Vasoactive intestinal peptide expression in the vaginal anterior wall of patients with pelvic organ prolapse

Jie-Mei Hu a,b, Xiao Cheng b, Lin Wang b, Jie-Ning Zhu a, Li-Hua Zhou b,*

a Department of Obstetrics and Gynecology, Guangdong General Hospital and Guangdong Academy of Medical Sciences, Number 106 Zhongshan Road 2, Guangzhou 510080, China
b Department of Anatomy, Zhong Shan School of Medicine, Sun Yat-Sen University, Number 74 Zhongshan Road 2, Guangzhou 510080, China

Accepted 19 January 2012

Abstract

Objective: Perimenopausal women are at high risk for pelvic organ prolapse (POP) and stress urinary incontinence (SUI) diseases. In the present study, the expression of VIP in the vaginal epithelium of 70 perimenopausal women was correlated with the severity of POP with or without SUI.

Materials and Methods: Seventy biopsy specimens from the anterior vaginal epithelium were obtained from postmenopausal patients. Immunohistochemical labeling for vasoactive intestinal peptide (VIP) and hematoxylin and eosin staining were performed. The VIP innervation was then compared between eight patient groups. Semiquantitative analysis of VIP protein by Western blotting was performed and compared between the eight patient groups.

Results: The results of the immunohistochemical study showed that the intensity of VIP-immunoreactivity (VIP-ir) in the eight groups was as follows (in decreasing order): Control; POPI; POP II; POP II + SUI; POP III; POP IV and POP III + SUI; and POP IV + SUI. The intensity of VIP-ir was obviously weak and similar among the POP IV, POP III + SUI, and POP IV + SUI groups. This result was validated by the Western blotting analysis. The level of the VIP peptide also deceased in POP patients and was as follows (in decreasing order): Control; POPI; POP II and POP II + SUI; POP III and POP III + SUI; and POP IV and POP IV + SUI.

Conclusion: The present study found that reduced VIP innervation in the vaginal epithelium of the perimenopausal women was correlated with the severity of POP with or without SUI.

Copyright © 2013, Taiwan Association of Obstetrics & Gynecology. Published by Elsevier Taiwan LLC. All rights reserved.

Keywords: human vaginal tissue; menopause; pelvic organ prolapse; vasoactive intestinal peptide

Introduction

Pelvic organ prolapse (POP) is frequently encountered in middle-aged and elderly women, and approximately one of every nine women will undergo surgery to correct POP and associated bladder and bowel dysfunction [1]. Stress urinary incontinence (SUI) is another pelvic dysfunction disease with an etiology similar to that of POP [2]. In general, POP is a major problem associated with altered anatomy of the pelvic floor. It may be caused by direct injury to the levator ani (LA) muscle, denervation of the pelvic floor musculature, or fascial damage incurred during traumatic childbirth [3,4]. Reproductive functions are under the influence of a peptidergic component of the parasympathetic and sympathetic branches of the autonomic nervous system [5]. Vasoactive intestinal peptide (VIP)-containing nerves have been described throughout the human female genital tract and are most abundant in the vagina, cervix, and clitoris [6,7]. Therefore, we hypothesized that VIP secreted by neuropeptidergic fiber terminals parties involved in the pathogenesis of POP. To explore the relationship between VIP innervation and the degree of POP, in the present study we tested for changes in VIP innervation in the epithelium of the anterior vaginal wall in patients with POP.

* Corresponding author. Department of Anatomy, Zhong Shan School of Medicine, Sun Yat-Sen University, Number 74 Zhongshan Road 2, Guangzhou 510080, China.
E-mail address: zhoulih@mail.sysu.edu.cn (L.-H. Zhou).
Materials and methods

Subjects

Seventy patients were enrolled. They had a mean age of 61 years (age range, 30–70 years) and were treated at the Department of Obstetrics and Gynecology of the Guangdong General Hospital between August 2008 and June 2009. Following gynecologic examinations, the patients were divided into four groups in accordance with the Pelvic Organ Prolapse Quantification (POP-Q) system [8]. There were 15 patients in POP I, 16 patients in POP II, 14 patients in POP III, and 15 patients in POP IV. Stress urinary incontinence was diagnosed by patient history, gynecologic examination, pad test, and urodynamic examination in accordance with the International Continence Society Standard [7]. Eleven patients in POP II, 6 patients in POP III, and 9 patients in POP IV had accompanying SUI, which placed them in the POP II + SUI, POP III + SUI and POP IV + SUI groups, respectively. Nine patients with benign gynecological conditions but no history of POP or SUI were placed in the control group. The study was approved by the Hospital Scientific Committee. All patients consented to participate in the clinical trial and agreed to have biopsies performed.

