invasions, which is reversed upon E2 supplementation. Discovering markers representing a specific subset of vaginal epithelial cells, including marker changes, may represent targeted therapy in vaginal epithelial cell atrophy. The nerve growth factor, a member of the death receptor signaling, was mainly expressed in the normal basal layer or atrophied vagina. E2 treatment is well-known for activated Wnt signaling via crosstalk with NGF. It has been shown to activate TrkA, triggering self-renewal of the vaginal epithelial cell (Ali et al., 2020; Chow et al., 2019). This mechanism may also hijack targeted points for menopausal women for the future approach.

CONCLUSION

Vaginal epithelial cell lining changed after OVX, followed by decreased expression of CGRP, VEGF, and NGF, which did not occur in rat models without OVX. The OVX model given E2 showed an increase in the vaginal epithelial cell layer followed by an improvement in CGRP, VEGF, and NGF expression. Thus, it may be concluded that a possible mechanism underlying the OVX-induced vaginal atrophy may be related to the downregulation expression of CGRP, VEGF, and NGF in vaginal tissue.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Fithri AN	Yueniwati Y	Arsana IW	Khotimah H	Nurwidyaningtyas W
Concepts or ideas	x	x	x	x	
Design	x	x	x	x	
Definition of intellectual content	x	x			
Literature search	x				
Experimental studies	x				
Data acquisition	x				x
Data analysis		x			x
Statistical analysis					x
Manuscript preparation					x
Manuscript editing	x				x
Manuscript review	x	x	x	x	x

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