Tissue extraction

For consistency, 70 biopsy specimens of the midline anterior vaginal cuff were obtained from 70 patients undergoing intravaginal panhysterectomy, anterior/posterior colporrhaphy, and pelvic floor function reconstruction. None of the patients took hormonal drugs during the 3 months before surgery. Excluded from the study were women who had previous endometriosis, adenomyosis, uterine fibroids, connective tissue disorders, pelvic inflammatory conditions, or pelvic surgery.

Sample preparation

After rinsing with saline to remove remaining blood, samples 3-mm to 5-mm thick and measuring 100–300 mg were obtained from each specimen. Half of each sample was placed in 10% formalin for VIP immunohistochemistry (IHC) and routine hematoxylin-eosin (HE) staining. The other half was prepared for Western blotting (WB) analysis. Formalin-fixed specimens were embedded in paraffin, sectioned (3 μm), and fixed to glass slides. Paraffin sections were deparaffinized, rehydrated with xylene and a graded series of ethyl alcohol, and rinsed with phosphate-buffered saline (PBS) three times.

Hematoxylin-eosin staining

Hematoxylin-eosin staining was performed in accordance with standard protocols for examination for routine light microscopy.

Immunohistochemistry for VIP

Immunohistochemistry procedures were performed according to our previous studies [9–12]. The slides were boiled in 0.01 mol/L sodium citrate buffer (pH 6.0) for 15 minutes in a microwave oven. Sections were washed three times with 0.01 M PBS for 10 minutes, incubated in 3% peroxide in methanol at room temperature for 20 minutes to quench endogenous peroxidase activity, and then rinsed three times with PBS. The sections were then incubated in 2% bovine serum albumin (BSA) and 0.2% Triton X-100 in 0.01 M PBS at room temperature for 1 hour, followed by incubation overnight with mouse anti-VIP antibody (1:100; Santa Cruz Biotechnology, Dallas, Texas, USA). After washing with PBS, biotinylated anti-mouse IgG antibody (1:200; Gene Tech, Shanghai Company Limited, China) was added to the sections and incubated at room temperature for 35 minutes. The primary or secondary antibody and the tissue sample were replaced with PBS as the negative control and with small intestine as the positive. The VIP was visualized with 3,3-diaminobenzidine (Sigma, Saint Louis, MO, USA) as the chromogen. The slides were rinsed with distilled water and counterstained with HE.

Semi-quantitative analysis of VIP immunoreactivity

Images were acquired with a camera mounted on an Olympus microscope (Carl Zeiss, Germany) using 10×, 40×, and 100× objective lenses. Under a microscope using a 40× lens or a 100× lens, VIP-immunoreactive (VIP-ir) particles were counted, if necessary, by two independent observers. The evaluation of immunoreactivity was semiquantitatively assessed, based on staining intensity and distribution. A four-point ordinate scale was adopted, based on previous studies [5,7,13]. Estimation of VIP nerve supply was graded by VIP-ir staining, as follows: “++” indicates a dense supply (i.e., showing strong brown staining with a ring-like distribution); “+” indicates a moderate supply (i.e., showing brown staining with concentrated distribution); “−” indicates a scarce supply (i.e., showing weak brown staining with sparse distribution); and “−−” indicates no detectable immunoreactivity.

Western blotting analysis

The procedures of the WB in this study were similar to the procedure used in our previous studies [14]. Using a grinder by hand, the vaginal tissues were homogenized in radio immunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail and 1mM phenylmethanesulfonyl fluoride (PMSF). The homogenates were lysed in ice for 1 hour, and then centrifuged at 15,000 r.p.m. for 30 minutes at 4 °C. The supernatant was decanted. The protein concentration was determined by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were loaded and separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were then blocked for 1 hour at room
temperature with 5% nonfat dry milk in tris-buffered solution followed by overnight incubation at 4 °C with the mouse anti-VIP (1:1000). The membranes were washed three times in tris-buffered saline and then incubated for 2 hrs at room temperature with anti-mouse IgG (1:4,000, Amersham Biosciences, Piscataway, NJ), and then detected by electrochemiluminescence (Pierce, Rockford, IL, USA). The same membrane was stripped and reblotted with a mouse anti-β-actin antibody (1:1000; Boster, China) for normalization. The blots were exposed to X-ray film (FUJIFILM, Japan). All studies were performed a minimum of five times, using independent samples from different groups. The changes of the VIP protein levels ratio, corresponding to β-actin level, were determined by the optical density measurements on Western immunoblot. The relative levels of proteins in the different lanes were compared by analyzing scanned images using the NIH IMAGE program (National Institutes of Health, MA, USA). The intensity was quantified by the corresponding β-actin.

Statistical analysis

Statistical calculations and data handling were performed using SPSS version 16.0 (Statistical Product and Service Solutions, LA, USA) by two persons who were blinded to the different groups. All data for continuous variables were expressed as the mean ± standard deviation (X ± SD) along with ranges. The Kruskal-Wallis test was applied to detect differences among groups for ranked data [7,9]. Semi-quantitative changes in the VIP level relative to the β-actin level in the POP groups were determined by optical density measurements. Data are shown as the mean ± SD (n = 3 independent blots from 3 separate experiments) and expressed as the fold changes in comparison to the controls. One-way analysis of variance (ANOVA) with a Bonferroni post-hoc multiple comparisons test was applied to detect differences between the groups. The null hypothesis was rejected at p < 0.05.

Results

General conditions of the studied patients

Age, number of postmenopausal years, SUI complications, parity, and body mass index (BMI, calculated as weight in kilograms divided by height in meters squared) were compared across the four groups of POP patients and the control group (Table 1). The F values for these data were estimated by the Kruskal-Wallis test. For each comparison, p was greater than 0.05, suggesting that differences in age, BMI, parity, and years post menopause were not significant between the four POP groups and the control group. Thus, the study groups were similar and comparable with regard to these factors.

Histology of the anterior vaginal wall of POP patients

Fig. 1 shows the HE-stained microphotos that characterize the morphologic appearance of the anterior vaginal wall of POP patients. Three layers were identified across the vaginal wall under light microscopy: the mucous layer, the muscular layer, and the fibrous tissue membrane layer. The vaginal mucosa consisted of squamous epithelium and the underlying lamina propria. The remaining vaginal wall (approximately 70–75% of the total wall thickness) consisted of the muscular layer and the adventitia. Smooth muscle, small blood vessels, capillaries, and nervous tissue were situated in the lamina propria, which was principally composed of connective tissue (Fig. 1A). The muscularis contained superficial and deep layers demarcated by changes in muscle orientation or by a layer of perforating vessels. In the control group, the smooth muscles were thick and well arranged like a braid and without fissures in the muscle fibers (Fig. 1A). In POP I, the connective tissues remained neat and compact, but local alterations in density were apparent. Small vessels and capillaries were abundant and their walls demonstrated thinning (Fig. 1B). In POP II, the epithelium appeared to vary in thickness, and the connective tissue was not as organized. The number of blood vessels and the number of cells in vessel walls were both reduced (Fig. 1C). The muscularis and muscle bundles from POP III and POP IV women were poorly organized, with the collagen fibers loosely dispersed throughout (Fig. 1D and E). The vessel wall was thin with poor elasticity and with clot deposition (Fig. 1E). In women with POP + SUI, the lamina propria tissue was more loosely dispersed and disorganized (Fig. 1F–H) than in patients with POP without SUI. In POP IV + SUI, some of the lamina propria tissue was broken down and the number of cells was decreased (Fig. 1H).

VIP innervation in the anterior vaginal wall of POP patients

The VIP immunoreactivity in the subepithelial connective tissue of the vaginal epithelium in all control and POP groups was measured. Fig. 2 shows results. The VIP-ir was detected

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Case</th>
<th>Age (y, mean ± SD)</th>
<th>Menopausal (n, %)</th>
<th>With SUI (n, %)</th>
<th>Parity</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>57.34 ± 4.26</td>
<td>2 (20%)</td>
<td>0 (0%)</td>
<td>1.32 ± 0.34</td>
<td>22.74 ± 2.09</td>
</tr>
<tr>
<td>POP I</td>
<td>15</td>
<td>59.76 ± 7.23</td>
<td>12 (80%)</td>
<td>0 (0%)</td>
<td>2.20 ± 0.83</td>
<td>24.29 ± 2.03</td>
</tr>
<tr>
<td>POP II</td>
<td>16</td>
<td>60.26 ± 6.55</td>
<td>14 (88%)</td>
<td>11 (69%)</td>
<td>2.41 ± 0.85</td>
<td>21.16 ± 2.07</td>
</tr>
<tr>
<td>POP III</td>
<td>14</td>
<td>63.35 ± 7.86</td>
<td>14 (100%)</td>
<td>6 (43%)</td>
<td>2.67 ± 1.26</td>
<td>23.08 ± 2.05</td>
</tr>
<tr>
<td>POP IV</td>
<td>15</td>
<td>61.50 ± 8.98</td>
<td>13 (87%)</td>
<td>9 (60%)</td>
<td>3.34 ± 1.32</td>
<td>22.92 ± 2.41</td>
</tr>
</tbody>
</table>

BMI = body-mass index; POP = pelvic organ prolapse; SD = standard deviation; SUI = stress urinary incontinence.
in the vaginal epithelium of the patients. The VIP-ir was localized beneath the vaginal epithelium and mainly surrounded blood vessels (Fig. 2A–H). In the control group, the VIP-ir appeared as an intact ring surrounding the blood vessels; VIP-positive neurons and fibers could be detected (Fig. 2A). The VIP expression was different in each group of
Fig. 2. The distribution of vasoactive intestinal peptide (VIP) in the subepithelial connective tissue of the anterior vaginal wall cross-sections from an asymptomatic control and from patients with pelvic organ prolapse (POP) with or without stress urinary incontinence (SUI) (A) In the control group, VIP-immunoreactive (VIP-ir) nerves appear as an intact ring surrounding blood vessels. (B) In POP I, VIP-ir nerves, which are localized around the thicken arteries, show continuous dark brown beading changes. The VIP-ir nerves have a dispersed and weakly stained pattern in (C) POP II and in (D) POP III. (F) In POP II + SUI, the VIP-ir nerves are more weakly stained in comparison to the nerves in (C) POP II. (E) In POP IV, (G) POP III + SUI, and (H) POP IV + SUI, only a few scattered VIP-ir nerves with weak staining are present. (The scale bar = 100 μm. VIP immunohistochemistry plus hematoxylin used in all images.)

POP patients. In POP I, VIP was expressed around thickened arteries with numerous dark brown particles found in beads (Fig. 2B). However, in POP II and POP III (Fig. 2C and D, respectively), the intensity and distribution of VIP-ir decreased and showed disperse and weak staining. In POP IV, POP III + SUI, and POP IV + SUI (Fig. 2E, G and H, respectively), only scattered VIP-ir with weak staining was observed. Table 2 shows the results of the semi-quantitative analysis. With the progression of POP symptoms, the VIP innervation in the anterior vaginal epithelium was significantly downregulated. The intensity of VIP-ir in the eight groups was as follows (in decreasing order of intensity): control; POP I;
POPIV = pelvic organ prolapse; SUI = stress urinary incontinence; VIP = vasoactive intestinal peptide; VIP-ir = VIP-immunoreactivity.

POPII; POPII + SUI; POPIII; POPIV and POP IV and POPII + SUI; and POPIV + SUI. The intensity of VIP-ir was considerably weaker and similar in the POPIV, POP III + SUI, and POP IV + SUI groups (Table 2).

**VIP levels in the vaginal tissues of the anterior wall of patients with POP**

The level of VIP protein in the vaginal tissues of the anterior wall was measured in all control and POP groups.

**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Case</th>
<th>Staining</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>POPI</td>
<td>8</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>POPII</td>
<td>10</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>POPIII</td>
<td>15</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>POPIV</td>
<td>8</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>POPII + SUI</td>
<td>7</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>POPIII + SUI</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>POPIV + SUI</td>
<td>5</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 3 shows the results. There was obvious expression of VIP in the control group. With the progression of POP symptoms, the level of VIP decreased significantly. Semi-quantitative analysis showed that the level of VIP protein in vaginal tissue was 89% ± 7.2% in POP I; 76% ± 6.3% in POP II; 71% ± 6.9% in POP II + SUI; 25% ± 4.1% in POP III; 23% ± 6.9% in POP III + SUI; 14% ± 3.5% in POP IV; and 11% ± 3.5% in POP IV + SUI. The significantly different levels of VIP in the eight groups were as follows (in decreasing order): control; POP I; POP II and POP II + SUI; POP III and POP III + SUI; POP IV and POP IV + SUI. For all comparisons, p < 0.05 (Fig. 3).

**Discussion**

In the present study, we hypothesized that, in addition to the mechanical stress on the pelvic floor, some women have differences in VIP innervation that predispose them to POP. Therefore, we undertook an analysis to assess VIP innervation in the vaginal epithelium of 70 postmenopausal women. The results showed that the expression of VIP-ir neurons and fibers decreased significantly with the progression of POP symptoms.

From our random samples, the proportion of POP combined with SUI was as follows: POP II, 69% (11/16); POP III, 43% (6/14); and POP IV, 60% (9/15); these results are consistent with a previous study [15]. In the human female genital tract, peptide-containing nerve fibers exist near blood vessels and smooth muscles and the fibers line the epithelium [6,16–18]. Epidemiologic data have suggested that disruption in the innervation of the pelvic floor, including dysregulation of VIP, may be an important factor in the etiology of SUI and POP [19–22]. Some reports have also shown age-related changes in VIP levels in patients with POP [7,13]. Our present study further supports these results, indicating that decreased VIP innervation of the anterior vaginal epithelium is associated with the severity of POP with or without SUI.

Our results show that the expression of VIP in the vaginal epithelium decreased significantly in the POP II + SUI and the POP III + SUI groups, compared to the corresponding groups without SUI. These results are consistent with previous studies showing that the expression of neuropeptide Y (NPY) was significantly reduced in the epithelium of the posterior vaginal wall in women with SUI [13]. The lower VIP-ir in patients with SUI may either be related to the lower neuronal production of VIP [13] or to age-induced nerve degeneration [7]. In the present study, the expression of VIP in the epithelium was reduced because of the decreased production of VIP, as indicated by our WB data, which proved that the VIP level in the same tissue was reduced in POP patients with or without SUI. Our WB data were consistent with previous studies showing that VIP levels were significantly decreased in the anterior vaginal wall of premenopausal and postmenopausal patients with SUI or POP. Furthermore, those studies found that VIP levels were inversely correlated with age and menopausal status in patients with SUI or POP [23]. Our results showed that VIP expression and VIP levels in the anterior vaginal epithelium were reduced with increased severity of POP with or without SUI.
SUI, which supports the findings of previous studies. However, another study has shown that VIP expression has no correlation with age or menopausal status in patients with SUI, but is negatively correlated with age and menopausal status in patients with POP [13]. These results suggest that the role of VIP innervation in POP may be different from its role in SUI. Vasoactive intestinal peptide is a neurotransmitter in the vasculature of the genitalia. It has been suggested that the endogenous neurotransmitter mediating genital blood flow may be a substrate for VIP [24]. Our data further confirm that VIP innervation occurs mainly near blood vessels located beneath the vaginal epithelium. With increasing severity of POP (with or without SUI), the distribution of VIP-ir particles changed from a circle that surrounded the vessel wall to particles that were scattered along the vessel wall.

The present data did not show significant differences between POP IV and POP IV + SUI groups in VIP innervation or in the VIP level in the anterior vaginal epithelium. As our HE data show, pathological changes such as lamina propria tissue breakdown and loss of a considerable number of cells in the vaginal epithelium were most severe in these two groups. There was only scattered VIP-ir expression with weak staining in the POP IV and the POP IV + SUI groups. It was difficult to analyze the significance of differences between the two groups with such low VIP expression. A previous study has demonstrated that the denervation of the vaginal epithelium provides a barrier to compensatory remodeling and repair in this situation [25,26]. Because the number of samples was limited, the present investigation has not explicitly proven that the proportion of POP cases accompanied by SUI is correlated with the severity of POP disease. Therefore, we should expand our sample size and gain more evidence.

In summary, the present study constituted the most definitive assessment of the relationship between VIP expression and the POP-Q score. The present data could not determine whether differences in VIP innervation are the cause of POP or whether they result from the pathological changes caused by POP. Further studies are needed in an animal model to investigate whether manipulating VIP levels in vaginal tissue could interfere with the progression of POP. Such analyses would give additional insights into what experiments are needed to confirm the relationship between VIP and the POP-Q score indicated in the present study.

Acknowledgments

The grant sponsors of this work were the National Natural Science Foundation of China (81070995) and Research Foundation of Department of Science and Technology of Guangdong Province (Grants 2008B050100011 and 2010B031600037). We thank Dr Zhi-Gang Li, Dr Yue-Wei Zuo, the residents and faculty of the Department of Obstetrics and Gynecology of Hospital for assistance in tissue acquisition. We also thank Mr Qiu-Xiong Hu and Miss Xiao-Hong Li for skillful technical assistance. We acknowledge the tissue core facility (sponsored by Zhong Shan School of Medicine) for its aid in tissue procurement.

References


